Research Article

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Fermented goat milk consumption improves cardiovascular health during anemia recovery

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Abstract

BACKGROUND: Iron (Fe) plays a crucial role in several fundamental processes, including erythropoiesis, cellular metabolism, and in cardiovascular disease. The aim of this work was to contribute to a better understanding of the physiology of and recovery from Fe deficiency by studying how fermented milk consumption affects vascular biomarkers during Fe repletion.

RESULTS: The deleterious cardiovascular biomarkers cytokine-induced neutrophil chemoattractant 1, connective tissue growth factor (CTGF), interleukin-6, monocyte chemoattractant protein-1 (MCP-1), inhibitor of tissue plasminogen activator 1 total, metallopeptidase inhibitor 1 (TIMP-1), tumor necrosis factor alpha, vascular endothelial growth factor (VEGF), sE-selectin, and soluble intercellular adhesion molecule 1 (sICAM-1) decreased after fermented goat milk consumption in groups of fed animals either with normal Fe or Fe overload with respect to rats fed with fermented cow milk. The beneficial cardiovascular biomarkers caveolin-1 and adiponectin were higher in both control and anemic rats fed fermented goat milk either with normal Fe or Fe overload with respect to fermented cow milk. Anemia decreased TIMP-1 in rats fed fermented goat milk with Fe overload, whereas there was increased CTGF and MCP-1 in animals fed fermented cow milk with either normal or Fe overload. In addition, Fe overload increased VEGF.

CONCLUSION: Fermented goat milk consumption improves hematological status and promotes beneficial metabolic responses, which may attenuate cardiovascular risk factors during anemia recovery and iron overload to lessen the inflammatory response, macrophages activation and atherosclerosis development.

Keywords: fermented milks; ferropenic anemia; iron overload; cardiovascular cytokines

INTRODUCTION

Iron (Fe) plays a crucial role in several fundamental processes, including erythropoiesis and cellular metabolism. In this sense, Fe status has been implicated in cardiovascular disease (CD).[1] The detrimental effect of higher Fe status on cardiovascular risk, a reduced incidence of heart disease in premenopausal women, compared with men and postmenopausal women, has been attributed to lower levels of stored Fe.[2] Higher Fe stores have also been positively associated with risk factors for CD, such as type 2 diabetes mellitus.[3] In addition, Fe deficiency is one of the major risk factors for disability and mortality worldwide, and it was identified as a common and ominous comorbidity in patients with heart failure.[4] The heart is a major organ where Fe accumulates in situation of overload. Fe accumulation is associated with cardiomyopathy, which is a major cause of morbidity and mortality. The pathophysiology of Fe-overload cardiomyopathy is multifactorial, including oxidant-mediated injury, interference with cardiac electrical function, and promotion of vascular fibrosis. Fe deficiency also has adverse consequences on the heart and blood vessels, organs with high energy demands. Some epidemiologic studies in the general population have demonstrated an association between increased heme Fe intake, body Fe stores, and cardiovascular risk.[5] Current European Society of Cardiology guidelines for the diagnosis and management of acute and chronic heart failure recommend assessment of Fe parameters, with ensuing Fe therapy in cases of Fe deficiency.[6]

In general, the origin of Fe deficiency is related to reduced Fe intake, increased Fe losses, and/or the abnormal Fe distribution when it is functionally not available for the body.[7] Patients with CD demonstrate reduced Fe intake in their diet,[8] but its significance in the development of overt Fe deficiency seen in circulating biomarkers has not been studied yet. It has been established that the inflammatory state characterizing several chronic diseases (including CD) is considered to be responsible for impaired Fe absorption, recycling, and release from body stores.[9] It has been anticipated that the pathogenesis of Fe deficiency in the course of heart failure resembles the pathomechanisms (related directly with inflammatory status).[10] Moreover, in patients with CD,

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Jankowska *et al.*[11] demonstrated that extremely low hepcidin was circulating, which suggests that Fe deficiency seen in the course of CD is the consequence of depleted Fe stores in the body.

On the other hand, a substantial proportion of the CD can be prevented by a healthier lifestyle to manage cardiovascular risk factors. In this sense, although dairy products can be high in saturated fat, it is estimated that dairy products (excluding butter) contribute to 25–30% of the saturated fat intake of the European diet.[12] Paradoxically, it has been suggested that the consumption of dairy products can ameliorate characteristics of the metabolic syndrome, which has a key role on CD.[13] Our research group has previously reported that goat milk consumption has positive effects on bile composition and secretion, leading to a reduction in the transaminases and triglycerides and stimulating the excretion of cholesterol via bile, leading to a healthier cardiovascular lipid profile.[14] In addition, consumption of fermented goat milk is more beneficial in overcoming the effects of Fe deficiency, increasing key proteins of intestinal Fe metabolism, enhancing Fe digestive and metabolic utilization, increasing Fe deposits in target organs and favoring the recovery of hematological parameters.[15] Finally, it has also been reported that fermented goat milk consumption induced a protective effect in the tissues, increasing total antioxidant status (TAS) and decreasing the oxidative damage biomarkers, protecting main cell bioconstituents (lipids, protein, prostaglandins, and DNA) from evoked oxidative damage during anemia recovery,[16] and it is well known that oxidative stress is one of the major triggers for developing CD.[17] However, in spite of the role of Fe deficiency on CD, to date no studies have directly tested the effect of fermented milks on cardiovascular health during anemia recovery.

Taking into account all these considerations, the aim of this work was to contribute to a better understanding of the influence of Fe deficiency and recovery on the physiology of the cardiovascular system by studying how fermented milk consumption affects cardiovascular health during Fe repletion.

