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Black Raisins Improved Experimentally Induced Iron Deficiency Anemia. Biochemical and Histological Evidence

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Iron deficiency anemia is a challenging clinical problem with a profound impact on general health. Administration of iron-containing preparations were reported to be associated with many side effects. The current study aimed to evaluate black raisins' therapeutic role against experimentally induced iron deficiency anemia in rats. Forty female rats were divided into 4 groups (n=10); control, anemia, anemia + iron, and anemia + raisins. Anemia was induced by bleeding and an iron-deficient diet for 4 weeks. Anemic rats were treated with ferric sulfate (200 mg/kg) or raisin extract (375 mg/kg) daily for 12 weeks. Complete blood count (CBC), blood films, body weight, splenic weight, and index were assessed. The spleens were processed and stained by hematoxylin and eosin (H & E) and immunohistochemically stained for CD3+ and CD68. Raisins contain a considerable amount of iron, vitamin B, phenolics, and flavonoid antioxidants. In raisins treated rats, CBC parameters displayed a significant increase compared to the anemic rats. Most of the RBCs in blood films showed normal shape, size, and central pallor. The spleen of raisins and iron-treated rats showed a marked increase in the area of white pulp. Their spleens also showed a significant increase in the CD3+ PALS area compared to anemic rats. Iron and raisins significantly decreased splenic CD68 macrophages. Consumption of black raisins could be considered an excellent natural source for flavonoids and iron to be used as an adjuvant supplement to iron for anemic patients with increased splenic T lymphocytes.

Keywords: Anemia; Raisins; macronutrients; flavonoids; spleen; histology.

1. INTRODUCTION

Anemia "is a condition in which there is a low level of hemoglobin in blood either because of diminished red blood cells and/or increase blood loss" [1]. Iron was previously thought to be essential for normal human physiology. It's required for a variety of proteins and enzymes that keep living human cells healthy. Iron is also a necessary component of proteins involved in hemoglobin's oxygen transport in red blood cells [2]. While iron is a plentiful mineral, it has low bioavailability, and dietary deficiency is among the most popular poor nutrition globally, with serious health consequences [3]. Iron deficiency anemia can strike at any age, but it is more common in pregnant women and children [4].

The total body iron content of the average adult male (70 kg) is approximately 4 g, which remains relatively constant during adult life. Iron homeostasis is based on the close connection between intestinal iron absorption and total body iron requirements since there are no significant physiologic mechanisms to control iron loss [5].

Anemia is the most apparent symptom of iron deficiency, and it has been shown to harm the immune system [6]. Iron is, however, a necessary component of molecules that trigger oxidative stress conditions in cells [7]. Toxicities associated with drug-induced oxidative stress can be found in a variety of tissues in the clinical setting. For the treatment of iron deficiency anemia, oral or parental iron supplements is the most common method [8]. On the other hand, overdosing on iron has been linked to increased oxidative stress, which can lead to tissue damage [9].

The "United States Department of Agriculture (USDA)" has listed seedless raisins as one of the key foods, implying that they are a major source of nutrients relevant to health issues in the US people [10,11]. Black or red raisins have 359.5 mg total phenols per 90 g serving, relative to 142.2 mg for green grapes and 198.5 mg for red grapes, both of which in 90 g servings [12,13]. Raisins are rich in metals like iron and copper; vitamins like vitamin B are essential for erythropoiesis [14].

There are few studies on the effect of dietary intervention in the treatment of iron deficiency anemia. The current research aims to assess black raisins' therapeutic role in reducing the adverse effects of experimentally induced iron deficiency anemia on blood profile, erythrocyte morphology, and histological changes in the spleen and its T lymphocyte population in adult female rats.

2. MATERIALS AND METHODS

2.1 Preparation of Raisins Extract

Raisins were brought from a hypermarket in Jeddah. A modified extraction of pigments was

used [15,16]. The collected samples were stored at a -80°C freezer. The materials were frozen in liquid nitrogen and blended for 30 seconds using a 250 mL stainless steel mini sample tube. The samples were then mixed with 50 mL acetone for 120 seconds. The acetone portion was decanted and filtered using a disposable sterile filter membrane (Corning incorporated) made in (USA) and a funnel/ 500 mL separator funnel. The raisin extract was divided into sterile Eppendorf tubes and stored until use. The chemical analysis of the extract was done in the Biochemical Unit, Assuit University, Assuit, Egypt.

2.2 Animals and Study Design

Forty healthy adult female rats (150 - 200 g) were obtained from King Fahd Medical Research Center (KFMRC), Jeddah, Saudi Arabia. The rats were kept for one week for acclimatization at a temperature of 25 ± 2oC. The experimental protocol was carried according to rules approved by the Committee for the Care of Animals, King Abdulaziz University, Jeddah, Saudi Arabia.

