

Excessive iron in diets promotes kidney fibrosis via disordering the inflammation factors in Sheep

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Abstract

Iron is a mineral that the body acquires from the environment in order to maintain physiological function; however, excessive iron causes disease. The aim of this work is to investigate the processes by which excessive iron in diets induces kidney fibrosis in sheep by disorders their inflammatory factors. All sheep were fed a basal diet supplemented with 50 (CON), 500 (L-iron), 1000 (M-iron), and 1500 (H-iron) mg Fe/kg as ferrous sulfate monohydrate ($\text{FeSO}_4 \cdot \text{H}_2\text{O}$), respectively. Hematoxylin-Eosin (H&E), Perls, and Masson staining were used to observe histopathological alterations; Western blotting, ELISA, and quantitative real-time polymerase chain reaction (qRT-PCR) were used to detect changes in the amounts of key inflammatory factor genes and proteins. As iron intake rose, glomerular cell contents dropped, tubular inflammatory cells infiltrated and fibrin was secreted, collagen fiber deposition and fibrosis have noticeable symptoms. Interleukin-1 β (IL-1 β), tumour necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) activities were decreased, while interleukin-2 (IL-2), interleukin-6 (IL-6), nuclear factor- κ B (NF- κ B), and transforming growth factor- β 1 (TGF- β 1) activities were increased. Excessive iron disorders inflammatory factor secretion, triggers an inflammatory response, and promotes the development of renal fibrosis, according to this study.

Introduction

Iron is a mineral that the body obtains from the environment in order to maintain its physiological activity, and the majority of it is found in the form of hemoglobin in red blood cells [1]. Iron homeostasis imbalances can cause significant disorders by disrupting mitochondrial activity, as well as DNA repair and synthesis [2]. Metabolic changes and altered skeletal muscle function can be caused by iron insufficiency [3]. Iron excess can cause oxidative stress and iron deposition in parenchymal organs [4], which can harm the organism [5]. Traditionally, inorganic iron sulphate has been added to the feed to promote normal growth in animals and to prevent clinical deficiencies caused by low iron utilization [6]. The NCR advises a basic iron intake of 30 mg/Kg of feed dry matter for sheep [7], with a maximum tolerance of 500 mg/Kg of feed dry matter [8].

The kidneys are an essential endocrine organ that play an important role in maintaining fluid balance and normal organ function [9]. Diets supplemented with various iron dosages were found to have a close linear association with iron contents in the kidneys in studies. The kidneys complete the transport and reabsorption of iron through divalent metal ion transporters and transferrin receptors [10, 11], and unused iron excretion in urine [12]. As a result, the kidney plays a crucial role in maintaining iron homeostasis and preventing excessive iron. Excessive iron intake can persist in kidney tubular cells [13], affecting the innate immune response and even leading to cell death [14]. Inflammation induces the creation of iron regulatory proteins, which govern iron homeostasis [15], and disorder of inflammatory factors may lead to dysregulation of tissue repair and fibrosis [16]. These findings imply that in vivo iron levels are linked to inflammation and fibrosis. While it is well established that excessive iron causes liver inflammation and fibrosis [17], and the certainty of this relationship is enhanced by the reduction of serum iron levels in the follow-up of inflammation-induced experimental infections [18], the association between excessive iron

and kidney inflammation and fibrosis in sheep is unclear and need to investigate further. Our study provides a theoretical foundation for further research into the harmful effects of excessive iron and the rational use of iron in sheep feeding management.

Inflammation is a defense mechanism in the body that is triggered by irritation or infection [19], inflammatory mediators play a role in cellular interactions and signal transduction. Heparin is a key factor in iron homeostasis management, which can suppress the expression of IL-1 β , IL-6, NF- κ B and TNF- α [20], and it is a major product released in response to IL-6 [21]. Excessive iron stimulation resulted in increased expression and release of IL-6 in the liver, according to previous studies [22]. IL-1 β stimulates heparin expression via the same mechanism that activates IL-6 mediated heparin transcription [23]. Chelation reduces intracellular iron, which inhibits NF- κ B induction of TNF- α and other cytokines. NF- κ B is an important regulator of the inflammatory response, and the NF- κ B pathway can be induced by TNF- α [24]. Iron can phosphorylate the inhibitor protein κ B (IKB) [25] and dissociate it from NF- κ B, resulting in NF- κ B activation and inflammatory disease [26], which can be inhibited by the use of an iron chelating ligand (deferasirox) [27]. IL-2 is a pro-inflammatory factor that impacts cell proliferation and differentiation and is essential for immune cell proliferation, activation, and dynamic homeostasis. IFN- γ and TNF- α are important regulators of macrophage iron status and immunological function, causing cytokine-mediated effects that contribute to the inflammatory response [28].

Fibrosis is a pathological process in which inflammation causes necrosis of the parenchymal cells of certain organs and abnormal tissues, but it is unclear whether inflammation play a role in the development of kidney fibrotic disease in sheep [29]. However, previous studies have demonstrated that IL-1 β stimulates macrophages and neutrophils [30], promotes cellular conversion to the epithelial mesenchyme, expresses collagen, and induces fibrosis [31], and induces tubulointerstitial renal tubular disease by promoting proximal tubular damage and fibrosis [32]. TNF- α is the primary cytokine responsible for pro-inflammatory cell recruitment and cellular inflammasome activation after macrophage activation[30]. IL-1 β and TNF- α induce NF- κ B translocation from the nucleus to active NF- κ B, which is involved in pro-inflammatory and pro-fibrotic production [33]. TNF- α , IL-1 β , and IL-6 activate the NF- κ B pathway, which causes fibroblast activation and kidney fibrosis [34]. TGF- β 1 is activated and released by macrophages, promotes intrinsic cell activation, which results in the production of large amounts of collagen and extracellular matrix [35], it is a crucial mediator in the recruitment of peripheral cells and the development of renal fibrosis in chronic kidney disease [36], and treatment of mouse renal tubular epithelial cells causes kidney fibrosis [37]. As a result, inflammatory factor disorders are virtually usually the cause of kidney fibrosis.