MATERIALS AND METHODS

Fermentation and dehydration of the milks

Fermented milks were prepared according to the method previously described by Moreno-Fernandez *et al.*[18] Both milk types were inoculated with traditional yoghurt starters *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (initial concentration 1×10^{11} colony-forming units (CFU) mL^{-1}; 10 mL L^{-1} inoculum) and incubated at 37 °C for approximately 24 h. Subsequently, fermented milk samples were subjected to a smooth industrial dehydration process, until the final moisture ranged between 2.5 and 4.5%.

Total nitrogen, dry matter, ash, total fat, lactose, and minerals contents

The nitrogen content was measured using the Kjeldahl method.[19] Dry matter, ash, total fat, and lactose were determined according to the AOAC method.[20] Mineral content in the fermented milks and diets was assessed by multielemental analysis by inductively coupled plasma optical emission spectrometer (ICP-OES). Samples were previously mineralized by a wet method in a sand bath (J.R. Selecta, Barcelona, Spain) using nitric acid followed by a mixture of 69% nitric acid–70% perchloric acid (v/v; Merck KGaA, Darmstadt, Germany; ratio 1 : 4, v/v) until the total elimination of organic matter. Calcium, phosphorus, Fe, zinc, copper, magnesium, sodium, and potassium analysis was undertaken using ICP-OES with an Optima 8300 instrument (PerkinElmer Inc. Waltham, MA, USA) with a segmented-array charge-coupled device high-performance detector.

Animals

Eighty male Wistar albino breed rats (21 days of age and weighing about 42 ± 5 g), purchased from the University of Granada Laboratory Animal Service (Granada, Spain), were used during the study. Animal assays were carried out in the breeding unit of the Centre of Biomedical Research of the University of Granada in an area certified as free of pathogens and the animals were kept in conditions of high biological safety, with sanitary and environmental rigorously controlled parameters. All animal care procedures and experimental protocols were approved by the Ethics Committee of the University of Granada (Ref. 11022011) in accordance with the European Community guidelines (Declaration of Helsinki; Directive 2010/63/EU for animal experiments).

During the course of the study, the animals were housed in individual, ventilated, thermoregulated cages with an automatically controlled temperature (22–23 °C), humidity (55–65%), and a 12 h light–dark cycle (09:00 to 21:00). Diet intake was controlled, pair feeding all the animals (80% of the average intake), and bidistilled water was available *ad libitum*.

Experimental design

The experimental design of the study is illustrated in Fig. 1. *Pre-experimental period (PEP)*. Eighty rats were divided into two groups; the control group received the AIN-93G diet (44.72 ± 0.98 mg kg^{-1} by analysis) and the anemic group received a low-Fe diet (5.91 ± 0.36 mg kg^{-1} by analysis) for 40 days, to induce anemia experimentally by a method developed previously by our research group.[21] Blood samples (approximately 400 µL) from the caudal vein in tubes with ethylenediaminetetraacetic acid as anticoagulant were collected for hematological control of the anemia.

Experimental period (EP). After the induction of the anemia (day 40 of the study), the control ($n = 40$) and anemic groups ($n = 40$) were further fed for 30 days with fermented cow milk or a fermented goat-milk-based diet, with normal Fe content (45 mg kg^{-1}) or Fe overloaded (450 mg kg^{-1}) to induce chronic Fe overload,[22] prepared with fermented cow (Holstein breed) or fermented goat milk (Murciano-granadina breed) powder (20% protein and 10% fat) (Table 1). The Fe content in the diets by analysis was as follows. The normal-Fe diets were 43.98 ± 0.39 mg kg^{-1} (fermented cow milk-based diet) and 44.28 ± 0.76 mg kg^{-1} (fermented goat milk-based diet); the Fe-overload diets were 469.82 ± 2.25 mg kg^{-1} (fermented cow milk-based diet) and 470.81 ± 2.35 mg kg^{-1} (fermented goat milk-based diet).

At the end of the EP, animals were anesthetized intraperitoneally with sodium pentobarbital (Sigma Diagnostics, St Louis, MO, USA) and totally bled out by cannulation of the abdominal aorta; blood aliquots with ethylenediaminetetraacetic acid were analyzed to measure the hematological parameters, and the rest of the blood was centrifuged ($1500 \times g$, 4 °C, 15 min) to measure plasma cardiovascular cytokines. The remaining blood was centrifuged without anticoagulant to separate the red blood cells from the serum and for subsequent analysis of Fe, total Fe binding capacity (TIBC), transferrin saturation, ferritin, and hepcidin.

