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RESEARCH ARTICLE

Erythrocyte antioxidant defenses as a potential biomarker of liver mitochondrial status in different oxidative conditions

M. Pilar Valdecantos[1], Patricia Pérez-Matute[1,2], Pedro Luis Prieto-Hontoria[1], Elena Sánchez-Campayo[1], María Jesús Moreno-Aliaga[1], and J. Alfredo Martínez[1]

[1]*Department of Nutrition, Food Science, Physiology and Toxicology University of Navarra, Pamplona, Spain, and* [2]*HIV and Associated Metabolic Alterations Unit, Infectious Diseases Area, Center for Biomedical Research of La Rioja (CIBIR), Logroño, Spain*

Abstract

The need for minimally invasive biomarkers to predict the progression of non-alcoholic fatty liver disease to non-alcoholic steatohepatitis is a priority. Oxidative stress and mitochondrial dysfunction contribute in this physiopathological process. The aim of this study was to analyze the potential role of erythrocytes as surrogate biomarkers of hepatic mitochondrial oxidative status in an animal model under different dietary oxidative conditions. Interestingly, we found that erythrocyte antioxidant status correlated with triglyceride content ($p < 0.05$–$p < 0.001$), thiobarbituric acid reactive species levels ($p < 0.001$) and with liver mitochondrial antioxidant levels ($p < 0.001$). These data suggest that erythrocyte antioxidant defenses could be used as sensitive and minimally invasive biomarkers of mitochondrial status in diverse oxidative conditions.

Keywords: High fat diet, obesity, NASH, oxidative stress, steatosis

Introduction

Currently, the diagnosis of non-alcoholic steatohepatitis (NASH) requires liver biopsy with corresponding drawbacks of sampling and interpretation errors (Adams & Feldstein 2011). Hence, the need for minimally invasive methods to cover the whole spectrum of non-alcoholic fatty liver disease (NAFLD) is a priority. In fact, several investigations have focused on diagnostic methods, such as breath markers (Solga et al. 2006; Millonig et al. 2010), but the results are inconclusive and further studies are needed. It is also necessary for future research to develop less invasive biomarkers to identify patients at risk of liver disease progression.

The NAFLD is generally considered as one of the main causes of hepatic dysfunction linked to obesity and an important manifestation of metabolic syndrome (Rector et al. 2010; Cohen et al. 2011). In obese individuals, the increased supply of free fatty acids from diet, from adipose tissue and through increased "*de novo*" lipogenesis all serve to promote hepatic steatosis (Donnelly et al. 2005). Moreover, visceral adipose tissue seems to play a major role by secreting hormones and adypokines that contribute to the progression of NAFLD to NASH (Wree et al. 2011).

The role of oxidative stress as a key contributor to the transition from NAFLD, usually asymptomatic, to NASH has been widely accepted (Pessayre 2007). On the other hand, another major factor involved in the pathophysiological evolution of NAFLD to NASH is the impairment in mitochondrial function, which is linked with an increase of oxidative stress.(Perez-Carreras et al. 2003; Rector et al. 2010). In this sense, the mitochondrion is a major cellular site involved in energy metabolism (Brand 2010), but it is also the main source of reactive oxygen species (ROS). Furthermore, and in order to counterbalance the negative effects of ROS, mitochondria also contain enzymatic antioxidant defenses, such as manganese superoxide dismutase (SOD2) (Brunet et al. 2004), mitochondrial glutathione peroxidase (mtGPx) (Lowes & Galley 2011), and mitochondrial glutathione (Canto et al. 2009). When

Address for Correspondence: J. Alfredo Martínez, Department of Nutrition and Food Sciences, Physiology and Toxicology, University of Navarra, Irunlarrea 1, 31008 Pamplona, Spain. Tel: +34 948425600 (6424). Fax: +34 948425649. E-mail: jalfmtz@unav.es

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Abbreviations

ANOVA, analysis of variance;
GPx, glutathione peroxidase;
GSH, reduced glutathione;
GSSG, oxidized glutathione;
HFD, high-fat diet;
LA, α-lipoic acid;
MDA, malondialdehyde;
mtDNA, mitochondrial DNA;
mtGPx, mitochondrial glutathione peroxidase;
NAFLD, non-alcoholic fatty liver disease;
NASH, non-alcoholic steatohepatitis;
PCR, polymerase chain reaction;
q-RTPCR, quantitative real time polymerase chain reaction;
ROS, reactive oxygen species;
SOD2, manganese superoxide dismutase;
SOD, superoxide dismutase;
TBARS, thiobarbituric acid reactive species;
TG, triglyceride;
WAT, visceral white adipose tissue;

the balance between mitochondrial ROS generation and the antioxidants occurs, an increase in mitochondrial oxidative stress is observed, which leads to impaired mitochondrial function (Simula & De Re 2010; Ozden et al. 2011).