In these studies, in vivo experiments were used to assess the development of the inflammatory response in the sheep kidney to verify the extent of inflammatory factor disruption. The inflammatory response was triggered by increasing the amount of iron in the diet, which resulted in increased secretion of IL-2, IL-6, TGF- β 1, and NF- κ B and decreased secretion of IL-1 β , IFN- γ , and TNF- α . Excessive iron leads to disturbance of the balance of inflammatory factors, causing tissue fibrosis to worsen.

Material And Methods

Animals and Treatments

Sixteen healthy 8-month-old German Merino sheep (Institute of Animal Husbandry, Heilongjiang Academy of Agricultural Sciences, China) weighing around 40 kg were chosen for this experiment. The 16 sheep were randomly divided into four groups (n = 4) and 50 (CON), 500 (L-iron), 1000 (M-iron), and 1500 (H-iron) mg $\text{FeSO}_4 \cdot \text{H}_2\text{O}/\text{kg}$ were added to the basal diet. All of the sheep in the study were euthanized after 75 days of storage, and kidney samples were collected. For the collected sheep kidney samples, A portion of the sheep kidney samples was fixed in neutral formalin and examined with H&E to see lesions, Perls staining to assess iron accumulation, and Masson staining to see tissue fibrosis. The rest is kept frozen at -80°C for future gene and protein assays. All animal experiments were carried out in accordance with the NEH Animal Care Agency and Institutional Committee rules for the protection and use of laboratory animals (SRM-11).

Pathological Examination

The fixed kidney was cut to $0.5 \times 0.5 \times 0.5$ cm in size and embedded with embedding agent. sections were prepared using the paraffin sectioning method, and serial sections were taken for staining.

Hematoxylin-Eosin Staining

The following were the basic H&E staining procedures: xylene (2×5 min), 100% ethanol (2×1 min), 95% ethanol (1 min), 90% ethanol (1 min), 80% ethanol (1 min), 70% ethanol (1 min), distilled water (3 min), Hematoxylin (15 min), differentiation (to pink), anti-blue (wash with running water to blue-gray), 70% ethanol (10 s), 80% ethanol (20 s), 95% ethanol (2×30 s), 100% ethanol (2×1 min), xylene (2×5 min), neutral gum sealing (overnight drying at 37°C), microscopic examination.

Perls Staining

Before staining, the perls dye solution was produced according to the instructions, the basic Perls staining methods were as follows: distilled water (2×10 min), Perls stain (20 min), distilled water (5 min), nuclear solid red (8 min), distilled water (5 s), 70% ethanol (5 s), 80% ethanol (20 s), 95% ethanol (2×40 s), 100% ethanol (2×3 minutes), xylene (2×4 minutes), neutral gum sealing (overnight drying at 37°C), microscopic examination.

Masson Staining

For the Masson Trichrome Stain Kit (Solid Green Method), the following steps were completed: Weigert iron hematoxylin (5 min), acid ethanol differentiation solution (3 s), masson bluing solution (3 s), distilled water (1 s), ponceau-magenta (5 min), weak acid working solution (1 min), phosphomolybdic acid (1 min), weak acid working solution (1 min), solid green dyeing solution (2 min), weak acid working solution (1 min), 95% ethanol (3×7 s), xylene (3×1 min), microscopic examination.

Protein content detection

Determination of NF- κ B and TGF- β 1 Protein Levels by Western Blotting

In order to obtain the supernatant, 0.05 g of sheep kidney tissue was collected, lysed in 500 μ L of Radio-immunoprecipitation Assay (RIPA) buffer with protease inhibitor cocktail, pulverized for 60 seconds in a tissue homogenizer, and centrifuged at 12,000 rpm for 10 minutes at 4°C. Whole-tissue protein extracts were separated on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels after being quantified using the bicinchoninic acid (BCA) assay, and the proteins were transferred to the nitrocellulose filter (NC) membrane. The membrane was blocked with 5% bovine serum albumin (BSA)/Tris Buffered Saline Tween (TBST) buffer for one hour at room temperature, hybridized with primary antibody overnight at 4°C, incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody, and then exposed with ECL efficient chemiluminescent reagent and placed in the exposure instrument for exposure. Each western blot depicted is representative of three independent experiments.

Determination of IL-1 β , IL-2, IL-6, TNF- α and IFN- γ Protein Levels by ELISA

The samples were extracted with diluent (PBS PH = 7.4) at a weight-to-volume ratio (1 g: 9 ml) of 0.050 g. Using low-temperature grinding and centrifugation, the supernatant was removed. Sheep kidney tissues were collected for ELISA assay using IL-1 β , IL-2, IL-6, TNF- α , and IFN- γ ELISA kits, as directed by the manufacturer (R&D Systems, Minneapolis, MN, USA). A spectrophotometer was used to access the specific binding optical density at 450 nm right away.

Expression of Inflammatory Factors mRNA by qRT-PCR

Total RNA from sheep kidneys was extracted using trizol, and cDNA was generated from 1 μ g of RNA using the iscript cDNA synthesis kit (Bio-Rad, USA), with absorbance at 260/280 nm for mRNA purity at a ratio greater than 1.8 achieved for all samples used. The samples were amplified in 96-well plates, and qRT-PCR was done with a 20 μ L reaction system on a Roche LightCycler 480 II System (Roche, Switzerland). The following were the primer sequences :

Table 1
Sequences of the primers used for qRT-PCR.