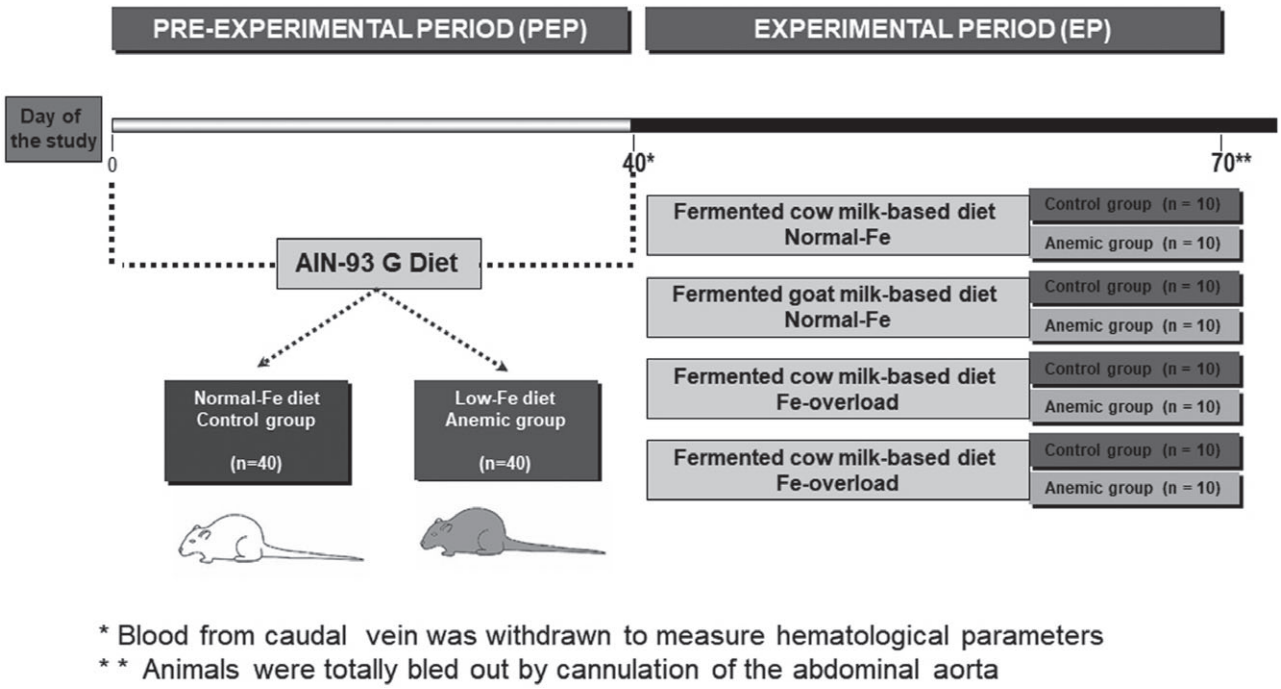

Figure 1. The study experimental design.

Table 1. Composition of the experimental diets

Component	Diet amount (g kg^{-1})
Pre-experimental period	
Standard (non-milk) diet[a]	
Casein	200
Lactose	0
Fat (virgin olive oil)	100
Wheat starch	500
Constant ingredients[b]	200
Experimental period	
Fermented-cow-milk-based diet[c]	
Protein	205
Lactose	295
Fat	100
Wheat starch	200
Constant ingredients[b]	200
Fermented-goat-milk-based diet[c]	
Protein	206
Lactose	291
Fat	100
Wheat starch	203
Constant ingredients[b]	200

[a] The diets were prepared according to the recommendations of the AIN-93G for control rats (45 mg Fe per kilogram of diet) (Reeves *et al.*, 1993) or with low Fe content (5 mg Fe per kilogram of diet) (Pallarés *et al.*[21]) for anemic groups.
[b] The constant ingredients (all per kilogram of diet) consisted of 50 g fiber (micronized cellulose), 100 g sucrose, 2.5 g choline chloride, 2.5 g L-cystine, 35 g mineral premix, and 10 g vitamin premix.
[c] Specific vitamin and mineral premixes supplements for fermented-goat-milk- and cow-milk-based diets were formulated taking into account the mineral and vitamin contents of the fermented milk powder supplied in order to meet the recommendations of the AIN-93G for normal-Fe diets (45 mg Fe per kilogram of diet) (Reeves *et al.*, 1993)[53] or Fe overload (450 mg Fe per kilogram of diet) (Raja *et al.*, 1993)[22].

Hematological test

All the hematological parameters studied were measured using an automated Sysmex K-1000D (Sysmex, Tokyo, Japan) hematology analyzer.

Serum Fe, TIBC, and transferrin saturation

Serum Fe concentration and TIBC were determined to calculate the rate of transferrin saturation, using Sigma Diagnostics Iron and TIBC reagents (Sigma Diagnostics). The absorbance of samples was read at 550 nm on a microplate reader (Bio-Rad Laboratories Inc., Hercules, CA, USA). The percentage of transferrin saturation was calculated using

$$\text{Transferrin saturation } (\%) = \frac{\text{Serum Fe concentration } \left(\mu g\ L^{-1}\right)}{\text{TIBC } \left(\mu g\ L^{-1}\right)} \times 100$$

Serum ferritin

Serum ferritin concentration was determined using the Rat Ferritin ELISA Kit (Biovendor GmbH, Heidelberg, Germany). The absorbance of the reaction was read at 450 nm using a microplate reader (BioTek Instruments Inc., Winooski, VT, USA). The color intensity developed was inversely proportional to the concentration of serum ferritin.

Serum hepcidin

Hepcidin-25 concentration was determined using a DRG ELISA Kit (DRG Instruments GmbH, Marburg, Germany). The microtiter wells were coated with a monoclonal (mouse) antibody directed toward an antigenic site of the hepcidin-25 molecule. Endogenous hepcidin-25 of a sample competed with a hepcidin-25–biotin conjugate for binding to the coated antibody. After incubation, the unbound conjugate was washed off and a streptavidin–peroxidase enzyme complex was added to each

well. After incubation, unbound enzyme complex was washed off and substrate solution was added. The blue color development was stopped after a short incubation time, turning the color from blue to yellow. The microplate was read at 450 nm on a microplate reader (Bio-Rad Laboratories Inc.) and the intensity of color developed was inversely proportional to the concentration of hepcidin in the sample.