At day 0: Blood was withdrawn from all animals before starting the experiment, and a complete blood picture (CBC) including (red blood cells (RBCs) count and hemoglobin (HB) concentration, HCT, MCV, MCH, MCHC) was performed. Then rats were divided into 2 main groups:

- **Group I (Control) (n=10):**

The rats were maintained on the standard pellets and water ad libitum.

- **Group II (n=30) (Iron deficiency anemia):**

The iron and hemoglobin reached a designated low level (109–112 g/L HB and 3 mg body iron by a low iron diet (3 mg/kg) and bleeding twice a week (2.5 ml/ time) via ”orbital puncture while rats were under light diethyl ether anesthesia” [17,18]. After 4 weeks, the CBC and serum iron were evaluated to confirm the occurrence of anemia. The animals, which developed iron deficiency anemia according to their blood picture and serum iron were divided into the following subgroups:

- **Anemia group (n=10):**

This rat’s group was maintained on water orally by gavage using a special plastic nasogastric tube.

- **Anemia + Iron group (n=10):**

An oral therapeutic dose of 200 mg/kg of ferrous sulfate, Sigma, USA, was given (the dose was adjusted by converting the human dose to the rat dose according to Paget and Barnes conversion tables) [19].

- **Anemia + Raisins (n=10):**

The rats in this group were orally given the prepared extract of black raisins (375 mg/kg) [11].

2.3 Sample Collection and Assessment of Body Weight, Spleen Weight, and Spleen Index

The body weights were measured after 12 weeks of treatments. All rats were sacrificed under light ether anesthesia. Blood samples were withdrawn for complete blood picture (CBC), and other biochemical measurements. Each spleen was excised and weighed using a digital balance and the organ index was calculated according to the following formula:

Organ index = Organ weight (g)/ body weight (g) [20].

Parts of spleen were fixed in 10% buffered formalin for the histopathological and immunohistochemical analysis.

2.4 Assessment of CBC, Serum Iron, total Iron Binding Capacity (TIBC) and Transferrin Saturation (TS)

The different CBC parameters were measured by using automated coulter. Serum iron concentration and TIBC were measured using iron and TIBC kits. TS was calculated from the ratio of serum iron to TIBC.

2.5 Assessment of RBCs Appearance Under the Light Microscope

Blood smears were prepared from all groups stained by Leishman stain and examined by light microscope and photographed to compare size and width of central area.

2.6 Histopathological Analysis (hemato-xylin and eosin (H & E) stain)

After 12 weeks, all groups were sacrificed under light ether anesthesia. The dissected spleens

were fixed in 10% buffered formalin and processed by an automatic processor (LYNX II Automated Tissue Processor). The 4-µm-thickness paraffin sections were prepared and stained with H&E. The stained slides were then examined under the light microscope (Olympus BX51, Japan) for any pathological change and photographed by the Olympus DP20 digital camera.

2.7 Immunohistochemical Analysis

Some spleen sections were placed on charged slides to be stained immunohistochemically for CD3+ and CD68 markers for T lymphocytes and macrophages respectively. Immunostaining was performed using an avidin–biotin–peroxidase technique for detecting the splenic T lymphocytes using anti CD3 (rabbit monoclonal antibodies from Ventana). For the splenic macrophages anti CD68 (mouse monoclonal antibody from Ventana) was used at a dilution of 1:250 for 1h. Secondary antibody (Ventana) was applied to sections for 30 min. The DAB solution (Ventana) was used for 15 min as a chromogen. Finally, the slides were counterstained with Mayer's hematoxylin. Positive controls using the thymus gland (for T lymphocytes) and liver tissue (for macrophages) were processed according to the same protocol. Negative controls were processed according to the same protocol after omitting the step of the primary antibody. The examination of slides was done using the compound light microscope (Olympus BX51, Japan) and photographed by the Olympus DP20 digital camera.

2.8 Morphometric Assessment of Optical Density (Od) of Cd68 and Areas Percent of the Cd3+ Immunopositive Cells

The digital images were collected and analyzed using computer-based image analysis software (Image processing and analysis in Java (ImageJ) free software on net (http://imagej.nih.gov/ij/), was used to determine OD of CD68 positively stained cells in the white pulp's marginal zone and the areas percent of CD3+ positively stained cells in periarteriolar sheath (PALS).

2.9 Statistical Analysis

The recorded quantitative data were analyzed using SPSS version 16. The data was presented as mean ± standard deviation. The unpaired student t-test was used to compare between the different experimental groups. The level of significance was considered at $p < 0.05$.