Genes	GenBank number	Primer Sequences(5'-3')	Product length (bp)
β-actin	U39357.2	F:AGATGTGGATCAGCAAGCAG R:CCAATCTCATCTCGTTTTCTG	135
IL-1β	NM_001009808.1	F:GGAGAATGTGGTGATGGTGACAGC R:ATTGGCAATGGCTTCCAGGTCATC	103
IL-2	NM_001009806.1	F:GCACTAACTCTTGCACTCGTTGC R:GCATCCTGGAGAGCTTGAGGTTC	148
IL-6	NM_001009392.1	F:ACACTGACATGCTGGAGAAGATGC R:GCCGCAGCTACTTCATCCGAATAG	132
TNF-α	DQ153000.1	F:CTGGTGCCTCAGCCTCTTCT R:GGGACTGCTCTTCCCTCTGG	104
IFN-γ	NM_001009803.1	F:AAGTTCTTGAACGGCAGCTCTGAG R:TTGGCGACAGGTCATTCATCACC	130
NF-κB	XM_015100238.2	F:TCGAGGTTTCGGTTCTACGAGGATG R:AACACGGTTACAGGACGCTCAATC	145
TGF-β1	NM_001009400.2	F:ACTACTACGCCAAGGAGGTCACC R:CACAGGTTTCAGGCACTGCTTCC	140

95°C for 2 minutes, 46 cycles of 95°C for 10 seconds, annealing at 58.7°C for 30 seconds, and extension 72°C for 15 seconds were used in the qRT-PCR amplification method. During the annealing extension, fluorescent signals were detected. As an internal reference control, β-actin was used. The relative levels of mRNA expression for each gene were normalized by glyceraldehyde-3-phosphate determined by Ct values and assessed using the relative quantification-cation ($2^{-\Delta\Delta CT}$ method). All of the experiments were carried out in triplicate.

Statistical Analysis

The mean ± standard deviation (SD) of at least three independent experiments were used to calculate the results. GraphPad Prism 6 Demo was used to examine the statistical significance of the differences with the Student's Two-way ANOVA. $P < 0.05$ was considered statistically significant (* or $P < 0.05$ indicates a significant difference; ** or $P < 0.01$ indicates an extremely significant difference; $P > 0.05$ indicates no difference).

Results

Histological Observation of Kidney Pathology after Excessive Iron in Sheep

Under a 100× magnification microscope. The tubular interstitial space was widened and more loosely arranged in the excessive iron group after H&E staining; under high magnification, localized tubular lesions of M-iron and H-iron were found, and tubular epithelial cells were excreted and necrotic, and the tubular interstitial space was accompanied by fibrin exudation and a small amount of inflammatory cell infiltration (Fig. 1). After Perls staining, no dispersion of iron-containing hemoglobin was found. The structural integrity of the glomeruli and tubules was observed in the control group following Masson staining, without significant collagen fibril hyperplasia. Glomerular pyknosis was observed in the excessive iron group, along with an iron dose-dependent increase in collagen fibril area. The glomerular basal membrane wrinkled, tubular collagen fibers exuded, and the lumen narrowed. Fibrous necrosis of the small artery walls of the entrance glomerulus (Fig. 2).

Changes of Protein Expression in Kidney of Excessive Iron-Fed Sheep

As shown in Table 2, the protein content of kidney IL-1 β , TNF- α , and IFN- γ decreased with increased iron addition in excessive iron sheep, and IL-1 β was extreme significantly lower in the M-iron and H-iron groups than in the control group ($p < 0.01$), TNF- α and IFN- γ were significantly lower than in the control group ($p < 0.05$) (Fig. 3).

With increasing iron addition, the protein contents of IL-2, IL-6, NF- κ B, and TGF- β 1 in sheep's kidneys increased (Table 2, Table 3). IL-2, IL-6, NF- κ B, and TGF- β 1 were significantly or extreme significantly higher in the M-iron and H-iron groups than in the control group ($p < 0.05$ or $p < 0.01$); IL-6 and NF- κ B were significantly or extreme significantly higher in the L-iron group than in the control group ($p < 0.05$ or $p < 0.01$); IL-2 and TGF- β 1 were not significantly different in the L-iron group compared to the control group ($p > 0.05$) (Fig. 3, Fig. 4).

Table 2
Effect of excessive iron on the kidney IL-1 β , TNF- α , IFN- γ , IL-2 and IL-6 protein contents in sheep(μ mol/L).

Item	CON	L-iron	M-iron	H-iron
IL-1 β	241.13 \pm 31.325	222.62 \pm 7.943	162.59 \pm 4.956**	79.70 \pm 8.980**
TNF- α	361.51 \pm 12.424	331.76 \pm 19.224	310.19 \pm 11.895*	292.94 \pm 30.677*
IFN- γ	460.04 \pm 13.647	456.16 \pm 0.172	392.45 \pm 7.799*	366.97 \pm 28.799*
IL-2	209.52 \pm 4.099	231.95 \pm 17.050	271.81 \pm 4.016*	277.05 \pm 20.418**
IL-6	1087.53 \pm 11.601	1244.64 \pm 98.899*	1271.78 \pm 83.336*	1321.60 \pm 73.163*

Compared with the control group, the values with asterisk significantly difference (* or $p < 0.05$), extreme significantly difference (** or $p < 0.01$), and no significantly ($p > 0.05$). Data is expressed as mean \pm SD. Same as blow.

