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Iron and exercise induced alterations in antioxidant status. Protection by dietary milk proteins

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Abstract

Lipid peroxidation stress induced by iron supplementation can contribute to the induction of gut lesions. Intensive sports lead to ischemia reperfusion, which increases free radical production. Athletes frequently use heavy iron supplementation, whose effects are unknown. On the other hand, milk proteins have *in vitro* antioxidant properties, which could counteract these potential side effects. The main aims of the study were: (1) to demonstrate the effects of combined exercise training (ET) and iron overload on antioxidant status; (2) to assess the protective properties of casein *in vivo*; (3) to study the mechanisms involved in an *in vitro* model.

Antioxidant status was assessed by measuring the activity of antioxidant enzymes (superoxide dismutase (SOD); glutathione peroxidase (GSH-Px)), and on the onset of aberrant crypts (AC) in colon, which can be induced by lipid peroxidation. At day 30, all ET animals showed an increase in the activity of antioxidant enzymes, in iron concentration in colon mucosa and liver and in the number of AC compared to untrained rats. It was found that Casein's milk protein supplementation significantly reduced these parameters. Additional information on protective effect of casein was provided by measuring the extent of TBARS formation during iron/ascorbate-induced oxidation of liposomes. Free casein and casein bound to iron were found to significantly reduce iron-induced lipid peroxidation. The results of the overall study suggest that Iron supplementation during intensive sport training would decrease anti-oxidant status. Dietary milk protein supplementation could at least partly prevent occurrence of deleterious effects to tissue induced by iron overload.

Keywords: *Exercise, iron overload, milk proteins, peroxidation, rat*

Introduction

Intensive practice of sports increases the risk of lipid peroxidation. During exercise, blood flow is redistributed to metabolically active muscles leading to ischemia of the splanchnic bed; at the end of the exercise, reperfusion induces a burst of reactive oxygen species (ROS) [1]. Iron excess is a potent inducer of peroxidation, which can overwhelm antioxidant defences and lead to tissue lesions; as such it contributes to the development of colon tumorigenesis [2]. However, there is no yet evidence that iron supplementation may improve performances in athletes, except in the case of iron deficiency [3]. High doses of iron are seldom used by athletes to

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increase haemoglobin synthesis, O_2 transport and, ultimately, to improve performance [4]. Effective and promising molecules have been elaborated to treat severe anaemia, but they are sometimes used for doping purpose. An iron supplementation is usually added to these drugs to enhance erythropoietin-induced erythropoiesis [4] and to prevent iron deficiency related to drug-induced increased erythropoiesis [5]. In the study performed by Deugnier et al. [6], one third of the French elite road cyclists were found to have hyperferritinemia at antidoping control test performed during the "Tour de France" race in 1998. Other reports showed similar results [7]. It is not known what health consequences may be associated with iron stores that elite sportsmen present long time after iron supplementation has been stopped [6,7].

Dietary antioxidants protect gut against iron-induced lipid peroxidation [8,9]. Milk is known to contain antioxidant factors [10]. It has been shown that caseins, the main cow milk proteins, exhibit antioxidant properties as inferred from *in vitro* experiments [11–16]. Several main protective mechanisms against oxidation reactions have been proposed. One involves that iron complexation by phosphoserine residues inducing resistance to enzyme digestion and pH variations preventing changes in Fe valence and free radical production [17,18]. It may be added that antioxidant properties of caseins are not lost once they are dephosphorylated [11,14]. Caseins can also bind Fe by their carboxyl groups, providing antioxidant properties and are able to inhibit oxygenases [11,14]; in addition, caseins have been shown to prevent lipoprotein peroxidation [19].

The objectives of this *in vivo* study were first to demonstrate that oxidative stress induced by dietary imbalance between antioxidant (vitamin E) and pro-oxidant (iron supplementation and exercise training (ET)) could overwhelm antioxidant defences and lead to tissue lesions. Secondly, we assessed the protective effect of a dietary intake of milk proteins (casein). Casein was given either free or bound to Fe, to assess the intraluminal effects of free iron which are prevented by its chelation.