3. RESULTS

3.1 Nutritional and Phytochemical Constituents of Raisins

The analysis reference of black raisins, which indicates that every 100 g of it contains carbohydrates constitute 83% of the total macronutrients, fibers comprise 7%, 5% moisture, 3% protein, and 2% monounsaturated fatty acids (MUFAs). The raisins contain no polyunsaturated fatty acids (PUFAs). Potassium is the major mineral constituent of raisins (85%), followed by phosphorus (8%), 5% calcium, 2% iron, and magnesium. Each 100 g raisins contain 89% of its vitamin content vitamin E, 5% vitamin B3, 3% vitamin C, 2% vitamin B2, and 1% vitamin B1. Phenolic compounds present in high amounts (98%) of raisins active constituents and only 1% flavanones and 1% anthocyanin (Fig. 1).

3.2 Effect of raisins on body weight, spleen weight, and spleen index determined in anemic rats

In comparison to control rats, anemia induction was associated with a substantial decrease in body weight and a significant increase in spleen weight and index. Treatment of anemic rats with iron and raisins significantly increased body weight and decreased spleen weight and index compared to anemic rats (Table 1).

3.3 Effect of raisins on RBC histological alteration

Blood smear examination from control rats showed normal appearance of rounded RBCs with narrow central pale area. In the anemic rats, erythrocyte sibling and increase the central pale area were observed confirming the presence of iron deficiency anemia. Rats treated with iron and raisins showed a nearly similar picture to those observed in control rats blood films (Fig. 2).

3.4 Effect of raisins on routine blood tests

In comparison to control rats, anemia induction was associated with a substantial decrease in all blood parameters, including RBCs count, HB

concentration, HCT, MCV, MCH, and MCHC. Treatment of anemic rats with iron and raisins results in a significant increase in all blood parameters, including RBCs count, HB concentration, HCT, MCH, and MCHC compared to the anemic rats (Table 2).

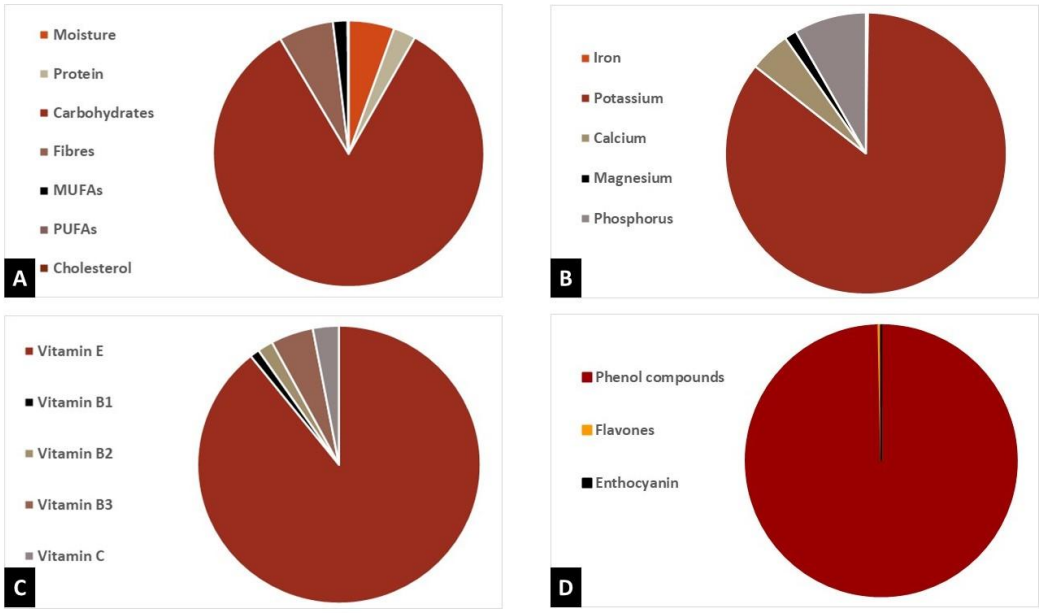

Fig. 1. Pie charts represent the nutritional and phytochemical constituents of raisins. A: Macronutrients; B: Minerals; C: Vitamins; D: Phytochemicals

