Folic acid supplemented goat milk has beneficial effects on hepatic physiology, haematological status and antioxidant defence during chronic Fe repletion

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The aim of the current study was to asses the effect of goat or cow milk-based diets, either normal or Fe-overloaded and folic acid supplement on some aspects of hepatic physiology, enzymatic antioxidant defence and lipid peroxidation in liver, brain and erythrocyte of control and anaemic rats after chronic Fe repletion. 160 male Wistar rats were placed on 40 d in two groups, a control group receiving normal-Fe diet and the Fe-deficient group receiving low Fe diet. Lately, the rats were fed with goat and cow milk-based diets during 30 d, with normal-Fe content or Fe-overload and either with normal folic or folic acid supplemented. Fe-overload increased plasma alanine transaminase and aspartate transaminase levels when cow milk was supplied. Dietary folate supplementation reduced plasma transaminases levels in animals fed goat milk with chronic Fe overload. A remarkable increase in the superoxide dismutase activity was observed in the animals fed cow milk. Dietary folate supplement lead to a decrease on the activity of this enzyme in all the tissues studied with both milk-based diets. A concomitant increment in catalase was also observed. The increase in lipid peroxidation products levels in rats fed cow milk with Fe-overload, suggest an imbalance in the functioning of the enzymatic antioxidant defence. In conclusion, dietary folate-supplemented goat milk reduces both plasma transaminases levels, suggesting a hepatoprotective effect and has beneficial effects in situation of Fe-overload, improving the antioxidant enzymes activities and reducing lipid peroxidation.

Keywords: Goat and cow milk, folic acid supplementation, antioxidant enzymes, lipid peroxidation: chronic iron repletion.

According to the World Health Organisation (WHO), iron deficiency anaemia (IDA) is one of the most common health problems worldwide, but in contrast, the recommendations of this mineral are higher in some populations (Ekström et al. 2002), leading in many cases to Fe-overload. Hepatotoxicity is common in patients with Fe supplements, followed by many other pathologies involving cardiovascular, endocrine and muscular systems. Fe excess in the body leads to generation of reactive oxygen species and severe damage to cell constituents (Piloni et al. 2013)

Fe is a necessary cofactor in many metabolic processes in the central nervous system, including oxidative phosphorylation, myelin synthesis, neurotransmitter production, nitric oxide metabolism and oxygen transport (Díaz-Castro et al. 2012). The liver has a key role in metabolism, eliminating xenobiotics and endogenous metabolites, and the antioxidant enzymes protect the cells (including hepatocytes) from the oxidative stress (Díaz-Castro et al. 2013).

Our research group has previously reported that goat milk consumption has positive influence on enzymatic antioxidant defence, even in situation of Fe-overload in comparison with cow milk (Díaz-Castro et al. 2012). In addition, goat milk fat has a higher nutritional quality than cow milk fat (Alférez et al. 2001) and improves Fe metabolism (Nestares et al. 2008). However, in spite of its nutritional quality and healthy benefits, goat milk folic acid content is much lower than in cow milk (Haenlein, 2001). The folic acid is an essential water-soluble vitamin that can regulate many cellular pathways such as growth and differentiation, DNA repair, apoptosis and carcinogenesis prevention, and in

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addition, has antioxidant effects and the capacity of scavenging the excessive free radicals output (Gursu et al. 2004).

Taking into account the beneficial effects of goat milk previously mentioned and the low folic acid content in this type of milk, the present study was carried out to assess the influence of cow and goat milk-based diets, either with normal-Fe content or Fe-overload and the influence of nutritional folic acid supplement on haematological parameters, some aspects of hepatic physiology and enzymatic antioxidant defence in liver, brain and erythrocyte of control and anaemic rats.

Materials and methods

Animals

All animal care procedures and experimental protocols were approved by the Ethics Committee of the University of Granada in accordance with the European Community guidelines. One hundred and sixty male Wistar albino breed rats (21 d old and weighing 41 ± 5 g), purchased from the University of Granada Laboratory Animal Service (Granada, Spain) were used in the current study.

Experimental design

During a pre-experimental period (PEP) of 40 d rats were divided into two groups: the control group (n = 80) receiving a normal Fe diet (45 mg/kg diet) (Reeves et al. 1993), and the anaemic group (n = 80) receiving a low-Fe diet (5 mg/kg diet), to induce a severe dietary Fe-deficiency according to the method developed previously by us (Pallarés et al. 1993). During the course of the study, the animals were placed in individual metabolic cages in an environmentally controlled room with a constant temperature (22 ± 2 °C), humidity (55 ± 5%) and a 12-h light-dark cycle (9:00 to 21:00). Diet intake was controlled, pair feeding all the animals (80% of the average intake) and bidistilled water was available *ad libitum*. On d 40 of the PEP, all rats per group (controls and anaemics) were anaesthetized by intraperitoneal injection with sodium pentobarbital (Sigma Diagnostics, St Louis, MO, USA), peripheral blood samples from caudal vein with ethylenediaminetetraacetic acid (EDTA) were analysed for haematological parameters.