Antioxidant status was assessed by measuring the activity of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px); since biological consequences of lipid peroxidation imbalance are known. The present study was devoted to the assessment of the induction of colonic aberrant crypts (AC) as lipid peroxidation-induced cell lesions in colonic mucosa. It may be mentioned that aberrant crypt foci are considered as preneoplastic lesions of colon cancer in rat [20,21] and human [22,23]. Additional information on the protective properties of casein, either free or bound to iron against lipid peroxidation was gained from an *in vitro* study.

Materials and methods

Protocol of the in vivo *study*

The protocol lasted 30 days, during which animals were fed with experimental diets that involved deprivation in vitamin E, supplementation with iron and variation in their protein content. Half of the control and experimental groups experienced an ET, to increase the lipid peroxidative stress induced by iron. At the end of the experiment, blood was drawn by retro orbital punction under light anaesthesia for a red blood cell count and the measurement of SOD and GSH-Px activities. SOD catalyses the dismutation of superoxide radicals ($O_2^{\cdot -}$) into hydrogen peroxide (H_2O_2) whereas GSH-Px catalyses the reduction of hydroperoxides by glutathione [24,25]. Then the animals were killed, colon was halved as described [9]; the proximal colon and the liver were removed, to measure Fe concentration. AC were counted in the colon mucosa.

Animals and diet (Table I)

Adult Sprague-Dawley rats, 3 months old (250–300 g), were housed in ventilated rooms at 21°C with a 12 h light: dark cycle. Animals were fed with one of the five experimental diets or the control diet (diet groups). Half of these groups experienced an ET.

The experimental powder diet (Dietex France, St Gratien) was free of vitamin E and supplemented with iron at a level previously used (560 mg Fe/kg diet as $Fe_2(SO4)_3$ [26]). The source of protein was casein (100 g/kg); fat was a standard mix consisting of coco oil (110 g/kg), evening primrose oil (10 g/kg) and colza oil (10 g/kg).

Table I. Composition of diets and experimental groups

	Experimental groups				Control group
	Diet 1 (n=14)	Diet 2 (n=14)	Diet 3 (n=16)	Diet 4 (n=16)	Diet 5 (n=14)
Trained rats	Diet 1 ET	Diet 2 ET	Diet 3 ET	Diet 4 ET	Control ET
Untrained rats	Diet 1	Diet 2	Diet 3	Diet 4	Control
Iron mg/kg diet	560	560	560	560	136
Vit E IU/kg diet	0	117	0	0	117
Protein g/kg diet	100 (casein)	100 (casein)	200 (casein)	100 (casein) + 100 g casein/iron complex	220 (soja + fish)

A first group ($n = 14$) was fed an experimental diet free of vitamin E and supplemented with iron (560 mg Fe/kg). Protein was casein (100 g/kg) (Diet 1). The second group ($n = 14$) was fed with the basal diet supplemented with 117 IU/kg of vitamin E (Diet 2) [8]. The third group ($n = 14$) was given the basal diet supplemented with 100 g/kg of casein (total protein content: 200 g casein/kg) (Diet 3). In the fourth group ($n = 16$), iron was chelated to casein (total protein content: 200 g casein/kg) (Diet 4). Addition of protein resulted in a dilution of the basal diet, so that vitamin E and iron contents had to be adjusted later to their set point. It may be pointed out that diets were isocaloric (basal diet: 2960 kcal/kg; protein enriched diet: 3060 kcal/kg). Any addition to basal diet was made in the laboratory. Experimental diets were prepared weekly. A control group (Diet 5) ($n = 14$) was also studied; animals were fed with a standard balanced maintenance diet for adult rodents, containing 22.3% proteins (soy and fish), 114 mg/kg Fe and 117 IU vitamin E/kg (Dietex France, Saint Gratien, France).

Preparation of casein/iron complex

Binding iron to casein was performed by mixing the protein with $FeCl_2$ solution (Fe/phosphopeptides molar ratio = 4) for 1 h at 37°C. Free iron was removed by diafiltration of the solutions on a 3 kDa ultrafiltration membrane. The amount of Fe bound to the casein was determined by atomic absorption spectrometry (Varian, Model AA 1275, Les Ulis, France) of freeze-dried samples. Distilled and deionized water was used for this purpose. All glassware and the polyethylene tubes used for the samples were washed and rinsed with distilled water.