Cardiovascular health parameters

Caveolin-1 (CAV-1), cytokine-induced neutrophil chemoattractant 1 (CINC-1/GRO/KC), connective tissue growth factor (CTGF), interleukin (IL)-6, monocyte chemoattractant protein-1 (MCP-1), inhibitor of tissue plasminogen activator 1 total (tPAI-1), metallopeptidase inhibitor 1 (TIMP-1), tumor necrosis factor alpha (TNF-α), and vascular endothelial growth factor (VEGF) were determined using the RV1MAG-26K MILLIPLEX MAP Rat Vascular Injury Magnetic Bead Panel 1 (Millipore Corporation, St. Louis, MO, USA). Adiponectin, sE-selectin, and soluble adhesion molecule 1 (sICAM-1) were determined using the RV2MAG-26K MILLIPLEX MAP Rat Vascular Injury Magnetic Bead Panel 2 (Millipore). These are based on immunoassays on the surface of fluorescent-coded beads (microspheres), following the specifications of the manufacturer (50 events per bead, 50 µL sample, gate settings 8000–15 000, time out 60 s). The plate was read on a LABScan 100 analyzer (Luminex Corporation, Austin, TX, USA) with xPONENT software for data acquisition. Average values for each set of duplicate samples or standards were within 15% of the mean. Standard curve: CAV-1, 1.4–1000 ng mL^{-1}; CINC-1/GRO/KC, IL-6, MCP-1, PAI-1 (total), 0.1–100 ng mL^{-1}; CTGF, 0.3–200 ng mL^{-1}; TIMP-1, 0.2–150 ng mL^{-1}; TNF-α, 0.05–20 ng mL^{-1}; VEGF, 0.1–15 ng mL^{-1}; adiponectin, 0.3–250 ng mL^{-1}, sE-selectin, 0.1–50 ng mL^{-1}; and sICAM-1, 0.1–40 ng mL^{-1}. Analyte concentrations in plasma samples were determined by comparing the mean of duplicate samples with the standard curve for each assay.

Statistical analysis

Statistical analyses were performed using the SPSS computer program (version 24.0, 2016, SPSS Inc., Chicago, IL, USA). Differences between groups fed normal-Fe- or low-Fe-content diets during the PEP were tested for statistical significance with Student's *t* test. Individual means were tested by pairwise comparison with Tukey's multiple comparison test when main effects and interactions were significant. Data were analyzed by two-way analysis of variance (ANOVA) to determine the effects of type of diet, anemia, and Fe content in the diet. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

After Fe deprivation (5 mg kg^{-1} diet) for 40 days (PEP), all the hematological parameters in the anemic group were lower than those of the counterpart controls ($P < 0.001$) except for white blood cells, which remained unchanged after severe Fe deprivation (Table 2).

After 30 days feeding the normal-Fe or Fe-overload fermented milk-based diets (EP), the hematological parameters were fully recovered with both milk-based diets. Serum hepcidin was lower in control and anemic animals fed fermented goat milk either with normal Fe or Fe overload in comparison with fermented goat milk ($P < 0.001$). As expected, serum Fe was higher in the Fe-overload groups ($P < 0.01$). Fe overload also increased hemoglobin ($P < 0.001$), serum ferritin ($P < 0.01$), transferrin saturation ($P < 0.01$), and TIBC ($P < 0.01$) (Table 3).

Table 2. Hematological parameters of control and anemic rats (PEP)

	Normal Fe Control group ($n = 40$)	Low Fe Anemic group ($n = 40$)
Total blood		
Hb concentration (g L^{-1})	132.95 ± 2.91	61.35 ± 2.86*
RBCs (10^{12} L^{-1})	7.21 ± 0.21	3.10 ± 0.22*
Hematocrit (%)	40.21 ± 1.09	13.21 ± 1.29*
MCV (fL)	55.49 ± 0.51	36.82 ± 0.36*
MCH (pg)	19.45 ± 0.14	14.15 ± 0.64*
MCHC (g dL^{-1})	35.57 ± 0.35	30.18 ± 0.76*
RDW (%)	16.25 ± 0.34	19.22 ± 0.36*
Platelets (10^{9} L^{-1})	729 ± 70.22	2119 ± 116*
WBCs (10^{9} L^{-1})	8.89 ± 0.38	8.56 ± 0.76
Lymphocytes (10^{6} mL^{-1})	7.87 ± 0.56	5.74 ± 0.81*
Serum		
Fe (µg L^{-1})	1334 ± 98.76	605 ± 56.03*
TIBC (µg L^{-1})	2674 ± 185	17 876 ± 587*
Transferrin saturation (%)	49.11 ± 5.86	3.95 ± 0.43*
Ferritin (µg L^{-1})	79.82 ± 2.11	49.23 ± 1.63*
Hepcidin (ng mL^{-1})	13.70 ± 0.36	15.54 ± 0.72*

Data are shown as the mean values plus/minus standard error of the mean.
*Significantly different from the control group ($P < 0.001$, Student's *t* test).
PEP, pre-experimental period; Hb, hemoglobin; RBCs, red blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular Hb; MCHC, mean corpuscular Hb concentration; RDW, red cell distribution width; WBCs, white blood cells; TIBC, total Fe-binding capacity.

In this study, a decrease in serum hepcidin was recorded in animals consuming fermented goat milk, and our research group have also previously reported[15] that hepcidin duodenal expression was found to be decreased in rats consuming fermented goat milk in comparison with rats consuming cow milk. This downregulation of hepcidin would increase Fe efflux from the duodenal cells, because this correlates inversely with the activity of ferroportin and Fe absorption, improving hematological parameters and promoting anemia recovery.