An experimental period (EP) of 30 d followed in which the control and anaemic groups were further fed with either goat milk (n = 80) or cow milk-based diets (n = 80) with normal folic acid content (2 mg/kg diet) or folic acid supplemented (40 mg/kg diet) (Achón et al. 2000) and either with normal Fe content (45 mg/kg diet) or Fe overloaded (450 mg/kg) to induce chronic Fe overload (Raja et al. 1994) (10 control and 10 anaemic rats for each of the eight milk-based diets; Table 1). On d 30 of the EP, the animals were kept fasting overnight anaesthetised intraperitoneally, totally bled out by cannulation of the abdominal aorta and blood aliquots with EDTA were analysed for haematological parameters.

Table 1. Composition of the experimental diets

Component	g/kg diet
Pre-experimental period, Standard (non-milk) diet†	
Casein	200
Lactose	0
Fat (virgin olive oil)	100
Wheat starch	500
Constant ingredients‡	200
Experimental period	
Goat milk-based diet§	
Goat milk protein	205
Goat milk lactose	290
Goat milk fat	100
Wheat starch	205
Constant ingredients‡	200
Cow milk-based diet§	
Cow milk protein	204
Cow milk lactose	295
Cow milk fat	100
Wheat starch	201
Constant ingredients‡	200

† The diets were prepared according to the recommendations of the AIN-93 for control rats (45 mg Fe/kg diet) (Reeves et al. 1993), or with low Fe content (5 mg Fe/kg diet) (Pallarés et al. 1993), for Fe-deficient groups
‡ The constant ingredients consisted of (g/kg diet): fibre (micronized cellulose) 50, sucrose 100, choline chloride 2·5, L-cystine 3, mineral premix 35, vitamin premix 10
§ Specific vitamin and mineral premixes supplements for goat milk and cow milk-based diets were formulated taking into account the mineral and vitamin contents of the skimmed milk powder supplied in order to meet these recommendations. The diets were prepared with normal-Fe content (45 mg Fe/kg diet) (Reeves et al. 1993), or with high Fe content (450 mg Fe/kg diet) (Raja et al. 1994) for Fe-overload groups, and both either with normal folic (2 mg/kg diet) or high folic content (40 mg/kg diet) for folic-acid-supplemented diets (Achón et al. 2000)

Subsequently, plasma was obtained by centrifugation (1500 **g**, 4 °C, 10 min). The remaining blood was centrifuged without anticoagulant to separate the red blood cells from the serum and subsequent analysis of serum Fe, ferritin and total Fe-binding capacity (TIBC). The liver and brain were removed and washed with ice-cold saline solution (0·9%, w/v, NaCl). Liver, brain and erythrocyte cytosolic fractions were prepared fresh the same day by successive differential centrifugations, preserving these fractions at −80 °C for further analyses of oxidative damage.

Iron determination in the diets

After mineralisation by a wet method in a sand bath (J.R. Selecta, Barcelona, Spain), Fe concentrations in the experimental diets were determined by atomic absorption spectrophotometry (PerkinElmer Analyst 1100B spectrometer with WinLab32 for AA software, Ueberlingen, Germany). To calibrate the measurements, samples of lyophilised bovine liver (certified reference material BCR 185; Community Bureau of References, Brussels, Belgium) were used to check the Fe recovery (Fe value = 209 (SEM 5·7) mg/kg, mean (SEM) of five determinations, certified value = 212 (SEM 5·1) mg/kg).

Haematological test

All the haematological parameters studied were measured and calculated by fully-automatic haematology analyser Sysmex K-1000D (Sysmex, Tokyo, Japan).

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 (Bio-rad Laboratories Inc., California, USA).

Serum iron, total iron binding capacity (TIBC) and transferrin saturation

To calculate the rate of transferrin saturation, serum Fe concentration and TIBC were determined using Sigma Diagnostics Iron and TIBC reagents (Sigma Diagnostics). The percentage of transferrin saturation was calculated from the following equation:

$$\text{Transferrin saturation (\%)} = \text{serum Fe concentration} \, (\mu g/l)/\text{TIBC} \, (\mu g/l) \times 100$$

Plasma transaminases

Plasma alanine transaminase (ALT) and aspartate aminotransferase (AST) activity were determinedusing a Transaminases kit (Sigma Diagnostics) as previously described (Bergmeyer et al. 1978).

Plasma creatinine and creatine kinase (CK)

Creatinine and CK were measured in plasma using a commercial colorimetric kit (Spinreact, Barcelona, Spain).

Liver, brain and erythrocyte cytosolic preparations

The liver, brain and erythrocyte samples were thawed and homogenates were freshly prepared the same day in a Potter homogeniser after the addition of 1·0 ml phosphate buffer per 0·1 g tissue. Successive differential centrifugations (Beckman Coulter Inc., CA, USA) were performed to separate cytosolic fractions, according to DeSandro et al. (1991). The erythrocyte cytosolic fractions were prepared according to Hanahan & Ekholm (1974). Cytosolic protein contents were measured as described by Lowry et al. (1951).