Table 3
Effect of excessive iron on the kidney NF- κ B and TGF- β 1 protein contents in sheep (Gray value ratio)

Item	CON	L-iron	M-iron	H-iron
NF- κ B	0.86 \pm 0.032	1.19 \pm 0.027**	1.42 \pm 0.021**	1.56 \pm 0.045**
TGF- β 1	0.94 \pm 0.015	0.96 \pm 0.006	1.02 \pm 0.006*	1.10 \pm 0.036**

Changes of Inflammatory Factor mRNA Expression in Kidney of Excessive Iron-Fed Sheep

As shown in Table 4, the expression of IL-1 β , TNF- α , and IFN- γ mRNAs, which are related inflammatory factors, decreased in sheep kidneys as iron addition increased. The expression of IL-1 β and IFN- γ was significantly lower in each excessive iron group than in the control group ($p < 0.01$), TNF- α was significantly lower in the M-iron and H-iron groups ($p < 0.01$), IL-1 β , TNF- α , and IFN- γ were significantly or extreme significantly lower in the H-iron group than in the L-iron and M-iron groups ($p < 0.05$ or $p < 0.01$) (Fig. 5).

With the addition of iron, the expression of IL-2, IL-6, NF- κ B, and TGF- β 1 mRNA increased. IL-2, IL-6, NF- κ B, and TGF- β 1 were significantly or extreme significantly higher in each excess iron group than in the control group ($p < 0.05$ or $p < 0.01$). Except for the IL-2 M-iron and H-iron groups, which were not significantly different, the H-iron group was significantly or extreme significantly higher than the L-iron and M-iron groups ($p < 0.05$ or $p < 0.01$) (Fig. 5).

Table 4
Effect of excessive iron on the kidney IL-1 β , TNF- α , IFN- γ , IL-2, IL-6, NF- κ B, and TGF- β 1 mRNA expression in sheep

Item	CON	L-iron	M-iron	H-iron
IL-1 β	1.00 \pm 0.000	0.84 \pm 0.017**	0.82 \pm 0.017**	0.40 \pm 0.004**
TNF- α	1.00 \pm 0.000	0.96 \pm 0.018	0.54 \pm 0.031**	0.15 \pm 0.027**
IFN- γ	1.00 \pm 0.000	0.48 \pm 0.006**	0.39 \pm 0.028**	0.28 \pm 0.020**
IL-2	1.00 \pm 0.000	4.36 \pm 0.434*	9.78 \pm 0.279**	12.86 \pm 0.575**
IL-6	1.00 \pm 0.000	3.238 \pm 0.464*	3.784 \pm 0.225*	5.50 \pm 0.166**
NF- κ B	1.00 \pm 0.000	3.85 \pm 0.336*	4.10 \pm 0.479**	7.05 \pm 0.154**
TGF- β 1	1.00 \pm 0.000	5.01 \pm 0.585**	8.63 \pm 0.143**	13.78 \pm 0.598**

Discussion

The body cannot produce iron spontaneously and has to come from external sources to maintain iron homeostasis [38]. The kidney is an important target organ for excess iron, and high amounts of iron in the renal tubules can cause acute kidney injury [38, 39]. As a foundation for illness prevention and control, it is critical to investigate the detrimental effects of too much iron in the body. In this study, in vivo trials were carried out on sheep's kidney. H&E staining and Perls staining were used to observe histopathological alterations with iron-containing haematoxylin distribution, as well as masson staining for the observation of fibrosis. WB and ELISA were used to determine inflammatory factors protein expression levels, and qRT-PCR was used to confirm the results at the genetic level.

H&E pathology revealed that increased iron intake caused pathological changes in sheep kidneys such as glomerular envelope atrophy, inflammatory cell infiltration, and cytoplasmic vacuolisation. The glomeruli basal membranes were thickened in the L-iron group, and there was a small amount of inflammatory interstitial infiltration of the cells. The glomeruli in the M-iron group were solid as well as sclerotic and shrunken. The tubules in the H-iron group were vacuolated, granular, and degenerated, with interstitial fibrosis visible. This shows that excessive iron in the body creates pathological alterations in the kidney, affecting its physiological function. It also shows tubular epithelial cell lesions, glomerular and interstitial vascular congestion, turbid degeneration of the distal tubules and collector ducts, luminal constriction, and inflammatory cell infiltration [40], which are all symptoms of fibrosis. In previous studies, an iron chelator was utilized to reduce cellular damage induced by high iron in renal proximal tubular epithelial culture cells [41]. Furthermore, kidney iron levels have increased dramatically in an established model of kidney injury due to excess protein [42]. It is hypothesized that ferrous iron affects the kidney, and that limiting iron intake reduces ferrous iron accumulation in the kidney and reduces renal tubular interstitial damage, which is mostly caused by iron-related protein metabolism abnormalities. As a result, the damage to the kidney caused by excess iron in this experiment could be attributable to disruptions in the

metabolism of iron-associated proteins rather than being directly caused by iron, as further confirmed by the lack of significant iron-containing hemoglobin deposits visible on Perls-stained kidneys. Excessive iron also resulted in considerable collagen deposition and severe fibrosis symptoms in the kidney, which matched the findings of previous studies in the liver by Shendge AK's team [43]. The glomerular was wrinkled, tubular collagen fibers were exuded, and the lumen was narrowed, the fibrous necrosis of the walls of the small arteries of the entrance glomerulus in our experiment, confirming that excessive iron is a direct factor in tissue fibrosis.