Fe plays a critical role in systemic oxygen delivery and utilization and contributes to erythropoiesis, and therefore Fe deficiency decreases the oxygen-carrying capacity of the red blood cells.[23] Fe is also an obligate component of enzymes involved in cellular respiration, oxidative phosphorylation, vascular homeostasis, nitric oxide generation, and the citric acid cycle.[24] Hence, cells with high energy demands, including skeletal, vascular, and cardiac myocytes, are particularly sensitive to depleted Fe stores.[25] Cardiac Fe-deficiency is present in patients with heart failure and is associated with impaired mitochondrial function,[26] abnormal sarcomere structure, and left ventricular systolic dysfunction;[23] however, in our study, after supplying the fermented milk diets, the hematological parameters recovered, revealing that Fe deficiency was recovered and therefore most of the parameters related to CD were unaffected at the end of the EP. However, CTGF and MCP-1 were still increased in anemic animals fed fermented cow milk either with normal-Fe or Fe-overload, and VEGF was also higher in anemic animals fed fermented goat or cow milk with Fe-overload, revealing that, although hematological status

Table 3. Hematological parameters from control and anemic rats fed for 30 days with fermented cow- or goat-milk-based diets with normal Fe content or Fe overload ($n = 10$ animals per group)

		Fermented cow milk diet		Fermented goat milk diet		Two-way ANOVA		
	Fe content	Control group	Anemic group	Control group	Anemic group	Diet	Anemia	Fe content
Hb concentration ($g\ L^{-1}$)	Normal	128.97 ± 2.87	129.31 ± 2.55	132.01 ± 2.75	129.22 ± 2.47	NS	NS	<0.001
	Overload	142.55 ± 2.62^{a}	141.15 ± 2.91^{a}	141.33 ± 2.99^{a}	146.98 ± 3.01^{ab}	<0.05	NS	
RBCs ($10^{12}\ L^{-1}$)	Normal	7.07 ± 0.17	7.08 ± 0.21	7.40 ± 0.20	7.22 ± 0.21	NS	NS	<0.05
	Overload	6.96 ± 0.18	7.18 ± 0.23	8.03 ± 0.29^{ac}	7.12 ± 0.21	<0.01	NS	
Haematocrit (%)	Normal	40.07 ± 1.18	39.01 ± 0.98	41.96 ± 1.22^{cc}	42.96 ± 0.99^{bb}	<0.01	NS	<0.01
	Overload	39.43 ± 1.32	44.88 ± 2.75^{bb}	44.84 ± 1.27^{acc}	45.47 ± 1.36^{a}	<0.05	NS	
MCV (fL)	Normal	57.55 ± 0.53	55.35 ± 0.58	57.33 ± 0.56	55.11 ± 0.53	NS	NS	NS
	Overload	56.81 ± 0.59	53.21 ± 0.56	56.45 ± 0.53	56.18 ± 0.52^{bb}	<0.05	NS	
Platelets ($10^{9}\ L^{-1}$)	Normal	932.89 ± 70.29	962.78 ± 66.56	926.15 ± 79.58	935.29 ± 67.01	NS	NS	NS
	Overload	940.01 ± 70.85	965.43 ± 72.17	934.03 ± 81.29	946.01 ± 70.34	NS	NS	
Serum Fe ($\mu g\ L^{-1}$)	Normal	1344 ± 85.91	1352 ± 86.21	1353 ± 87.76	1325 ± 93.49	NS	NS	<0.01
	Overload	1589 ± 99.03^{a}	1588 ± 99.78^{a}	1555 ± 100^{a}	1577 ± 96.93^{a}	NS	NS	
TIBC ($\mu g\ L^{-1}$)	Normal	2785 ± 156	2797 ± 136	2784 ± 144	2791 ± 159	NS	NS	<0.01
	Overload	3146 ± 178^{a}	3253 ± 174^{a}	3252 ± 171^{a}	3194 ± 167^{a}	NS	NS	
Transferrin saturation (%)	Normal	46.01 ± 0.89	45.47 ± 0.90	46.66 ± 0.75	46.38 ± 0.95	NS	NS	<0.01
	Overload	47.77 ± 1.31^{a}	47.85 ± 1.08^{a}	49.61 ± 0.97^{a}	48.92 ± 1.02^{a}	NS	NS	
Serum ferritin ($\mu g\ L^{-1}$)	Normal	83.27 ± 1.73	82.85 ± 1.66	84.29 ± 1.73	82.35 ± 1.79	NS	NS	<0.01
	Overload	87.68 ± 1.82^{a}	86.85 ± 1.89^{a}	87.83 ± 1.91^{a}	86.67 ± 1.95^{a}	NS	NS	
Serum hepcidin ($ng\ mL^{-1}$)	Normal	16.33 ± 0.59	16.41 ± 0.50	14.26 ± 0.55^{cc}	14.25 ± 0.57^{bb}	<0.01	NS	NS
	Overload	17.73 ± 0.61^{a}	16.88 ± 0.59	15.10 ± 0.61^{cc}	14.39 ± 0.62^{bb}	<0.01	NS	

NS, not significant; Hb, hemoglobin; RBCs, red blood cells; MCV, mean corpuscular volume; TIBC, total Fe-binding capacity.
[a] Mean values from the corresponding group fed with normal-Fe content were significantly different ($P < 0.05$, Student's t test).
[b] Mean values from anemic group fed with fermented cow milk diet were significantly different ($P < 0.05$, Tukey's test).
[c] Mean values from control group fed with fermented cow milk diet were significantly different ($P < 0.05$, Tukey's test).

was normal, Fe homeostasis and metabolism recovered better with fermented goat milk, as previously described.[15] Fermented goat milk upregulates enterocyte duodenal cytochrome b, divalent metal transporter 1, ferritin, and ferroportin 1 and downregulates transferrin receptor 1 and hepcidin antimicrobial peptide, improving Fe homeostasis and storage in erythropoietic and target organs and avoiding vascular damage due to Fe deficiency or overload.