Exercise protocol

Rats were familiarized and trained to swim for 10 min twice a day for 2 days before the beginning of the experiment. The ET consisted of 2 × 20 min swimming periods, 4 days per week during 30 days is derived from Metin [27].

Rats were used to swim by groups of 5, in a plexiglas pool, 50 cm in depth. Water temperature was maintained at 33–35°C. Every rat swam during the same time; care was taken to ensure that animals could not rest during swimming time.

Animals were at rest for 24 h before killing and subsequent blood sampling (day 30) in order to measure the effects of chronic training and not of an acute training.

Measurement of Cu, Zn-SOD activity

Since a positive correlation has been observed between isoenzymes of SOD [28], and as Cu, Zn-SOD is widely used in anti-oxidant studies [29,30], Cu, Zn-SOD activity was measured in blood. For this purpose, xanthine and xanthine oxidase were used to generate superoxide radicals that react with 2-(4-iodophenyl)-3-5-phenyltetrazolium chloride to form red formazan dye. SOD activity was then measured by assessing the extent of inhibition of the reaction (Kit RANSOD; Laboratoires Randox, Montpellier-Fréjorgues, France).

Measurement of GSH-Px activity

GSH-Px was measured in blood by an UV method adapted from the protocol developed by Paglia and Valentine [31]. Oxidation of GSH by GSH-Px was achieved using cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidised glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. GSH-Px activity was measured by the decrease in absorbance at 340 nm (Kit RANSEL, Laboratoires Randox, Montpellier-Fréjorgues, France). The use of cumene hydroperoxide as the substrate of GSH-Px allowed the determination of both seleno-dependent and non seleno-dependent GSH-Px activities.

Measurement of tissue iron

Fe was measured by atomic spectrometric absorption (Perkin-Elmer 3030, F-91945 Courtaboeuf, France) in liver and right colon mucosa after the tissues were digested with nitric acid at ambient temperature during 24 h. Ringer–Lavoisier solute was used as the blank.

Histological study

The colon was removed, opened along the longitudinal median axis and fixed in 10% buffered formalin for 24–48 h. Then the tissue was rolled up and embedded in paraffin, cut at 5 μm, and routinely stained with hematoxylin-eosin [32]. Crypts were considered aberrant when they showed epithelial thickening, slit-like lumen, nuclear atypia, stratification and loss of mucosecretion. An increased number of crypt mitoses was also observed in severely dysplastic crypts [33]. AC were quantified twice blindly, under the microscope, at a magnification of 40× [34]. As every colon contained a different number of crypts, results were expressed per 800 crypts really counted.

In vitro *study*

The antioxidant capacity of casein/iron complex was estimated by determining the 2-thiobarbituric acid

reactive substances (TBARS) using the malonaldehyde method [35]. The following concentrations were used:

3.3×10^{-4} M of iron either free or in a bound form and 8.4×10^{-5} M of caseins.
1.6×10^{-4} M of iron and 4.2×10^{-5} M of caseins.

Statistical analysis

In vivo study: The experimental and control groups were compared using an ANOVA method together with a *post hoc* test (Fisher test). Values were expressed as mean (± SEM) and the level of significance was set at $p \leq 0.05$. To assess the relationship between systemic antioxidant defences and mucosal lesions a regression analysis was performed between the count of AC and the activity of antioxidant enzymes.

A *t*-test was performed between diet and ET diet groups to assess the influence of exercise.

In vitro study: *t*-Tests were performed between the two iron concentrations; an ANOVA approach was performed between the 4 experimental forms of iron, followed by *t*-tests.

Results

No significant differences in animal weights were observed at the end of the experiment.

Figures 1 and 2 display the measured activity of antioxidants enzymes (SOD and GSH-Px). Iron load and ET were found to lead to a significant increase in antioxidant enzyme activity for all experimental groups. The increase in antioxidant activity level that was correlated with iron load and ET was not observed in the control group. Vitamin E and casein's supplementation was found to modulate the effects of ET and iron load on antioxidant status.