Besides histopathological observation of excessive iron induced kidney fibrosis, we also verified the presence of reaction to inflammatory factors through the expression of inflammatory factors and changes in protein content in this study. Previous studies have shown that inflammatory factors such as interleukins (ILs) [44], IFN, and TNF [45] can mutually induce and enhance their effects, and that this mutual regulation mechanism is closely related to immunity and inflammation. TNF- α and IL-1 β can activate the NF- κ B pathway during inflammation [46], and activated NF- κ B can up-regulate IL-6 expression [47]. TGF- β 1 is a significant pro-fibrotic cytokine [48], therefore it could be a target for anti-fibrotic treatment. In these studies, *in vivo* experiments were used to assess the development of the inflammatory response in the sheep kidney to verify the extent of inflammatory factor disruption, the amounts of IL-1 β , TNF- α , IFN- γ , IL-2, and IL-6 proteins were assessed using the WB method in separate groups, and ELISA was used to determine changes in TGF-1 and NF- κ B protein levels. The inflammatory response was initiated by increasing the amount of iron in the diet, which resulted in a boost in IL-2, IL-6, TGF- β 1, and NF- κ B protein secretion as well as a decrease in IL-1 β , IFN- γ and TNF- α . Inflammatory factors are disturbed as a result of this process, worsening the inflammatory response and fibrosis symptoms. Changes in gene levels were evaluated using qRT-PCR to further validate this conclusion, and the results were consistent with the trend in protein levels.

In conclusion, excessive iron promotes an inflammatory response in a sheep's kidney, resulting in kidney fibrosis. The molecular network involved in this study reveals a novel picture of kidney inflammation in sheep that may be caused by excessive iron, revealing new information about the mechanisms and treatment of kidney fibrosis.

Declarations

Author Contribution Xue-li Gao and Fengjiao Sun conceived and designed the experiments. Fengjiao Sun wrote the main manuscript text. Yuzhi An and Liangyu Zhang finished all the figures. Chaonan Liu and Xiaoping Lv did all the tables of the result. Xue-li Gao responded for the whole manuscript checking. All authors reviewed the manuscript.

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Data Availability The data of this study will be made available on reasonable request.

Code Availability Not applicable.

Ethics Approval and Consent to Participate All the animal experiments involved in this paper have been approved by the Animal Research Ethics Committee of Northeast Agricultural University.

Conflict of Interest The authors declare no competing interests.

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Figures

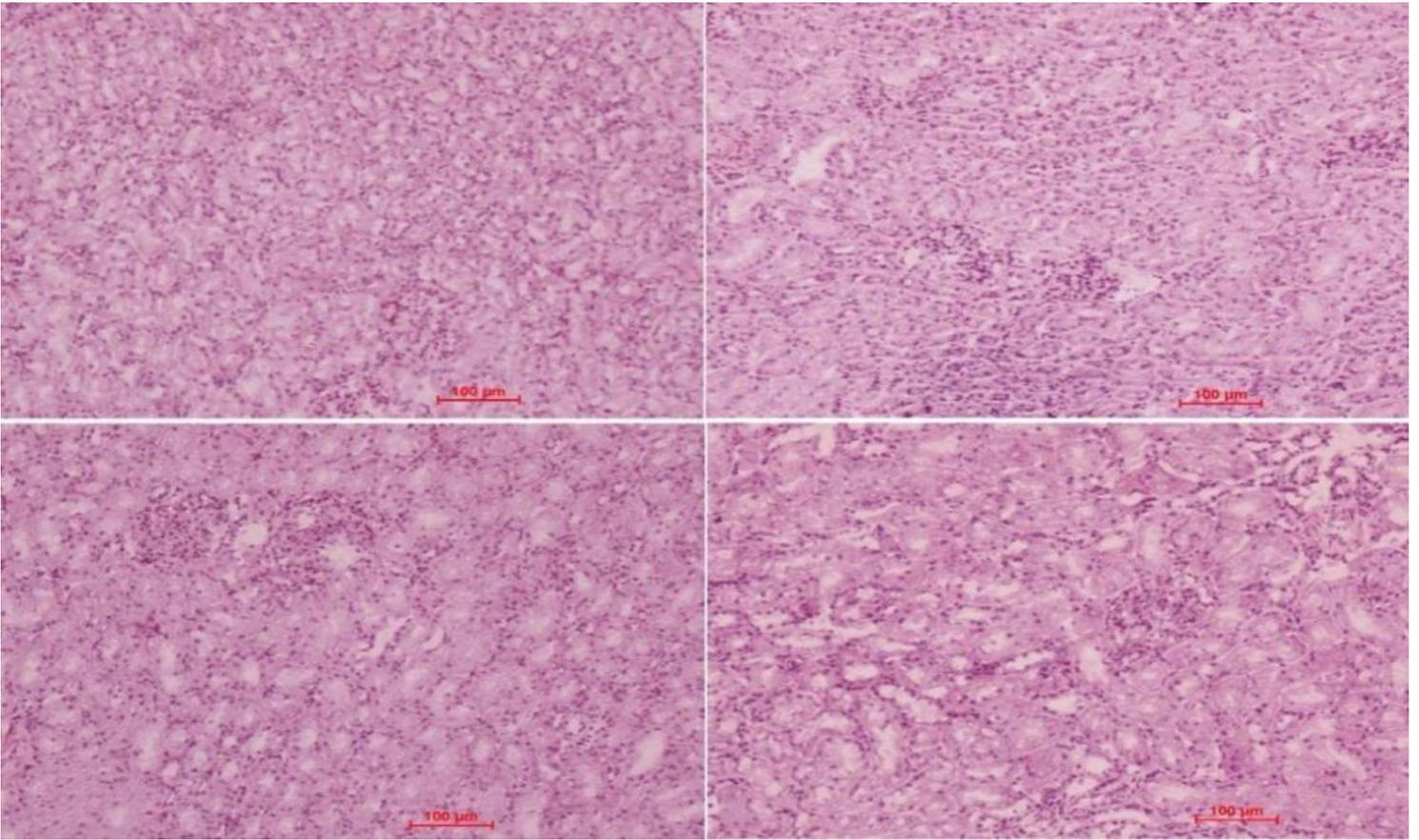


Figure 1

Pathological changes in the kidney of sheep after feeding excessive iron (H&E $\times 100$). **a** CON group. **b** L-iron group. **c** M-iron group. **d** H-iron group.