A better understanding of the cardiovascular physiology and the link with Fe metabolism should facilitate new nutritional strategies to improve hematological status and cardiovascular health. In the current study, using a severe Fe deficiency model, several biomarkers related to cardiovascular health have been studied to demonstrate whether fermented goat milk consumption has a positive influence on vascular physiology and hematology. The baseline test was completed after a PEP during which severe Fe deficiency was achieved; after that, Fe replenishment under Fe-overload conditions was carried out. Therefore, the benefits observed with fermented goat milk do not merely represent an improvement in hematology, but also a decrease in several deleterious biomarkers and an increase in beneficial cytokines for the cardiovascular, indicating additional benefits of fermented goat milk in the course of Fe replenishment.

Table 4 shows that, after 30 days of feeding the milk-based diets (EP), fermented goat milk consumption in both groups of animals, either with normal Fe or Fe overload, decreased the deleterious cardiovascular biomarkers (CINC-1/GRO/KC, IL-6, MCP-1, tPAI, TIMP-1, VEGF, TNF-α, sE-selectin, and sICAM-1) ($P < 0.001$ for CINC-1/GRO/KC, tPAI, TIMP-1, sICAM-1; and $P < 0.01$ for IL-6, MCP-1, TNF-α, and VEGF).

Anemia decreased TIMP-1 in animals fed fermented goat milk with Fe overload ($P < 0.001$) and increased CGTF and MCP-1 in animals fed fermented cow milk either with normal Fe ($P < 0.05$) or Fe overload ($P < 0.01$ for CGTF and $P < 0.05$ for MCP-1), showing no effect on the other biomarkers.

Finally, Fe overload decreased TIMP-1 in anemic animals fed both fermented milks ($P < 0.05$), increased VEGF in all the groups fed both fermented milks ($P < 0.001$), increased CTGF in anemic animals fed fermented cow milk ($P < 0.001$), and increased sICAM-1 and adiponectin in control and anemic animals fed fermented cow milk ($P < 0.001$).

Fermented goat milk consumption decreased IL-6, TIMP-1, and TNF-α in all experimental conditions, which act as proinflammatory cytokines and they are expressed by cardiac myocytes, fibroblasts, endothelial cells, smooth muscle cells, and monocytes/macrophages. It is well known that TNF-α, IL-6, and TIMP-1 are involved in all stages of the atherosclerosis process, from endothelial dysfunction to plaque rupture and thrombosis,[27] and they are key factors in the development of CDs.[28] The better nutritional characteristics of fermented goat milk, in comparison with fermented cow milk,[18] play a potential role of this dairy product as a high nutritional value food, with anti-inflammatory properties,

Table 4. Cardiovascular risk biomarkers in plasma from control and anemic rats fed for 30 days with fermented cow- or goat-milk-based diets with normal Fe content or Fe overload ($n = 10$ animals per group)

Biomarker (ng mL^{-1})	Fe content	Fermented cow milk diet		Fermented goat milk diet		Two-way ANOVA		
		Control group	Anemic group	Control group	Anemic group	Diet	Anemia	Fe content
CAV-1	Normal	0.703 ± 0.045	0.684 ± 0.044	0.946 ± 0.088[a]	0.961 ± 0.087[b]	<0.001	NS	NS
	Overload	0.685 ± 0.035	0.711 ± 0.049	0.856 ± 0.068[a]	0.953 ± 0.095[b]	<0.001	NS	
CINC-1/ GRO/KC	Normal	0.459 ± 0.075	0.508 ± 0.053	0.289 ± 0.051[a]	0.175 ± 0.020[b]	<0.001	NS	NS
	Overload	0.452 ± 0.025	0.540 ± 0.033	0.303 ± 0.022[a]	0.185 ± 0.035[b]	<0.001	NS	
CTGF	Normal	0.335 ± 0.041	0.791 ± 0.085[c]	0.391 ± 0.039	0.296 ± 0.065[b]	<0.01	<0.05	<0.05
	Overload	0.351 ± 0.052	1.079 ± 0.125[cd]	0.362 ± 0.083	0.309 ± 0.044[b]	<0.01	<0.05	
IL-6	Normal	0.483 ± 0.048	0.409 ± 0.044	0.309 ± 0.051[a]	0.316 ± 0.042[b]	<0.01	NS	NS
	Overload	0.439 ± 0.021	0.423 ± 0.035	0.337 ± 0.018[a]	0.327 ± 0.031[b]	<0.01	NS	
MCP-1	Normal	0.575 ± 0.031	0.767 ± 0.052[c]	0.458 ± 0.028[a]	0.507 ± 0.044[b]	<0.001	<0.01	NS
	Overload	0.580 ± 0.033	0.697 ± 0.048[c]	0.466 ± 0.039[a]	0.504 ± 0.052[b]	<0.001	<0.05	
tPAI-1	Normal	0.183 ± 0.019	0.169 ± 0.026	0.122 ± 0.027[a]	0.142 ± 0.035	<0.001	NS	NS
	Overload	0.178 ± 0.028	0.163 ± 0.031	0.125 ± 0.031[a]	0.135 ± 0.022[b]	<0.001	NS	
TIMP-1	Normal	15.771 ± 0.852	17.861 ± 0.836	7.543 ± 0.509[a]	7.579 ± 0.602[b]	<0.001	NS	<0.05
	Overload	15.581 ± 0.651	14.554 ± 0.798[d]	7.055 ± 0.582[a]	5.899 ± 0.585[bcd]	<0.001	<0.05	
TNF-α	Normal	0.086 ± 0.045	0.092 ± 0.049	0.061 ± 0.037[a]	0.063 ± 0.058[b]	<0.01	NS	NS
	Overload	0.085 ± 0.036	0.088 ± 0.033	0.063 ± 0.031[a]	0.062 ± 0.036[b]	<0.01	NS	
VEGF	Normal	0.864 ± 0.088	0.957 ± 0.091	0.537 ± 0.089[a]	0.651 ± 0.087[b]	<0.001	NS	<0.001
	Overload	1.345 ± 0.123[d]	1.556 ± 0.154[d]	0.856 ± 0.101[d]	1.181 ± 0.152[bd]	<0.001	NS	
Adiponectin	Normal	44.412 ± 2.891	44.771 ± 3.823	162.225 ± 3.342[a]	159.823 ± 5.011[b]	<0.001	NS	<0.01
	Overload	58.221 ± 3.325[d]	56.325 ± 2.925[d]	158.532 ± 3.825[a]	160.325 ± 4.925[b]	<0.001	NS	
sE-Selectin	Normal	123.243 ± 10.121	126.181 ± 12.254	50.325 ± 1.321[a]	49.221 ± 1.925[b]	<0.001	NS	NS
	Overload	124.113 ± 9.981	129.223 ± 10.321	51.225 ± 1.632[a]	50.876 ± 2.321[b]	<0.001	NS	
sICAM-1	Normal	13.225 ± 1.324	12.671 ± 1.223	8.825 ± 1.731[a]	8.941 ± 1.025[b]	<0.001	NS	<0.01
	Overload	17.154 ± 1.189[d]	19.654 ± 1.561[d]	10.123 ± 1.658[a]	10.547 ± 1.723[b]	<0.001	NS	