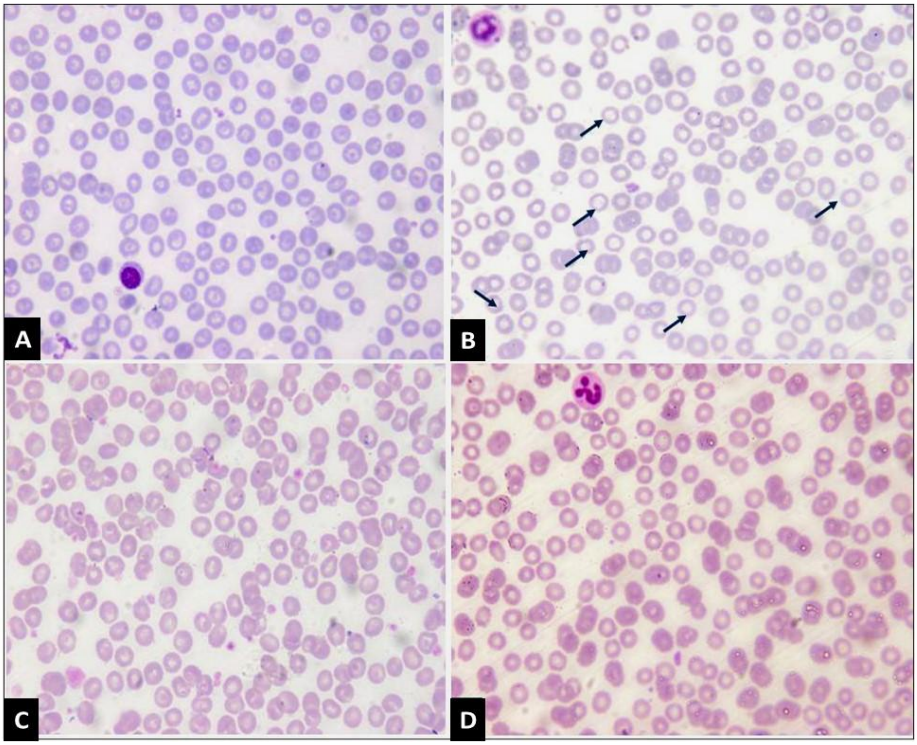

Fig. 2. Blood smears from rats (Leishman stain X1000) showing: A: Control; B: Anemia; C: Anemia + Iron; D: Anemia + Raisins

Table 1. Effect of raisins on body weight, spleen weight, and spleen index determined in anemic rats

	Control	Anemia	Anemia + Iron	Anemia + Raisins
Body weight (g)	200 ± 7.0	163 ± 7.1[a]	198 ± 33.7[b]	182 ± 13.15[b]
Spleen weight (g)	0.79 ± 0.17	1.40 ± 0.17[a]	1.03 ± 0.57[b]	0.61 ± 0.24[b]
Spleen index	0.39 ± 0.02	0.87 ± 0.02[a]	0.50 ± 0.02[b]	0.30 ± 0.02[b]

Data are presented as mean ± SD

[a] Significant difference compared to the control group ($p < 0.05$, unpaired student t-test, n=10);
[b] Significant difference compared to anemia group ($p < 0.05$, unpaired student t-test, n=10)

Table 2. Effect of raisins on routine blood tests determined in anemic rats

	Control	Anemia	Anemia + Iron	Anemia + Raisins
RBCs (10^6 cells/mm^3)	5.3 ± 0.58	3.6 ± 1.4[a]	6.1 ± 0.4[b]	6.6 ± 0.2[b]
Hemoglobin (g/dL)	15.8 ± 60.1	9.7 ± 3.0[a]	12.4 ± 1.4[b]	12.2 ± 1.8[b]
HCT (%)	33.1 ± 8.24	26.2 ± 1.2[a]	34.8 ± 1.0[b]	41.4 ± 3.1[b]
MCV (μm^3)	78.0 ± 2.3	60.2 ± 3.3[a]	60.5 ± 3.9	60.2 ± 4.2
MCH (pg/cell)	28.2 ± 2.0	20.1 ± 1.5[a]	25.0 ± 2.5[b]	22.8 ± 2.4[b]
MCHC (g/dL)	33.4 ± 2.4	23.9 ± 1.2[a]	33.0 ± 1.4[b]	30.4 ± 1.1[b]

Data are presented as mean ± SD

a Significant difference compared to the control group ($p < 0.05$, unpaired student t-test, n=10);
b Significant difference compared to anemia group ($p < 0.05$, unpaired student t-test, n=10)