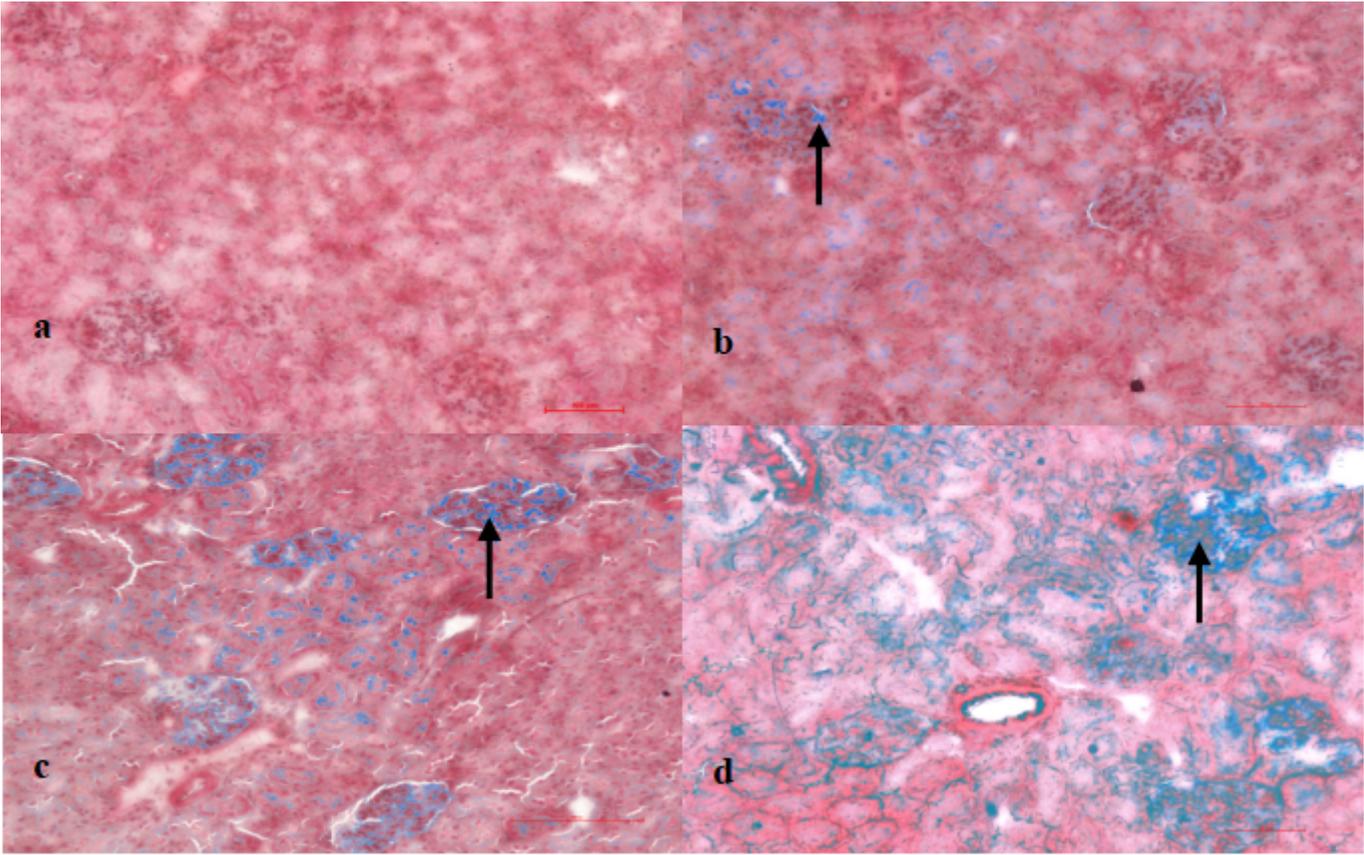


Figure 2

Fibrosis in the kidney of sheep after feeding excessive iron (Masson $\times 100$). **a** CON group. **b** L-iron group. **c** M-iron group. **d** H-iron group. '↑' showed collagen fiber exudation.