The iron concentrations in liver and right colon mucosa are reported in Tables II and III. Iron load was found to increase iron liver content in all experimental groups. In contrast, the presence of free casein led to the decrease in the iron liver accumulation level compared to vitamin E (Diet 2 group). ET was found to increase iron liver content in Diet 1 and 2 groups. Iron colon content was shown to be only increased in Diet 1 and Diet 1 ET groups.

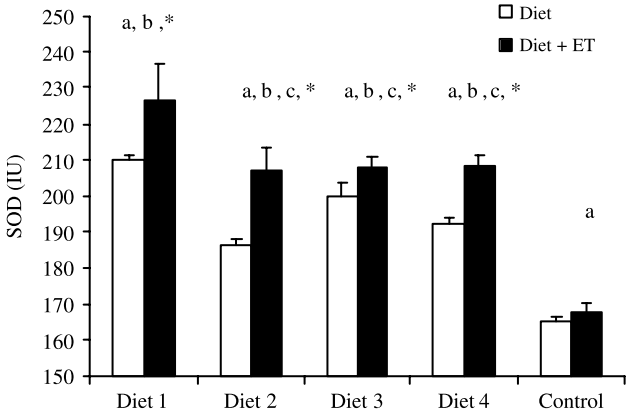
The number of measured AC in right colon for the different diets is shown in Figure 3. The number of AC in colonic mucosa was found to be increased by iron loading and ET. This increase was efficiently prevented by either vitamin E or free protein supplementation. Iron-chelated proteins had an increased efficiency compared to free casein, with and without ET.

The onset of colon AC was found to be significantly correlated to Fe concentrations into liver and colon and to SOD activity (Table III and Figure 4).

The results of the *in vitro* study are reported in Figure 5. The extent of peroxidation of liposomes was higher at 3.3×10^{-4} M than at 1.6×10^{-4} M iron. Peroxidation levels were similar for the two iron chlorides but inhibited by caseins. Free caseins were found to exhibit higher antioxidant properties than iron bound caseins.

Discussion

High iron supplementation that is often provided to sportsmen results in high tissue storage [6,7].



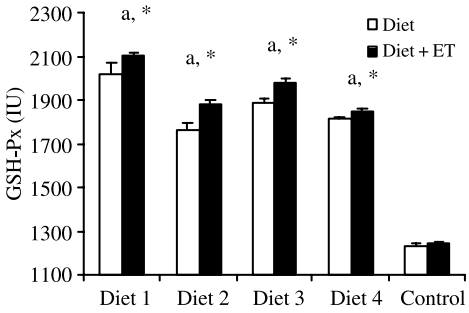
ANOVA treatment of experimental and control groups in each row; Fisher test between groups: ($p<0.05$).

Diet: F= 543; $p<0.0001$; [a] all groups different from each other

Diet + ET: F= 121; $p<0.0001$; [b]≠ Control group; [c]≠ Diet 1.

Student test between groups with and without ET: SOD *: Diet ≠ Diet + ET ($p<0.05$)

Figure 1. Activity of SOD.



GSH-Px:

Diet: F = 950; p<0.0001; [a] all groups different from each other

Diet + ET: F = 4388; p<0.0001; [a] all groups different from each other

Student test between groups with and without ET: GSH-Px *:Diet ≠ Diet + ET(p<0.05)

Figure 2. Activity of GSH-Px.

However, there is almost a complete lack of information on the biological consequences of this process. The present study shows that a simple imbalance between antioxidant (vitamin E) and pro-oxidant agents (iron and ET) was sufficient to promote preneoplasic lesions, in the absence of any chemical carcinogen. It may be added that the effects of iron overload were increased by the oxidative stress caused by physical exercise.

As expected, vitamin E deficiency associated to iron load was found to increase the level of activity of endogenous antioxidant enzymes including SOD and GSH-Px [26,35–38]. These changes may be explained by iron loading [13,34,38–40]. Vitamin E was shown to reduce antioxidant response [9], and also to decrease Fe tissue storage in liver and colon mucosa. This suggests that iron metabolism and ROS may interact. Thus, chronic oxidative stress may modulate iron uptake and storage, leading to a self-sustained spiral of cytotoxic and mutagenic event response catalysed by free Fe itself [33]. Recent findings by Theurl et al. [41] provided confirmation that experimental iron overload results in iron accumulation in hepatocytes. The same authors also observed that the transfer of iron from the gut to the circulation was diminished by hepcidin action [41]. Thus, free iron should cause deleterious effects on tissue leading to the induction of lesions [42].