Antioxidant enzymes activity in cytosolic fractions of liver, brain and erythrocyte

Glutathione peroxidase (GPx) activity was measured by the method of Flohé & Günzler (1984), which is based on the instantaneous formation of oxidised glutathione during the reaction catalysed by GPx. The oxidised glutathione is continually reduced by an excess of glutathione reductase and NADPH. The subsequent oxidation of NADPH to $NADP^+$ was monitored spectrophotometrically (Thermo Spectronic, Rochester, USA) at 340 nm.

Superoxide dismutase (SOD) activity was determined according to Crapo et al. (1978) based on its inhibition in the reduction of cytochrome c, measured in a spectrophotometer (Thermo Spectronic, Rochester, USA) at 550 nm. Catalase (CAT) activity was determined following the method described by Aebi (1984), monitoring at 240 nm in a spectrophotometer (Thermo Spectronic, Rochester, USA) the H_2O_2 decomposition, as a consequence of the catalytic activity of CAT.

Thiobarbituric acid−reactive substances (TBARS) measurement

The extent of lipid peroxidation was evaluated on liver, brain, erythrocyte and plasma by measuring the concentration of TBARS according to the methods of Yagi (1976) & Ohkawa et al. (1979). The reaction product was extracted and measured by spectrophotometric analysis (Thermo Spectronic) at 532 nm.

Statistical analysis

Data are reported as means ± standard error of the mean (SEM) during the PEP and means ± residual standard deviation (RSD) during the EP. Statistical analyses were performed using SPSS (SPSS, version 20.0, 2012; SPSS Inc., Chicago, IL, USA). The data were analysed by the use of a complete randomised block experiment design, with factorial arrangement ($2 \times 2 \times 2 \times 2$), 2 (type of diet: goat *vs.* cow milk diet) × 2 (group of animal: control *vs.* anaemic) × 2 (Fe content in the diet: normal-Fe or Fe-overload) × 2 (folic acid content in the diet: normal *vs.* folic acid-supplemented), and by the least squares method (Steel et al. 1997). Differences between groups (control *vs.* anaemic, normal-Fe *vs.* Fe-overload and normal-folic acid *vs.* folic acid-supplemented) were tested for statistical significance with Student's *t* test. Variance analysis by one-way methods was used to compare the different diets supplied. Individual means were tested by pairwise comparison with Tukey's multiple comparison test when main effects and interactions were significant. Differences were considered significant at $P < 0·05$.

Results

After rats had consumed the low-Fe diet for 40 d, haematological parameters were dramatically different from the controls ($P < 0·001$) and control rats fed normal-Fe diet had all the haematological parameters within normal limits for this species at the end of the PEP (Table 2). After 30 d feeding the milk-based diets (d 70), the haematological parameters were fully recovered with both milk-based diets, either with normal-Fe or Fe-overload. As expected, serum Fe was higher

Table 2. Haematological parameters in the pre-experimental period. Values are means ± SEM for $n = 80$ per group

Haematological parameter	Normal-Fe control group	Low-Fe anaemic group
Hb concentration (g/l)	128·87 ± 2·75	73·48 ± 2·66*
RBCs (10^{12}/l)	7·11 ± 0·14	6·13 ± 0·15*
Haematocrit (%)	39·75 ± 0·68	27·54 ± 0·51*
Platelets (10^9/l)	723 ± 25·05	1388 ± 67·74*
MCV (fl)	55·63 ± 0·24	38·87 ± 0·55*
Serum Fe (µg/l)	1375 ± 119	703 ± 55*
Serum ferritin (µg/l)	82·95 ± 2·53	49·93 ± 1·33*
Transferrin saturation (%)	47·03 ± 3·10	3·85 ± 0·32*
TIBC (µg/l)	2852 ± 198	17642 ± 725*

Hb, haemoglobin; RBCs, red blood cells; MCV, mean corpuscular volume; TIBC, total Fe-binding capacity
* Significantly different ($P < 0·001$) from the control group by Student's t test

in the Fe-overload groups ($P < 0·01$). Fe-overload also increased haemoglobin ($P < 0·001$), serum ferritin ($P < 0·01$), transferrin saturation ($P < 0·01$) and TIBC ($P < 0·01$). Folic supplementation increased RBC count in the normal-Fe groups ($P < 0·01$) and reduced MCV ($P < 0·001$) (Table 3).

Fe-overloaded cow milk consumption increased ALT and AST levels in comparison with goat milk, in both animal groups ($P < 0·001$). In addition, Fe-overload lead to an increase of ALT and AST with cow milk-based diets compared with normal-Fe content in all experimental conditions ($P < 0·01$), meanwhile no effect of Fe-overload was observed with goat milk. Folate supplement markedly reduced transaminases plasma levels with goat milk-based diet ($P < 0·001$). In addition, supplemental folic acid significantly increased creatinine and CK with both milk-based diets and under all the experimental conditions ($P < 0·001$) (Table 4).