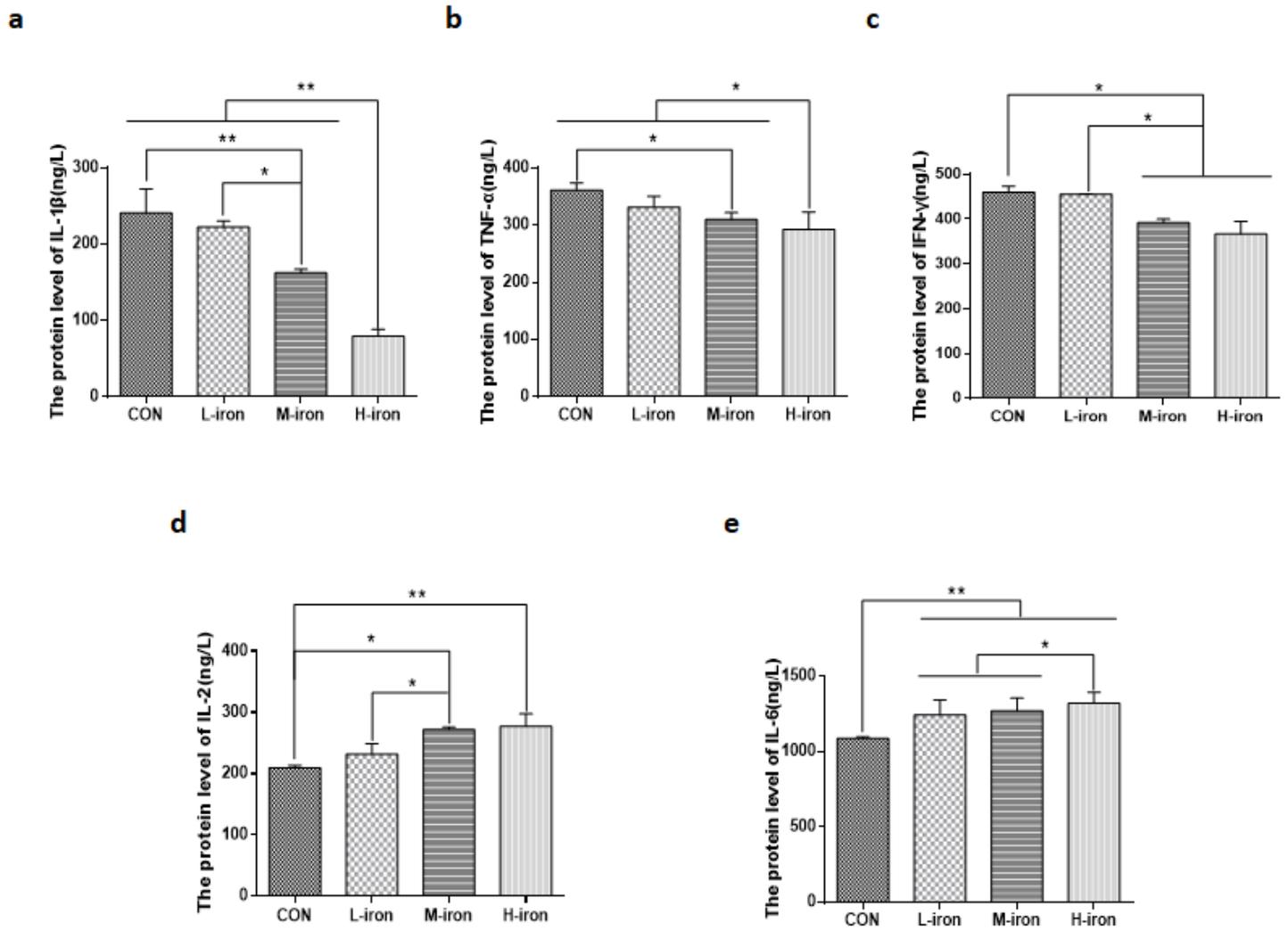


Figure 3

Kinetic changes in inflammatory factors protein expression in the kidney. **a** IL-1 β protein expression. **b** TNF- α protein expression. **c** IFN- γ protein expression. **d** IL-2 protein expression. **e** IL-6 protein expression. Data is expressed as mean \pm SD.