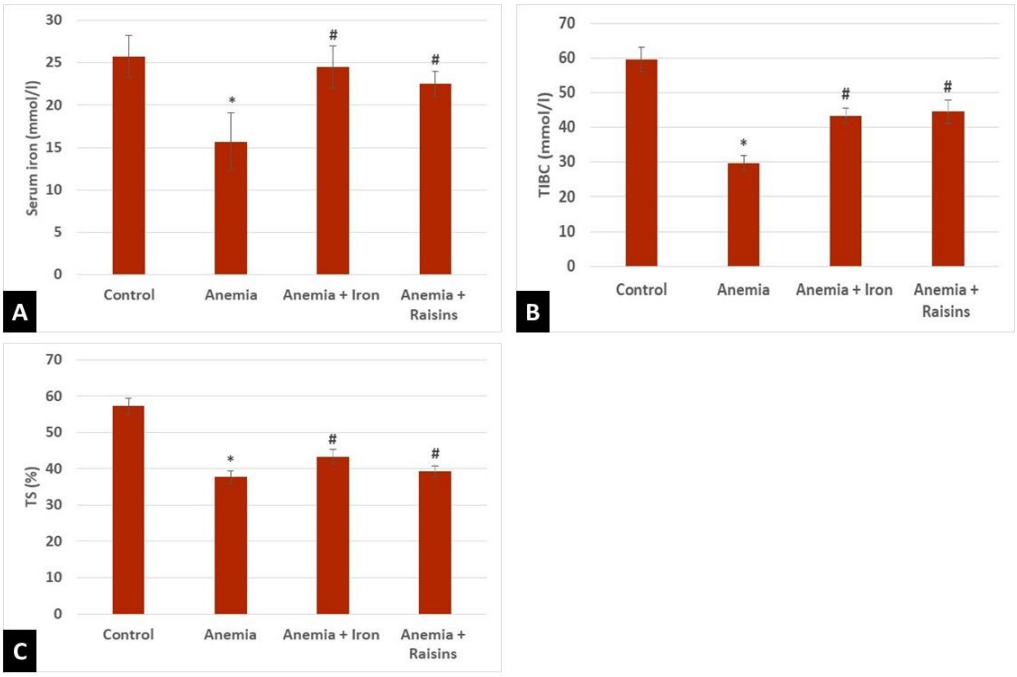

Fig. 3. Bar charts represent the effect of raisins on A: Serum iron concentration; B: Total iron-binding capacity (TIBC); C: Transferrin saturation (TS) determined in anemic rats. Data are presented as mean ± SD

a Significant difference compared to the control group ($p < 0.05$, unpaired student t-test, n=10)
b Significant difference compared to anemia group ($p < 0.05$, unpaired student t-test, n=10)

3.5 Effect of raisins on the concentration of serum iron, TIBC, and TS

In the anemic rats, the recorded serum iron, TIBC, and TS were significantly decreased compared to the control level. Treatment of anemic rats with iron and raisin significantly increased iron, TIBC, and TS concentrations compared to the anemic rats (Fig. 3).

3.6 Effect of raisins on rat splenic histological alteration by H&E stain

Examining the H & E-stained control spleen sections showed the distinct lymphatic follicles (white pulp) embedded in a highly vascular matrix (red pulp). The white pulp was subdivided into the PALS around the central arteriole, follicular and marginal zones. The PALS could be identified as the region around the central arteriole. The red pulp had a network of splenic cords and several venous sinusoids. Sections from the anemic rats showed marked dilation and congestion of red pulp sinusoids with an apparent decrease in the white pulp. In iron and raisins treated rats, the congested sinusoids were markedly decreased with an evident increase in the area of white pulp in the iron treated rats more than the raisins treated rats (Fig. 4).

3.7 Effect of raisins on rat splenic CD68 macrophages immunoexpression

Examination of spleen CD68 immunoexpression sections showed few CD68 positive macrophages in the control rats. In the anemic rats' sections, there was an evident increase in the CD68 positive macrophages occupying mainly the marginal zone compared to the control rats. In rats treated with iron and raisins, CD68 macrophages were decreased compared to the anemic rats. Using image J analysis, there was a significant increase in optical density (OD) of immunostained CD68 macrophages in anemic rats compared with the control rats. On the other hand, there was a significant decrease in the OD of immunostained CD68 macrophages in iron and raisins treated rats compared to the anemic rats (Fig. 5).