An increase in the SOD activity was observed in the animals fed cow milk diet in all the organs either with normal-Fe or Fe-overload with normal- folic content in comparison with goat milk ($P < 0·001$). Fe-overload also increased SOD activity in erythrocyte and brain for control animals and in liver for anaemic rats fed cow milk, in comparison with normal-Fe content ($P < 0·05$). Folic acid supplementation lead to a decrease on the activity of this enzyme in all the tissues studied except for plasma in the different experimental conditions ($P < 0·001$) (Table 5). A concomitant increment in CAT was also observed in all the tissues of control animals fed cow milk in comparison with goat milk with normal-Fe content ($P < 0·001$ for liver and erythrocyte and $P < 0·01$ for brain) and the same trend is observed for anaemic animals (Table 5).

In general in all the conditions assayed, Fe-overload increased CAT activity in liver and erythrocyte ($P < 0·01$), and no changes were observed in brain. Folate supplementation leads to an increase in CAT activity in liver and a decrease in erythrocyte and brain ($P < 0·001$). Anaemia diminished CAT activity in brain of animals fed cow milk-based diet either with normal or Fe-overload and normal

folic acid content ($P < 0·001$), while its activity is not affected by the folic acid supplementation. In general, GPx activity decreased in the animals fed cow milk with Fe-overload in comparison with goat milk with high Fe content in liver ($P < 0·001$), and erythrocyte ($P < 0·05$) (Table 5). Fe-overload in the milk-based diets decreased GPx activity, especially in liver and erythrocyte of the animals fed cow milk with normal-folate content ($P < 0·001$). Folate supplementation diminished GPx levels in liver ($P < 0·001$), and increased its activity in erythrocyte ($P < 0·001$). In general, anaemia had no effect on enzymatic activities in brain under the different experimental conditions (Table 5).

With regard to the oxidative stress-mediated damage to lipids (Table 5), in general, TBARS levels are higher in erythrocyte ($P < 0·05$), brain ($P < 0·01$) and plasma ($P < 0·05$) for control and anaemic animals fed with Fe-overloaded cow milk-based diet in comparison with goat milk. Anaemia had no effect on TBARS levels in all tissues studied under the different experimental conditions. Fe-overload had a negative influence with cow milk and normal-folic content, increasing lipid peroxidation in erythrocyte ($P < 0·05$), brain ($P < 0·01$) and plasma ($P < 0·05$). Furthermore, supplemental folic acid in the milk-based diets had a beneficial effect, reducing the extent of lipid peroxidation in all the tissues studied ($P < 0·001$) (Table 5).

Discussion

Fe-deficiency occurs because the rats with normal body Fe stores lose a large portion of body Fe before the haemoglobin falls, and that the stores were replenished after feeding the animals during 30 d EP with different milk-based diets either with normal-Fe or Fe-overload. Folic supplementation increased RBC count in the normal-Fe groups, a fact that can be explained by the key role of folate in cell growth and differentiation (Gursu et al. 2004). The reduction of mean corpuscular volume (MCV) in the folate-supplemented groups can be attributed to the higher cell turnover in this group, giving place to smaller cells (Cook, 2005).

After suppling cow milk with Fe-overload, ALT and AST levels increased, indicating hepatocellular damage and release of both transaminases into the bloodstream. Dietary folate supplementation reduced both plasma transaminases levels in animals fed goat milk, suggesting a hepatoprotective effect under basal conditions and more noticeably in situation of Fe-overload. This fact can be explained by the better Zn bioavailability and nutritive utilisation of goat milk fat, which provides lower substrate for hepatic lipid peroxidation and consequently reducing free radicals production in the animals consuming this type of milk (Díaz-Castro et al. 2012).

We have observed that plasma creatinine and CK increased in the groups with folic acid supplementation, probably because of increased production of creatine which is a major consumer of methyl groups and necessary for a correct folate metabolism (Gamble et al. 2007).