In human, a therapeutic dose of 80 mg represents at least four times the usual dietary intake (12–15 mg/day). The athletes' intake is often higher with doses which may higher than 200 mg/day [2,43]. In the present study, iron doses were three times above usual intakes. It may be noted that AC tissue lesions developed within 4 weeks, a period that is the shortest one reported in the literature. These results suggest that an imbalance between nutrients (more than iron loading alone) is responsible for the observed peroxidation-induced lesions and cancer. Intensive sport practice could contribute to reveal latent nutritional deficiencies. Consequently, it could be useful as a preventive action to enhance the protective potential of diet.

Table II. Colon and liver iron content

		Diet 1	Diet 2	Diet 3	Diet 4	Control
Liver	Diet	156 ± 8.4^{b}	$97 \pm 7^{b,c}$	$94 \pm 7^{b,c}$	$100 \pm 6^{b,c}$	49 ± 3
	Diet + ET	$181 \pm 9^{b,\star}$	$130 \pm 10^{b,\star}$	$101 \pm 11^{b,c,d}$	$109 \pm 9^{b,c,d,\star}$	49 ± 5
Colon	Diet	79 ± 8	61 ± 11^{c}	$64 \pm 15^{b,c}$	59 ± 12^{c}	51 ± 5^{c}
	Diet + ET	$114 \pm 19^{c,\star}$	57 ± 13^{c}	$63 \pm 4^{b,c}$	60 ± 11^{c}	51 ± 5

ANOVA treatment of experimental and control groups in each row; Fisher test between groups ($p < 0.05$).
Liver Iron: Diet, $F = 257$; $p < 0.0001$; [b] ≠ control group; [c] ≠ Diet 1. Diet + ET, $F = 254$; $p < 0.0001$; [b] ≠ control group; [c] ≠ Diet 1; [d] ≠ Diet 2.
Colon Iron: Diet, $F = 830$; $p = 0.0003$; [b] ≠ control group; [c] ≠ Diet 1. Diet + ET, $F = 41$; $p < 0.0001$; [b] ≠ control group; [c] ≠ Diet 1.
Student test between groups with and without ET: Liver Iron, Colon Iron: ⋆Diet ≠ Diet + ET ($p < 0.05$).

Table III. Factors associated with the increase in colon AC

	T	*p*
[Fe] liver	3.9	0.002
[Fe] colon	6.1	<0.0001
SOD J30	2	0.047
GSH-Px J30	0.4	NS

multiple regression between the number of AC and iron concentration [Fe] in liver, colon mucosa, blood SOD and GSH-Px activities at the end of the experiment ($F = 162$; $p < 0.0001$).

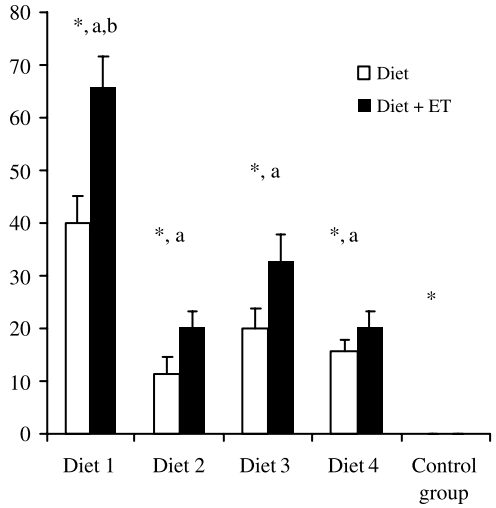
Dietary proteins could be one of these active components.