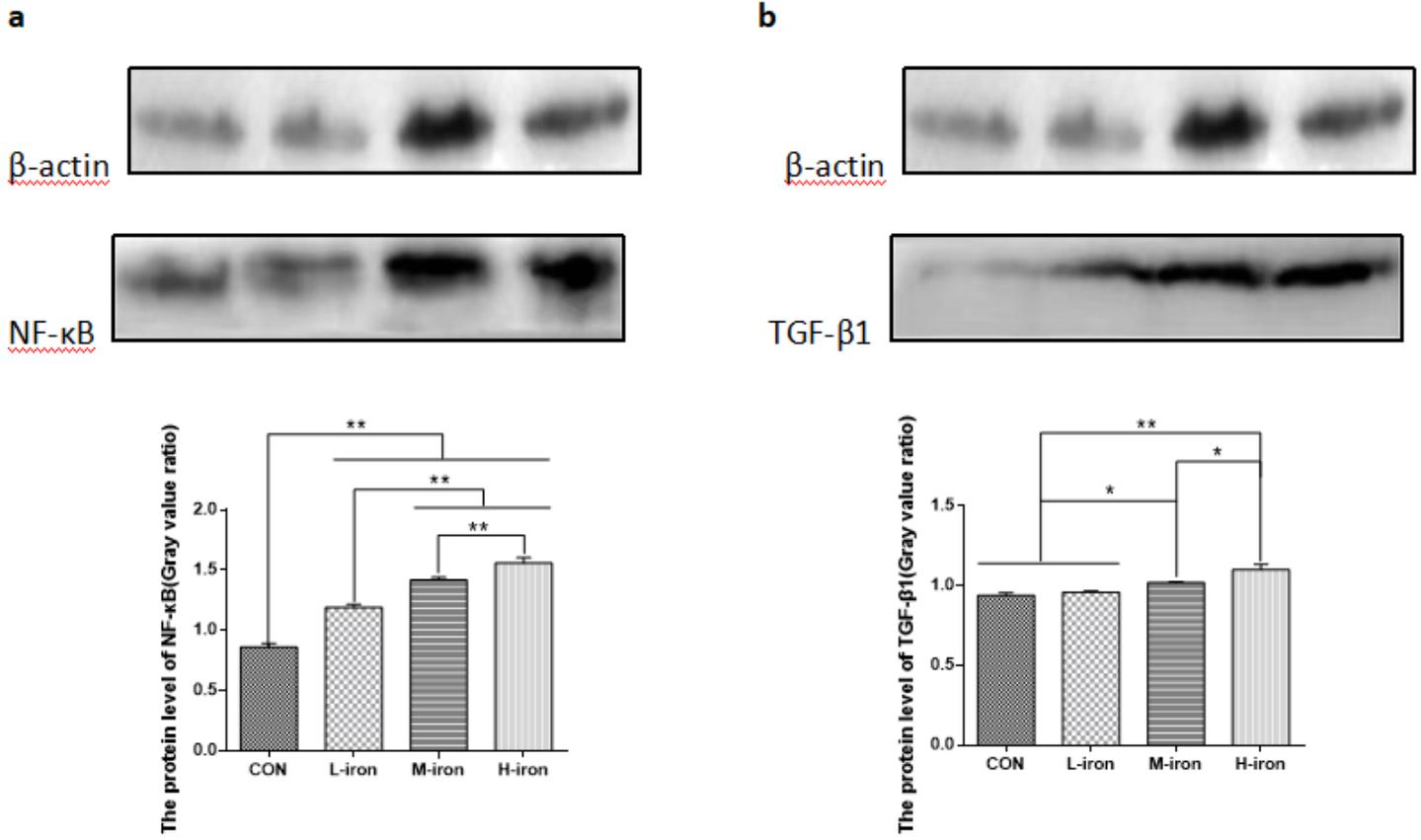


Figure 4

Kinetic changes of inflammatory factors protein expression (Gray value ratio). **a** NF- κ B protein expression. **b** TGF- β 1 protein expression. Data is expressed as mean \pm SD.

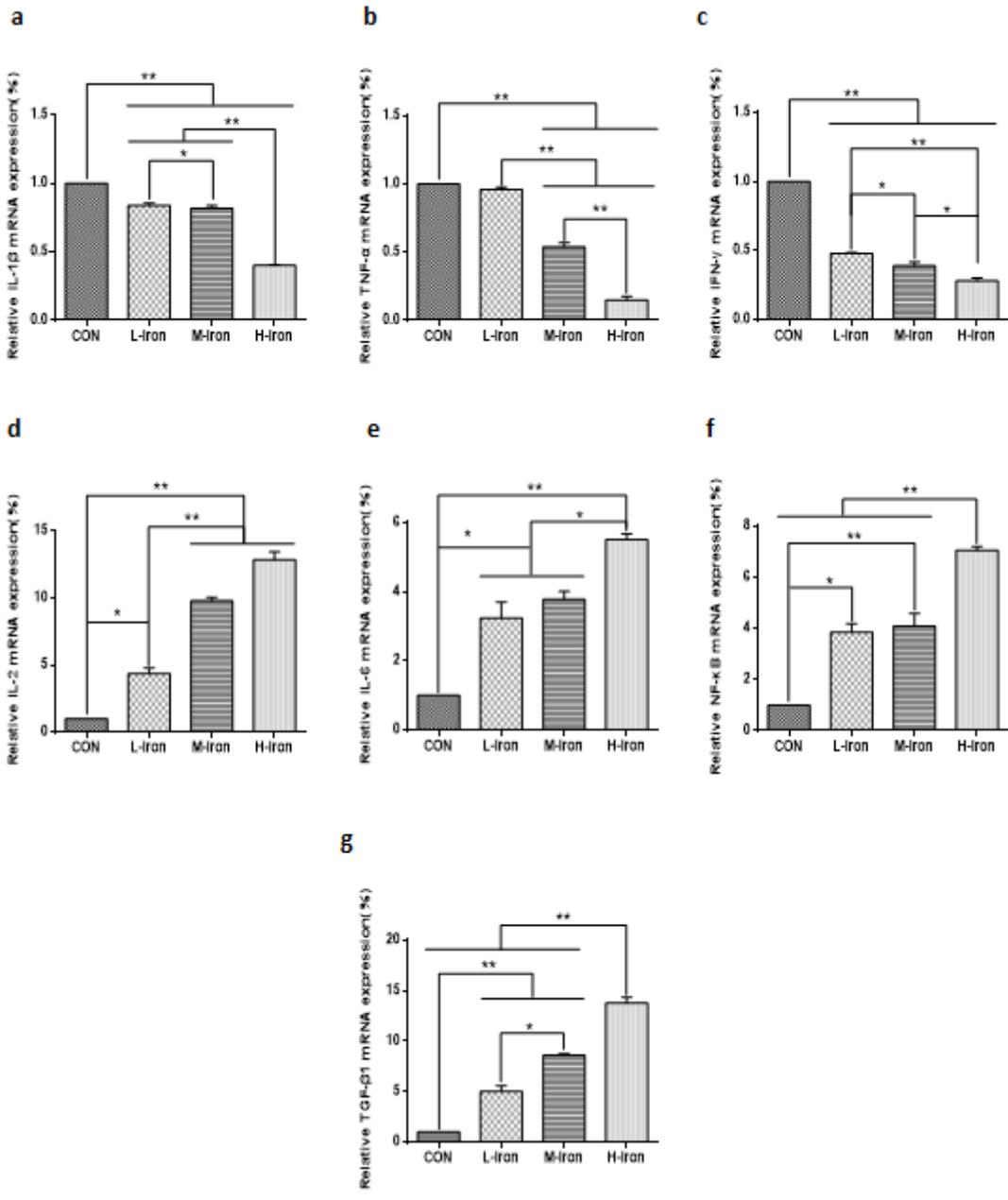


Figure 5

Kinetic changes in inflammatory factors mRNA expression. **a** IL-1 β mRNA expression in the kidney. **b** TNF- α mRNA expression in the kidney. **c** IFN- γ mRNA expression in the kidney. **d** IL-2 mRNA expression in the kidney. **e** IL-6 mRNA expression in the kidney. **f** NF- κ B mRNA expression in the kidney. **g** TGF- β 1 mRNA expression in the kidney. Data is expressed as mean \pm SD.