Table 3. Haematological parameters in the experimental period

Parameter	Fe content	Goat milk-based diet				Cow milk-based diet				Level of significance				
		Control groups		Anaemic groups		Control groups		Anaemic groups		Main effects				
		Folic-normal ($n=20$)†	Folic-supplemented ($n=20$)†	Folic-normal ($n=20$)†	Folic-supplemented ($n=20$)†	Folic-normal ($n=20$)†	Folic-supplemented ($n=20$)†	Folic-normal ($n=20$)†	Folic-supplemented ($n=20$)†	RSD	Diet	Anaemia	Fe content	Folic acid content
Hb concentration (g/l)	Normal	130·80	134·00[D]	129·80	129·30	132·60	133·25	128·50	133·43[D]	2·345	NS	NS	<0·001	<0·05
	Overload	143·80[C]	141·30[C]	148·00[AC]	146·00[C]	142·80[C]	142·13[C]	142·10[BC]	145·00[C]	2·984	<0·05	NS		NS
RBCs (10[12]/l)	Normal	7·40[a]	8·01[aD]	7·20[A]	8·04[AD]	7·05[a]	7·74[bD]	7·07[A]	7·68[BD]	0·183	<0·05	NS	<0·05	<0·001
	Overload	8·03[aC]	7·87[a]	7·10	7·98[AC]	6·92[b]	7·17[b]	7·20	7·49[B]	0·127	<0·01	NS		<0·05
Haematocrit (%)	Normal	42·38[a]	44·08[aD]	39·54[A]	42·48[D]	40·82[b]	38·52[bD]	38·94[B]	43·04[D]	0·568	<0·01	NS	<0·01	<0·01
	Overload	45·28[aC]	48·54[aCD]	45·36[C]	42·32	39·21[b]	40·23[b]	45·28[C]	42·59[D]	0·593	<0·05	NS		<0·05
Platelets (10[9]/l)	Normal	926·00	928·00	939·33	963·67	934·00	903·00	964·00	944·89	29·53	NS	NS	NS	NS
	Overload	909·67	936·56	968·00	984·12	940·25	916·75	968·50	913·25	32·21	NS	NS		NS
MCV (fL)	Normal	57·25	52·62[D]	55·04	53·44[D]	57·68	53·77[D]	55·34	52·48[D]	0·386	NS	NS	NS	<0·001
	Overload	56·44	52·39[D]	56·15[A]	52·00[D]	56·79	53·33[D]	53·18[B]	50·56[D]	0·402	<0·05	NS		<0·001
Serum Fe (µg/l)	Normal	1352	1347	1326	1345	1346	1364	1355	1353	58·65	NS	NS	<0·01	NS
	Overload	1456[C]	1445[C]	1476[C]	1489[C]	1491[C]	1478[C]	1487[C]	1454[D]	61·87	NS	NS		NS
Serum ferritin (µg/l)	Normal	84·33	83·60	82·34	80·26	83·25	83·87	82·97	82·60	1·925	NS	NS	<0·01	NS
	Overload	87·91[C]	88·40[C]	86·65[C]	87·53[C]	87·73[C]	88·06[C]	86·87[C]	88·71[D]	2·322	NS	NS		NS
Transferrin saturation (%)	Normal	46·65	47·34	46·37	46·25	45·98	46·21	45·32	45·18	1·543	NS	NS	<0·01	NS
	Overload	49·59[C]	48·95[C]	48·96[C]	48·85[C]	47·76[C]	48·59[C]	47·88[C]	47·76[C]	1·885	NS	NS		NS
TIBC (µg/l)	Normal	2785	2821	2789	2776	2787	2765	2798	2776	156·43	NS	NS	<0·01	NS
	Overload	3251[C]	3285[C]	3195[C]	3156[C]	3145[C]	3321[C]	3254[C]	3128[C]	189·54	NS	NS		NS

RSD, residual standard deviation; NS, not significant

† ($n=20$), 10 rats with Fe normal and 10 with Fe overload

[a,b]Mean values among groups of controls rats fed with different diet and different lower case letters in the same row indicated significant difference by one-way ANOVA (Tukey's test)

[A,B]Mean values among groups of anaemic rats fed with different diet and different upper case letters in the same row indicated significant difference by one-way ANOVA (Tukey's test)

[C]Mean values were significantly different from the corresponding group of rats fed with normal Fe content at $P<0.05$ by Student's t test

[D]Mean values were significantly different from the corresponding group of rats fed with normal folic acid content at $P<0.05$ by Student's t test

Table 4. Transaminases, creatinine and creatine kinase (CK) in the experimental period

Biochemical parameter	Fe content	Goat milk-based diet				Cow milk-based diet				Level of significance					
		Control groups		Anaemic groups		Control groups		Anaemic groups			Main effects				
		Folic-normal ($n=20$)†	Folic-supplemented ($n=20$)†	Folic-normal ($n=20$)†	Folic-supplemented ($n=20$)†	Folic-normal ($n=20$)†	Folic-supplemented ($n=20$)†	Folic-normal ($n=20$)†	Folic-supplemented ($n=20$)†	RSD	Diet	Anaemia	Fe content	Folic acid content
ALT (U/l)	Normal	37·59	24·57[aD]	33·55	26·82[AD]	40·38	37·22[b]	35·24	32·20[B]	1·537	<0·05	NS	<0·01	<0·01
	Overload	39·81[a]	27·39[aD]	36·43[A]	29·42[AD]	50·99[bC]	53·15[bC]	44·52[BC]	43·12[BC]	1·675	<0·001	NS	<0·01	<0·01
AST (U/l)	Normal	108·15	98·87[aD]	109·23	94·66[AD]	110·25	109·92[b]	108·73	107·65[B]	3·922	<0·05	NS	<0·01	<0·01
	Overload	112·20[a]	101·17[aD]	111·52[A]	95·19[AD]	124·32[bC]	122·57[bC]	123·41[BC]	124·08[BC]	4·129	<0·001	NS	<0·01	<0·01
Creatinine (µmol/l)	Normal	20·51	27·32[D]	21·32	28·32[D]	21·35	29·30[D]	23·45	29·22[AD]	1·107	NS	NS	NS	<0·001
	Overload	20·32	29·53[D]	20·59	29·54[D]	21·98	29·32[D]	22·34	30·18[AD]	1·113	NS	NS	NS	<0·001
CK (U/l)	Normal	71·81	95·93[D]	74·81	99·56[D]	73·95	101·23[D]	72·76	98·87[AD]	3·243	NS	NS	NS	<0·001
	Overload	72·12	101·91[D]	73·07	103·42[D]	76·86	100·57[D]	73·97	103·11[AD]	3·389	NS	NS	NS	<0·001