The antioxidant properties of caseins are known from previous *in vitro* investigations. In the present *in vivo* study, casein was found to decrease both Fe loading and AC development. The mechanisms of antioxidant action are complex, and are shared by whole proteins and their digestion-derived peptides. They are able to chelate Fe and favour the auto-oxidation of the metal by the phosphoseryl residues. In addition, other mechanisms than mineral-binding could be involved [12,13,44]: Caseins have the ability to scavenge free radicals and to inhibit the activity of lipoxygenases [11–14]. The chelation of iron enhanced the effects of casein; however, it did not change the iron content of tissues, suggesting that the latter protein does not mainly operate by decreasing absorption of iron, but rather by exerting an antioxidant activity. This could occur either in gut lumen [44] or in the body [19]. The relationship found in our study between the onset of ACF and liver iron concentration suggests an important role for systemic effects of casein: The *in vitro* study showed that either free or iron bound caseins behave as antioxidant against the peroxidation of a membrane model. The slight difference between the efficiency of free and bound protein observed in the *in vivo* and *in vitro* experiments suggests that the metabolism of casein could differ *in vivo*. Other properties, such as immuno-stimulating activity of peptides derived from casein could contribute to its protective effect on preneoplasic lesions [45].

Conclusions

An imbalance between pro- and anti-oxidants components of the diet gives rise to an oxidative stress which is able to promote the occurrence of preneoplasic lesions of the gut. ET was found exacerbates the deleterious of iron loading. The present results raise doubts on the iatrogenic effects of iron overload and EPO in all endurance sports; a follow up of iron and peroxidative status should be made in athletes supplemented with iron.

Dietary milk protein casein has a protective effect on AC and could be used by athletes as a diet component to reduce the consequences of iron overload associated with strenuous exercise.



ANOVA treatment of experimental and control groups in each row; Fisher test between groups: ($p<0.05$).

Diet: F= 343; $p<0.0001$; * all groups different from each other

Diet + ET: F= 317; $p<0.0001$; [a] Every experimental group ≠ Control group;[b] Diet 1 ≠ Every other group.

Figure 3. Number of AC in the right colon.

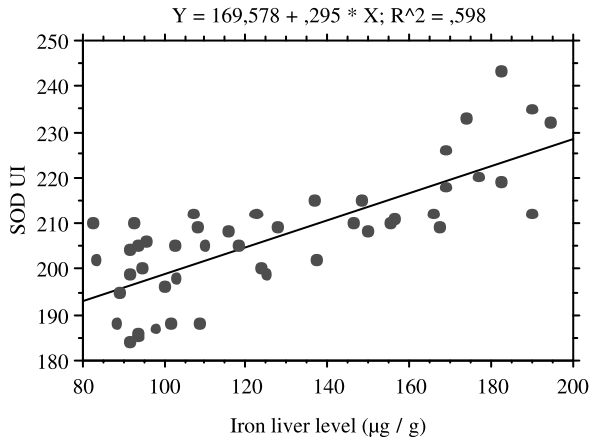


Figure 4. Relationship between iron liver content and SOD activity.

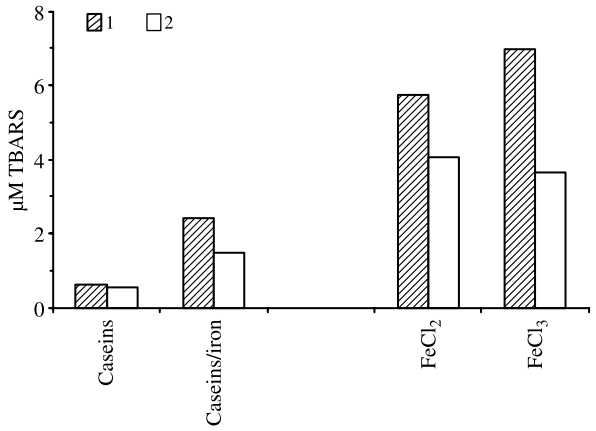


Figure 5. *In vitro* assessment of the protective effect of casein (free or bound to iron) against iron-induced peroxidation of liposomes. TBARS: 2-thiobarbituric acid reactive substances (1): 3.3×10^{-4} M of iron either free or in its complexed form and 8.4×10^{-5} M of caseins. (2): 1.6×10^{-4} M of iron and 4.2×10^{-5} M of caseins vs (2): $p < 0.05$ for caseins/iron, $FeCl_2$, $FeCl_3$. ANOVA: $p < 0.05$ *t*-tests: Caseins < caseins/iron < $FeCl_2$, $FeCl_3$.

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