NS, not significant. CAV-1, caveolin-1; CINC-1/GRO/KC, cytokine-induced neutrophil chemoattractant 1; CTGF, connective tissue growth factor; IL-6 interleukin-6; MCP-1, monocyte chemoattractant protein-1; tPAI-1, inhibitor of tissue plasminogen activator 1; TIMP-1, total metallopeptidase inhibitor 1; TNF-α, tumor necrosis factor alpha; VEGF, vascular endothelial growth factor.
[a] Mean values from control group fed with fermented cow milk diet were significantly different ($P < 0.05$, Tukey's test).
[b] Mean values from anemic group fed with fermented cow milk diet were significantly different ($P < 0.05$, Tukey's test).
[c] Mean values from the corresponding group of control rats were significantly different ($P < 0.05$, Student's *t* test).
[d] Mean values from the corresponding group of rats fed with normal-Fe content were significantly different ($P < 0.05$, Student's *t* test).

reducing the levels of these cytokines. The anti-inflammatory activities of lipids in goat products have been studied in traditional goat dairy products,[29] and their lipid fractions exhibited inhibitory activity toward platelet-activating-factor-induced platelet aggregation. The most biologically active lipid fractions contained sphingomyelin, phosphatidylcholine, and phosphatidylethanolamine lipid derivatives. However, these lipid fractions do not always present as a typical phospholipid structure; they share a similar structure to phosphatidylcholine derivatives, as reported by Nasopoulou *et al.*[30] Phosphatidylcholine has several anti-inflammatory effects.[31] Poutzalis *et al.*[32] compared the platelet-activating-factor-inhibiting properties of fermented goat products, and all goat samples possessed platelet-activating-factor inhibitors. The resulting data indicated an increasing trend of platelet-activating-factor inhibition during lipolysis (i.e., incubation of milk to yogurt and cheese). These results are in agreement with the results obtained in the current study in which tPAI was lower in control and anemic rats fed fermented goat milk either with normal Fe or Fe overload. This trend has been attributed to fermentation involving microorganisms such as *Lactobacillus delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, traditional fermentation starters used in the current study. Goat milk has also bioactive lipids with anti-inflammatory properties.[29,32] Finally, fermented goat milk is richer in oleic and linoleic acids,[18] and it is well known that oleic acid lowers total plasma cholesterol, low-density lipoprotein (LDL)-cholesterol, and triglycerides, and has a potential role preventing cardiac dysfunction and failure,[33] and linoleic acid reduces total cholesterol, protects against ischemic stroke, and exerts positive effects in reducing heart disease,[34] as these fatty acids have putative modulating effects on proinflammatory response.[35]

The expression of VEGF is associated with angiogenesis. This angiogenesis involves the invasion and formation of new vessels. This not only increases the hemodynamic instability and fragility of the plaque, but also allows further infiltration of inflammatory cells, thus additionally increasing the inflammation in the atherosclerotic lesion.[36] VEGF activates MCP-1 in endothelial cells and increases the permeability of the endothelial layer, leading to chronic low-level inflammation and monocyte infiltration.[37] In this sense, an accumulation of macrophages and their uptake of oxidized LDLs is an important feature in atherosclerosis. It is widely known that macrophages are major contributors to atherosclerotic progression.[38] Once recruited, monocytes infiltrate the intima with

the help of cellular adhesion molecules that are expressed on the surface of vascular endothelial cells and whose circulating counterparts are endothelium-derived factors, such as sE-selectin and sICAM-1. This cascade of events causes monocyte differentiation into dendritic cells or CD36-positive macrophages that interact with atherogenic lipoproteins.[39] Macrophages $CD36^+$ are important for scavenging and endocytosis of oxidized LDL-cholesterol and foam cell formation, which, in turn, produce and secrete proinflammatory cytokines, activating a vicious cycle.[40] All these molecules released during the atherogenesis process can serve as markers of this condition.[41] The decrease in VEGF, SICAM-1, sE-selectin, and MCP-1 recorded with fermented goat milk can be attributed to the beneficial nutritional character of this type of milk, which provides a lower substrate for lipid peroxidation and consequently decreases the generation of free radicals and monocyte migration and adhesion. Additionally, the ability of fermented goat milk peptides to inhibit deleterious changes caused by lipid oxidation appears to be related to certain amino acid residues in the peptides, such as tyrosine, methionine, histidine, lysine, and tryptophan, which are capable of chelating pro-oxidative metal, inhibiting lipid oxidation, reducing hydroperoxides output and contributing to the improvement in TAS, limiting oxidative damage to the biomolecules,[16] including blood-vessel walls.