RSD, residual standard deviation; NS, not significant

† ($n=20$), 10 rats with Fe normal and 10 with Fe overload

[a,b]Mean values among groups of controls rats fed with different diet and different lower case letters in the same row indicated significant difference by one-way ANOVA (Tukey's test)

[A,B]Mean values among groups of anaemic rats fed with different diet and different upper case letters in the same row indicated significant difference by one-way ANOVA (Tukey's test)

[C]Mean values were significantly different from the corresponding group of rats fed with normal Fe content at $P<0.05$ by Student's t test

[D]Mean values were significantly different from the corresponding group of rats fed with normal folic acid content at $P<0.05$ by Student's t test

Creatine increases the muscle mass and energy production. After creatine synthesis, the level of thiodiglycolic acid (TDGA) increases really fast (Navrátil et al. 2010). From an oxidative point of view, the increase in creatinine would be negative, because the oxidative degradation of xenobiotics via TDGA formation decreases the glutathione (GSH), one of the main intracellular antioxidant, therefore, this fact lead to a decrease of all metabolic pathways dependent on GSH (Navrátil et al. 2010). However, we postulate that folate supplementation reduces the GSH consumption and also improves hepatic antioxidant capacity, scavenging the excessive free radicals produced in the course of creatine metabolism, finding supported by the activities of SOD, CAT and GPx recorded in the current study.

In general, Fe-deficiency anaemia did not affect SOD, CAT and GPx activities and, as a consequence, lipid oxidative damage in the tissues studied remain unaltered, results which are in agreement with those reported previously by us, (Díaz-Castro et al. 2008).

A remarkable increase in the SOD and CAT activity was observed in the animals fed cow milk diet in all the organs either with normal-Fe or Fe-overload especially with folate supplementation for CAT. This finding indicates that the increase observed in the SOD activity induces a concomitant augmentation in the formation of hydroperoxides and the CAT is increased to scavenge these ROS, needing an increase in the GPx activity to remove the excess of these intermediary products. The lower levels of GPx in all the groups fed Fe overload diets, especially in liver and erythrocyte of the animals fed cow milk, can be due to the reduction of the enzyme in the process of neutralisation of free radicals generated. The beneficial nutritional characteristics of the goat milk previously mentioned (Díaz-Castro et al. 2012) can explain the lower affect on the liver and erythrocytes induced by the Fe overload in animals fed this type of diet. In the current study, folate supplementation decreased SOD, which is the first line of enzymatic defence against ROS. Therefore, oxidative status markers were modified by folic acid supplementation in the sense of lower oxidative stress, which has clear positive effects on enzymatic antioxidant defence.

On the other hand, ROS play a key role in several pathways of the nervous system, while antioxidants neutralise the oxidative stress (Uttara et al. 2009). The role of antioxidant defence in the brain is not clear and, in this sense, some authors report that the brain is not enriched in antioxidant defences (Uttara et al. 2009), while other studies report that antioxidants (enzymatic and/or non-enzymatic) protect the brain against induced oxidative damage (Ozturk et al. 2005), although the presence of antioxidant enzymes CAT, GPx and SOD in brain region has been previously documented (Alférez et al. 2011). In the current study, in brain and erythrocyte, folate-supplementation led to a reduction in the antioxidant enzymes studied, supporting earlier reports which suggest that the function of the key intracellular antioxidant enzymes may be interdependent (Valko et al. 2007). This finding can be due to the antioxidant

Table 5. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and thiobarbituric acid-reactive substances (TBARS) in the experimental period