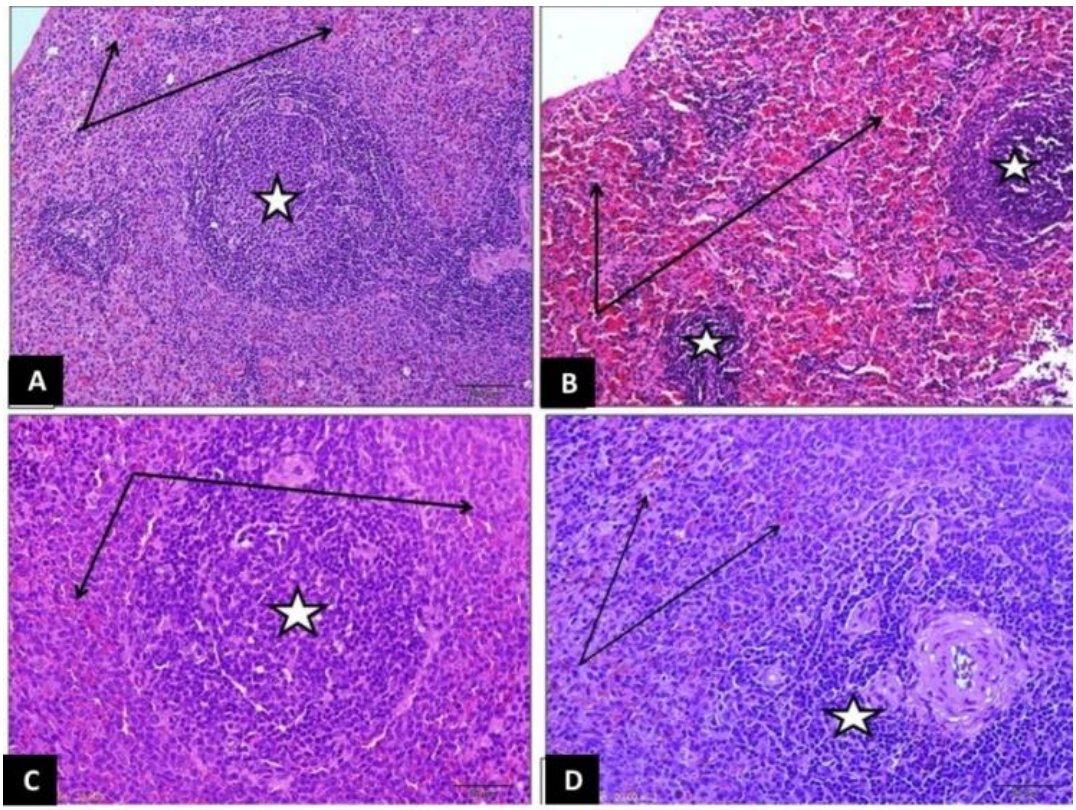

**Fig. 4. Photomicrographs of the H&E-stained spleen sections\
Photo A: control spleen showed the white pulp (stars) and the surrounded red pulp (arrows); Photo B: anemic rat spleen showed significant congestion of sinusoids (arrows) with an apparent decrease in the white pulp area (stars); Photo C: anemic rats treated with iron spleen showed a marked decrease in congested sinusoids (arrows); Photo D: anemic rats treated with raisins spleen showed a similar picture to the iron treated group with an apparent increase in the area of white pulp (star). Bar = 20 µm**