Erythrocyte antioxidant status has been described as a biomarker of general oxidative stress (Kalahasthi et al. 2006; Pavao et al. 2006). In fact, several studies have used antioxidant defenses, for example, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities as predictors in the development of oxidative stress linked to disorders, such as cancer (Monari et al. 2006) or neurodegenerative diseases (Torsdottir et al. 2010). Thus, we investigated if erythrocytes could also be potential biomarkers of antioxidant status using an animal model under different oxidative conditions: obesity and obesity-treated with alimentary antioxidant, α-lipoic acid (LA).

Methods

Animals and diets: Male Wistar rats ($n = 40$) aged 6 weeks were supplied from the Centre of Applied Pharmacology (CIFA, Pamplona, Spain). Animals were housed in cages in a temperature-controlled room (22 ± 2°C) with a 12-hour light–dark cycle, fed a pelleted chow diet and given deionized water *ad libitum* for an adaptation period of 5 days. After this period, rats were assigned to four experimental groups for 8 weeks. Control (C) and CLIP (control + LA) groups were fed a standard diet (Harlam Tekland Global Diets, USA) containing 4.6% of energy as a lipids per dry weight. The Obese (OB) and OLIP (obese + LA) were fed a high-fat diet (HFD) containing 60% of energy as a lipids per dry weight. The diet of the CLIP and OLIP subgroups was supplemented with α-LA (Sigma Aldrich, USA) in a proportion of 0.25 g/100 g of diet as previously described (Prieto-Hontoria et al. 2009).

Body weight and food intake were recorded every 2–3 days. At the end of the experimental period, rats were euthanized by decapitation and blood and tissue samples were immediately collected, frozen in liquid nitrogen and kept at –80°C until analysis. All experimental procedures were performed according to National and Institutional Guidelines for Animal Care and Use at the University of Navarra.

Tissue homogenization procedure and mitochondrial isolation: Livers were quickly excised after sacrificing the animals and either frozen in liquid nitrogen or placed in ice-cold buffer (250 mM sucrose, 1 mM EDTA, and 5 mM NaTES, pH = 7.4). Fresh tissue placed in buffer was used to prepare mitochondrial suspensions according to the method of Rickwood et al. (1987) with slight modifications as described elsewhere (Valdecantos et al. 2010).

Erythrocyte concentrates: Erythrocyte concentrates were obtained after centrifugation in Vacutainer tubes containing gel without additives (Vacutainer Gel SST II) and pellets were resuspended in PBS and kept at –80°C.

Hepatic triglyceride content: To determine the hepatic triglyceride (TG) content, 150 mg of these tissues were sonicated in a Branson 250 Sonifier (Duty cycle 40%; Output control 4; Hold Continuous) for 40 s in 1.5 mL of buffer (150 mM NaCl, 0,1% Triton and 10 mM Tris (pH8)) at 50°C. After centrifugation at 12,000*g* for 10 min, the obtained supernatants were used to measure the TG levels with a COBAS-Mira analyzer (Roche Diagnostics, Switzerland) using an enzymatic kit (Horiba ABX, USA) and a COBAS-Mira analyzer (Roche Diagnostics, Switzerland) as described elsewhere (Perez-Echarri et al. 2009).

Lipid peroxidation: The formation of thiobarbituric acid reactive species (TBARS) derived from the reaction with reactive aldehydes, such as malondialdehyde (MDA), from the decomposition of the unstable peroxides was spectrophotometrically quantified following their controlled reaction with thiobarbituric acid. Changes in absorbance were measured in liver homogenate with a commercial kit (Cayman Chemical, Michigan, USA) according to the manufacturer's instructions with slight modifications and using a Luminoskan Ascent spectrophotometer (Thermo Electron Corporation, USA). Results were corrected by the amount of tissue in milligrams (mg).

Real-time quantitative polymerase chain reaction: Total RNA was isolated from liver using Trizol® reagent (Invitrogen, Carlsbad, California, CA, USA) according to the manufacturer's instructions. The RNA concentrations were determined by Nanodrop 1000 (Thermo Electron Corporation, Foster City, California, CA, USA). To avoid contamination with genomic DNA, DNA digestion and inactivation were assessed using the DNAse kit (Ambion

Inc, St. Austin, Texas, TX, USA). A total of 4 μg of RNA were transcribed to cDNA using the M-MLV kit (Invitrogen) following instructions from the suppliers.