| | | Goat milk-based diet | | | | Cow milk-based diet | | | | Level of significance | | | | |
| | | Control groups | | Anaemic groups | | Control groups | | Anaemic groups | | Main effects | | | | |
Tissue	Fe content	Folic-normal (n=20)†	Folic-supplemented (n=20)†	Folic-normal (n=20)†	Folic-supplemented (n=20)†	Folic-normal (n=20)†	Folic-supplemented (n=20)†	Folic-normal (n=20)†	Folic-supplemented (n=20)†	RSD	Diet	Anaemia	Fe content	Folic acid content
SOD (U/mg protein)														
Liver	Normal	2·832[a]	1·253[E]	2·925[A]	1·281[E]	11·111[b]	1·296[E]	10·045[B]	1·398[E]	0·276	<0·01	NS	<0·05	<0·001
	Overload	3·075[a]	1·402[E]	3·234[A]	1·361[E]	12·325[b]	1·406[E]	12·121[BD]	1·380[E]	0·374	<0·01	NS		<0·001
Erythrocyte	Normal	2·931[a]	1·108[aE]	2·929[A]	1·116[AE]	9·187[b]	1·580[bE]	9·554[B]	1·402[BE]	0·282	<0·001	NS	<0·05	<0·001
	Overload	3·189[a]	1·169[aE]	3·032[A]	1·042[AE]	10·386[bD]	1·499[bE]	9·987[B]	1·221[BE]	0·217	<0·01	NS		<0·001
Brain	Normal	2·922[a]	2·174[E]	2·953[A]	2·017[AE]	6·322[b]	2·182[E]	6·434[B]	2·391[BE]	0·269	<0·01	NS	<0·05	<0·001
	Overload	3·165[a]	2·049[aE]	3·206[A]	2·007[AE]	7·434[bD]	2·433[bE]	7·567[BD]	2·604[BE]	0·388	<0·01	NS		<0·001
Plasma	Normal	0·316[a]	0·835[E]	0·325[A]	0·764[AE]	0·525[b]	0·842[E]	0·531[B]	0·892[BE]	0·083	<0·01	NS	NS	<0·001
	Overload	0·325[a]	0·809[E]	0·313[A]	0·747[E]	0·530[b]	0·824[E]	0·512[B]	0·757[E]	0·088	<0·01	NS		<0·001
CAT (U/ml cytosol)														
Liver	Normal	0·178[a]	0·293[aE]	0·183[A]	0·182[AC]	0·226[b]	0·547[bE]	0·320[BC]	0·557[BE]	0·032	<0·001	<0·05	<0·01	<0·001
	Overload	0·206[a]	0·397[aDE]	0·385[CD]	0·216[ACE]	0·324[bD]	0·646[bDE]	0·394	0·680[BDE]	0·042	<0·001	<0·05		<0·001
Erythrocyte	Normal	0·681[a]	0·388[aE]	0·620[A]	0·304[ACE]	0·850[b]	0·479[bE]	0·868[B]	0·576[BCE]	0·051	<0·001	<0·05	<0·01	<0·001
	Overload	1·075[D]	0·432[aE]	0·986[D]	0·331[ACE]	1·083[D]	0·526[bE]	0·962[D]	0·654[BCDE]	0·045	<0·001	<0·05		<0·001
Brain	Normal	0·151[a]	0·091[aE]	0·089[C]	0·139[E]	0·199[b]	0·166[bE]	0·092[C]	0·138[E]	0·019	<0·01	<0·05	<0·05	<0·001
	Overload	0·158[a]	0·109[aE]	0·113	0·129[AE]	0·221[b]	0·170[bE]	0·110[C]	0·142[BE]	0·017	<0·01	NS		<0·001
GPx (mmol/mg protein/ml)														
Liver	Normal	0·851	0·303[E]	0·876	0·375[E]	0·880	0·364[E]	0·822	0·339[E]	0·035	NS	NS	<0·01	<0·001
	Overload	0·585[aD]	0·312[E]	0·553[AD]	0·342[AE]	0·351[bD]	0·312[DE]	0·283[BD]	0·272[BDE]	0·041	<0·001	NS		<0·001
Erythrocyte	Normal	1·135	1·389[a]	1·189	1·665[ACE]	1·088	0·902[b]	1·175	1·077[B]	0·059	<0·05	NS	<0·05	<0·001
	Overload	1·070[a]	1·104	1·085[A]	1·237[DE]	0·845[bD]	1·201[DE]	0·860[BD]	1·137[E]	0·050	<0·05	NS		<0·001
Brain	Normal	0·316[a]	0·308	0·315	0·295	0·283[b]	0·283	0·292	0·303	0·022	NS	NS	NS	NS
	Overload	0·315[a]	0·323[a]	0·323[A]	0·301	0·281[b]	0·286[b]	0·289[B]	0·299	0·031	<0·05	NS		NS
TBARS (nmol/mg protein)														
Liver	Normal	5·79	2·58[E]	5·88	2·63[E]	5·63	2·77[E]	6·04	2·88[E]	0·221	NS	NS	<0·01	<0·001
	Overload	6·35[a]	2·68[E]	7·58[AD]	2·79[E]	8·54[bD]	2·89[E]	8·66[BD]	3·09[E]	0·225	<0·05	NS		<0·001
Erythrocyte	Normal	8·16[a]	3·88[aE]	8·98	3·96[E]	9·65[b]	4·87[bE]	8·96	4·55[E]	0·331	<0·05	NS	<0·05	<0·001
	Overload	9·59[a]	3·55[aE]	9·97[A]	3·99[E]	14·35[bD]	5·10[bE]	12·05[BD]	4·16[E]	0·376	<0·05	NS		<0·001
Brain	Normal	11·55	5·10[E]	11·05	4·88[AE]	11·07	5·11[E]	11·49	5·66[BE]	0·331	NS	NS	<0·01	<0·001
	Overload	11·94[a]	5·03[aE]	11·88[A]	5·03[AE]	19·95[bD]	6·55[bE]	17·81[BD]	6·24[BE]	0·355	<0·01	NS		<0·001
Plasma	Normal	3·11	1·41[E]	3·12	1·43[AE]	3·66	1·73[E]	3·01	2·23[BE]	0·329	NS	NS	<0·05	<0·001
	Overload	3·95[a]	1·52[E]	4·10[A]	1·40[AE]	5·91[bD]	1·75[E]	5·56[BD]	2·28[BE]	0·337	<0·05	NS		<0·001