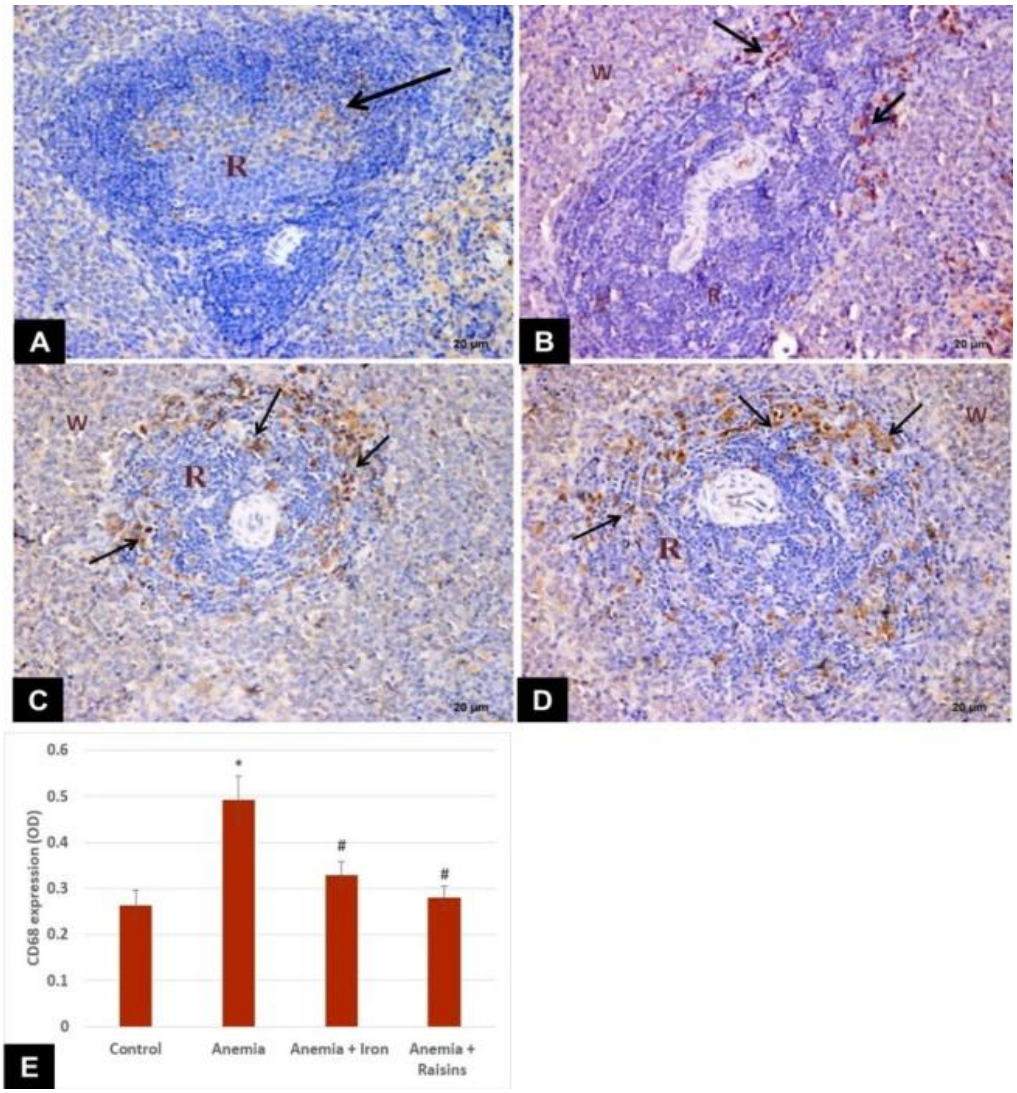

Fig. 5. Photomicrographs of macrophage (CD68) immunostaining of spleen sections Photo A: control spleen showed few CD68 positive macrophages; Photo B: anemic rat spleen showed a marked increase in the macrophages occupying the white pulp's marginal zone (arrows); Photo C and D: anemic rats treated with iron and raisins spleen showed similar macrophages less than the anemia photo; Photo E: A bar chart showed the mean gray value (OD) of CD68 expression, Bar = 20 µm

3.8 Effect of raisins on rat splenic CD3+ T lymphocyte immunoexpression

The examined sections of the spleen from control rats displayed an abundant population of CD3+ immunopositive T lymphocytes in the periarteriolar sheath region. On the other hand, anemic sections showed marked depletion of the CD3+ immunopositive T lymphocytes was evident around the central arteriole demarcating the PALS. In iron and raisins treated rats, the CD3+ immune positive T lymphocytes population increased to control rats (Fig. 6).

4. DISCUSSION

Anemia is the most common disease, especially iron deficiency, in vulnerable groups such as children and women [21,22]. As previously stated, iron deficiency anemia is the most common hematological problem, particularly among women during their reproductive years, and it is a global issue [23–25]. Many studies

have centered on the prevalence of anemia among Saudi females [26,27]. The present study aims to support an alternative natural nutritional source, namely raisins, to treat iron deficiency anemia induced in the rat model by repeated chronic bleeding. It was observed that causing iron deficiency anemia from a bled animal alone is difficult because the spleen compensates for blood loss in animals [28]. In the current research, iron deficiency anemia was produced in rats by frequent bleeding and feeding iron-deficiency diet. Rats developed anemia showing decreased body weight and most blood profile parameters such as RBCs count, HB, HCT, MCV, serum iron, TIBC, and TS. Previously, similar findings were observed in hamster and rat models [29,30]. When iron deficiency was corrected with raisin administration, body weight gain was observed. Another research showed a similar finding after the anemic status was corrected [31,32].

The detected histologically observed splenomegaly in the anemic rats could be explained by a rise in congested blood sinusoids of the red pulp, which was associated with a decrease in the size of the PALS white pulp. According to a previous study, anemia is associated with active erythropoiesis in the spleen of the pregnant mouse, which can cause spleen enlargement [33]. Expansion erythroid elements of red pulp were also described in the murine spleen in response to hemolytic anemia for maintenance of normal hematopoiesis in the adult and were attributed by the authors to the interaction of stem cell factor and its receptor c-kit [34,35].