CTGF is a key mediator of tissue fibrogenesis in various chronic diseases.[42] CTGF is involved in various biological processes, including extracellular matrix production, proliferation, apoptosis, chemotaxis, and angiogenesis. Many cell types express CTGF, including endothelial cells, vascular smooth muscle cells, fibroblasts, and cardiac myocytes.[43] CTGF is upregulated by stimuli involved in cardiovascular damage, including oxidative stress.[44,45] Our research group has previously reported that fermented goat milk induces a significant elevation of some antioxidant endogenous enzymes, together with an increase in TAS, a decrease in 8-hydroxy-2′-deoxyguanosine, reducing DNA strand breaks, hydroperoxides, 15-F2t-isoprostanes and protein carbonyl groups in tissues, revealing an improvement in both systemic and cellular antioxidant activity of plasma and tissues due to fermented goat milk consumption.[16] This protective antioxidant effect in the tissues, increasing TAS and decreasing the oxidative damage biomarkers, which directly correlates with the expression/activity of antioxidant enzymes, could explain why fermented goat milk reduces CTGF plasma levels, reducing the cardiovascular risk during anemia recovery.

With regard to the beneficial cardiovascular biomarkers studied (CAV-1 and adiponectin), these were higher in both groups of animals (control and anemic) fed a fermented goat milk diet either with normal Fe or Fe overload with respect to fermented cow milk diet ($P < 0.001$ for CAV-1 and adiponectin). Fe overload increased adiponectin in control and anemic animals fed fermented cow milk ($P < 0.001$).

Fermented goat milk consumption also increases CAV-1 levels, allowing blood vessels to sense, organize, and mediate signal transduction, thereby giving arteries the ability to change their physical properties and to maintain/regulate normal blood flow and pressure in the face of altered shear stress conditions,[46] reducing the blood vessels' fragility during anemia recovery.

Adiponectin is an adipose-tissue-derived hormone possessing anti-inflammatory properties that exerts a pivotal role in vascular protection through activation of multiple intracellular signaling cascades. Decreased plasma adiponectin levels were implicated in the pathogenesis of many CD and atherosclerosis cases.[47] Experimentally, it has been shown that higher adiponectin levels protect mice from developing arterial hypertension in response to hypoxia and inflammation.[48] The inverse relationship between hypertension and adiponectin concentration could be attributed to suppression of adiponectin secretion by the local adipose renin–angiotensin system, which is turned on once fat cell mass increases, leading to increased blood pressure.[49] In the present study, animals consuming fermented goat milk recorded higher levels of adiponectin. As already mentioned,[18] fermented goat milk is also richer in short- and medium-chain fatty acids. These have a positive effect on adiponectin secretion[50] and also reduce the synthesis of endogenous cholesterol and its intestinal absorption, which is able to pass through the mitochondrial membrane independently of carnitine, does not need re-esterification, and is oxidized in the mitochondria, providing fast energy discharge available for several metabolic pathways,[15] reducing lipogenesis *de novo* and therefore preventing fat mass expansion and adiponectin secretion. Additionally, fermented goat milk reduces adiposity, inducing leptin elevation and ghrelin reduction, upregulating irisin, which contributes to a favorable metabolic profile and the browning of adipose tissue,[51] a fact that could contribute greatly in the maintenance of adiponectin levels. On the other hand, CINC-1/GRO/KC is a strong chemotactic factor, and serum levels of this chemokine are elevated in obesity;[52] therefore, the reduction in lipogenesis and fat mass expansion linked to the adiponectin increase would also reduce the levels of this biomarker, also exerting a protective effect on cardiovascular health.

The results of the current study show there is a negative influence of Fe overload and Fe deficiency on blood vessels. This is in agreement with the report by Dev and Babitt[5] indicating that the influence of Fe overload includes oxidant-mediated injury, interference with cardiac electrical function, and promotion of vascular fibrosis. Fe deficiency also has adverse consequences on the heart and blood vessels. Pathogenic mechanisms suggested by some studies include promoting endothelial cell dysfunction, monocyte adhesion, and/or oxidative stress and atherosclerotic plaque instability.

CONCLUSIONS

This study demonstrated that fermented goat milk consumption improves hematological status and promotes beneficial metabolic responses, which may collectively attenuate cardiovascular risk factors during anemia recovery and Fe overload. Fermented goat milk consumption decreased TNF-α, IL-6, and TIMP-1, which are involved in all stages of the atherosclerosis process, from endothelial dysfunction to plaque rupture and thrombosis. Fermented goat milk consumption also led to a decrease in VEGF, SICAM-1, sE-selectin, MCP-1, and CTGF plasma levels, increasing adiponectin and CAV-1, reducing the cardiovascular risk and vascular damage during anemia recovery. Fermented goat milk is a natural candidate as a nutraceutical agent that may have a beneficial role on cardiovascular health during Fe-deficiency recovery to lessen the inflammatory response, macrophages activation, and atherosclerosis development.

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