RSD, residual standard deviation; NS, not significant

† (n=20), 10 rats with Fe normal and 10 with Fe overload

[a,b] Mean values among groups of controls rats fed with different diet and different lower case letters in the same row indicated significant difference by one-way ANOVA (Tukey's test)

[A,B] Mean values among groups of anaemic rats fed with different diet and different upper case letters in the same row indicated significant difference by one-way ANOVA (Tukey's test)

[C] Mean values that were significantly different from the corresponding group of control rats at $P < 0.05$ by Student's t test

[D] Mean values were significantly different from the corresponding group of rats fed with normal Fe content at $P < 0.05$ by Student's t test

[E] Mean values were significantly different from the corresponding group of rats fed with normal folic acid content at $P < 0.05$ by Student's t test

effects of folate and the capacity of scavenging the excessive free radicals output, a fact that can be directly correlated with the lower increase in SOD activity in these tissues, reducing the formation of hydroperoxides. With regard to Fe content in the diet, the slightly modified activities of SOD, CAT and GPx in the nervous tissue, reveals that it is relatively independent of the Fe variations in the organism (Moos et al. 2007).

The liver is a tissue especially vulnerable to the oxidative damage, which is reflected in the increase of the SOD activity in hepatic cytosol of animals Fed with Fe-overload diets. This hepatic Fe-overload promotes the generation of free radicals, so the upregulation of SOD would be directed to eliminate this high rate of generation of ROS (Díaz-Castro et al. 2012). This situation demands an excessive activation of the protective mechanisms and leads to the progressive inactivation of antioxidant enzymes, fact that might probably justify the reduction in the activity of GPx. The higher levels of CAT in the liver of folate-supplemented animals indicates that folate supplementation causes different effects on antioxidant enzyme activity in various tissues/organs owing to inherent differences in organ pathways regulating folate metabolism as well as antioxidant enzyme synthesis, function, or turnover. The remarkable increase observed in the SOD of normal-folate diets (especially with cow milk) leads to an increment of the formation of hydroperoxides and CAT is also increased to neutralise and to scavenge the excessive production of $O_2^{\bullet-}$ in this tissue. The higher levels of CAT found in liver in situation of folate-supplementation can be due to the antioxidant and scavenging effect of folic acid, which protects hepatocytes from oxidative stress by decreasing homocysteine levels that are associated with oxidation and auto-oxidation of homocysteine, and increases the antioxidant activity of CAT (Yamakura et al. 1998; Handy et al. 2005). In addition, folate may exert antioxidant effects independent of homocysteine lowering by inhibiting NADPH oxidase-mediated superoxide anion production (Woo et al. 2006) and improves the total plasma antioxidant capacity (Alvares Delfino et al. 2007), scavenging the excessive free radicals produced due to the increased SOD activity and avoiding the progressive inactivation of CAT. This situation would need a concomitant increase in the GPx activity to remove the excess of these intermediary products highly harmful to the cell, a fact that leads to the progressive inactivation of the antioxidant activities, justifying the reduction in the activity of GPx, especially in the Fe-overload groups.

The increased SOD activity observed in our study with cow milk and Fe-overload may be a compensatory mechanism of the body to cope with the increase in oxidative stress, however, the increase in lipid peroxidation products levels (TBARS) in control and anaemic groups fed cow milk with Fe-overload, suggest an imbalance in the functioning of the enzymatic antioxidant defence system. Nevertheless, this increase is more significant in the animals receiving cow milk diet that in those fed goat milk diet. In addition, folate supplement has beneficial effects, avoiding

lipid peroxidation with both milk-based diets. Our results are in agreement with previously reported results highlighting that folic acid scavenges free radicals and their metabolic products, maintaining normal cellular physiology very efficiently and avoiding lipid oxidative damage (Gursu et al. 2004).

In the light of these results, it would be really positive to include folic acid supplemented goat milk in the diet of population consuming oral Fe supplements, because this would avoid the undesirable effects of Fe excess in the tissues.

Conclusion

In conclusion, Fe-overloaded cow milk induces hepatocellular damage, releasing both transaminases into the bloodstream and also increases oxidative stress in all the tissues, an issue that is not observed with goat milk. Dietary folate-supplemented goat milk reduces both plasma transaminases levels, suggesting a hepatoprotective effect of this type of milk and has beneficial effects in situation of Fe-overload, protecting cell from free radicals oxidative damage, improving the antioxidant enzymes activities and limiting lipid peroxidation

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