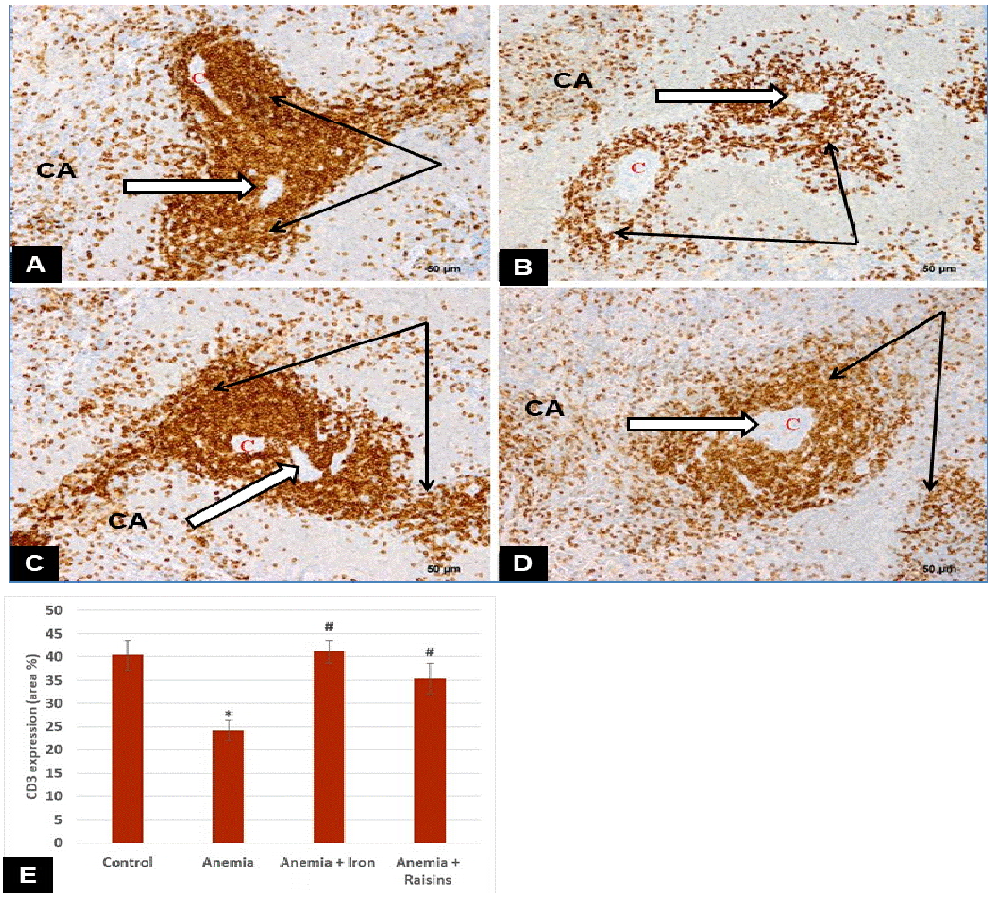

Fig. 6. Photomicrographs of T lymphocytes CD3+ immunostaining of spleen sections Photo A: control spleen showed an abundant population of the periarteriolar sheath with CD3+ T lymphocytes; Photo B: anemic rat spleen showed marked depletion in the CD3+ T lymphocytes of the periarterial lymphatic sheaths (PALS); Photo C and D: anemic rats treated with iron and raisins spleen showed a similar T lymphocytes population of the PALS, similar to the control photo; Photo E: A bar chart showed the area % of CD3 expression, Bar = 20 µm

T lymphocytes are known to congregate in the peri-arterial sheath, verified in this study by immunohistochemistry for CD3 positive cells [36]. In the current study, the decrease in CD3+ T lymphocyte population in anemic rats suggested the effect of iron deficiency anemia on the body's immune system [37]. Similar results were described in zinc deficiency and protein-energy malnutrition [38]. Anemia has been proposed to understand why the number of T cells in the body decreases when the body suffers from malnutrition [39,40]. A study on human children suffering from iron deficiency anemia demonstrated quantitatively altered T cell subsets [41].

A rise in splenic white pulp macrophages in anemic rats may be linked to reactive erythropoiesis. In anemia, macrophages function as sequestering cells, capturing iron and transporting it to the erythropoiesis site [42,43]. Regulation of iron metabolism was reported to be carried partially by macrophages [44,45]. Erythrophagocytic macrophages in the red pulp of the spleen were reported to have a role in providing iron originates from engulfed senescent RBCs to support heme biosynthesis in erythroblasts [46].

Iron therapy is the most well-documented treatment for long-term iron deficiency anemia [47,48]. Benefits and potential risk of iron therapy in different status of iron deficiency anemia was recorded [49,50]. Many aspects of oxidative stress and drug toxicity, including iron, were examined [51]. Iron is a known pro-oxidant that enhances the formation of hydroxyl radicals and inhibits nitric oxide signaling causing irreversible cell damage [52,53]. The oxidative stress results in the release of free radicles with subsequent tissue damage, as reported with iron therapy [54].

Raisins are the dried form of grape, and black grape, in particular, received interest from many researchers as a dietary supplement to promote healthy body status [14,55]. Raisins are high in bioactive phenolic compounds such as flavonoids and phenolic acids [56]. Flavonoids have been documented to protect cells from hemolysis [57]. The defensive effect of flavonoids against erythrocyte haemolysis was most likely due to their ability to neutralize free radicals [58]. According to various reports, human RBCs are a natural flavonoid reservoir [59].

Based on previous research, raisins supplementation improved both blood profile and histological changes in the splenic parenchyma in the current study. Thus, the improvement of anemic status in the present research upon black raisins administration could be due to providing essential nutrients, including iron, and most possibly due to supporting flavonoids content of RBCs. However, this suggestion needs further study to determine the role of this substance in enhancing red corpuscles production in hematopoietic tissues and increasing antioxidants in blood serum of anemic animal models. Similarly, the benefits of raisins supplement in enhancing HB level in iron deficiency anemia without the adverse effects seen with the administered iron formulations. It may also be used as a preventative measure to prevent iron deficiency in those who are at risk [60].

5. CONCLUSION

The current study showed the effectiveness of the intake of black raisins to treat iron deficiency anemia induced by chronic bleeding in a rat model with the protection of the spleen from its consequent complication. Black raisins as an antioxidant can be advised as adjuvant therapy to treat iron deficiency to avoid oxidative stress of iron supplementation and complication from giving large doses of iron supplementation therapy. Controlled clinical trials are indicated to prove raisins' efficacy for anemic patients.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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