Mitochondrial oxidative damage estimation: Mitochondrial DNA (mtDNA) oxidative damage was estimated as the ratio of 80-bp fragment (260–339 position), compared to a 162-bp fragment (260–421 position) of the same sample. For real-time polymerase chain reaction (PCR) reactions of both fragments, we used SYBR Green qPCR Master Mix with ROX as reference dye (Invitrogen, Carlsbad, California, CA, USA) in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, California, CA, USA). The primers used were HVII-FOR260 (5′-GCCACTTTCCACACAGACATCATA-3′), HVII-L421 (5′-AGTGCATACCGCCAAAA GATAAA-3′) and HVII-C339 (5′-TGTTTAAGTGCTGTGGCCAGA-3′) from Sigma Aldrich (Sigma Aldrich, St. Louis, Missouri, MO, USA). Both fragments were amplified in a total volume of 10 μL with 15 ng of total DNA, 5 μL SYBR Green Mix, and 10 μM of HVII-FOR260 and HVII-L421 for large fragments and 2.5 μM HVII-FOR260 and HVII-C339 for short fragments. The PCR conditions were 2 min at 50°C, 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min.

Measurement of antioxidant defences: Total SOD and GPx activities were measured in erythrocyte concentrates. The SOD2 and mitochondrial GPx activities were assessed in isolated rat liver mitochondria. Enzymatic colorimetric activity kits (Assay Designs Inc, Michigan, MI, USA) were used in these determination following the manufacturer's instruction with slight modifications as described previously (Valdecantos et al. 2010). Total, reduced (GSH) and oxidized (GSSG) glutathione levels were measured in the same samples using a colorimetric assay (Assay Designs Inc, Michigan, MI, USA). The ratio between GSH and GSSG was also calculated as a marker of antioxidant status in both erythrocytes and the hepatic mitochondrial compartment.

Statistical analysis: The effects of diet, LA treatment, or interaction were evaluated using a two-way analysis of variance (ANOVA) test. When interactions were significant, the effects were evaluated using one-way ANOVA followed by the Bonferroni post-hoc test. Relationships between variables were analyzed by calculating Pearson correlation coefficients as all variables followed a normal distribution as verified by two different methods (Kolmogorov–Smirnov and Shapiro–Wilk tests). All statistical analysis was performed using the GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). Differences were considered as statistically significant at $p < 0.05$. Variables analyzed and presented in figures are unique and did not require a multiple comparison correction.

Results

Effects of HFD and LA on the liver: As expected, significant increases in body weight gain and ectopic fat accumulation in the liver were observed. Increase in hepatic oxidative damage was also observed in obese rats after ingestion of a HFD. These effects were significantly prevented by LA supplementation (Table 1). Thus, LA supplementation of standard diet also diminished body weight gain, hepatic TG content, and TBARS levels as compared with the control group. Interestingly, a strong interaction between diet and treatment was observed in the actions of LA on ectopic fat storage in the liver and in hepatic oxidative damage (Table 1).

Effects of HFD and LA on oxidative generated mtDNA damage: We analyzed oxidative-generated mtDNA damage by quantitative real time polymerase chain reaction (q-RTPCR) and observed that, as expected, high fat intake induced an increase in oxidative injury to mtDNA (Table 1), which was completely reversed by LA treatment. This protective effect was independent of type of diet, as statistically significant differences between the C and CLIP group were found ($p < 0.001$).

Erythrocyte antioxidant defences in obesity and LA-treated animals: The analysis of antioxidant defences in erythrocytes revealed that the consumption of HFD induced a significant inhibitory effect (–51.32%) in SOD dismutase activity (Figure 1A), although no significant effects were observed in GPx activity (Figure 1B) or in the GSH:GSSG ratio (Figure 1C). In contrast, LA supplementation is able to reverse the effects of obesity in SOD activity (+122.44% *versus* OB group) as well as to induce an increase in the activity of this enzyme when

Table 1. Effects of diet and lipoic acid on obesity and oxidative injury markers.

					ANOVA 2×2			
	CONTROL	CLIP	OBESE	OLIP	DIET	LA	D*LA	ANOVA
Initial body weight (g)	215 ± 20.3	215 ± 22.3	218 ± 17.8	211 ± 21.6	n.s	n.s	n.s	0.9206
Body weight gain (g)	177 ± 26.3	106 ± 20.7	264 ± 36.9	167 ± 38.9	< 0.001	< 0.001	n.s	< 0.001
Liver TG s (%)	3.6 ± 1.05	2.5 ± 0.65*	9.2 ± 0.78***	2.5 ± 0.63$^{*,\#\#\#}$	< 0.001	< 0.001	<0.001	< 0.001
TBARS (μmoles/mg tissue)	4.6 ± 0.50	3.3 ± 0.50***	6.9 ± 7.74***	2.9 ± 0.47$^{***,\#\#\#}$	< 0.001	< 0.001	<0.001	< 0.001
mtDNA oxidative injury	0.76 ± 0.05	0.66 ± 0.05	0.84 ± 0.06	0.75 ± 0.04	< 0.01	< 0.001	n.s	< 0.001

Values are means ± SE; $^{*}p < 0.05$, $^{***}p < 0.001$ *versus* control group; $^{\#\#\#}p < 0.001$ *versus* obese group according to one way ANOVA followed by post-hoc multiple comparisons correction with Bonferroni test.

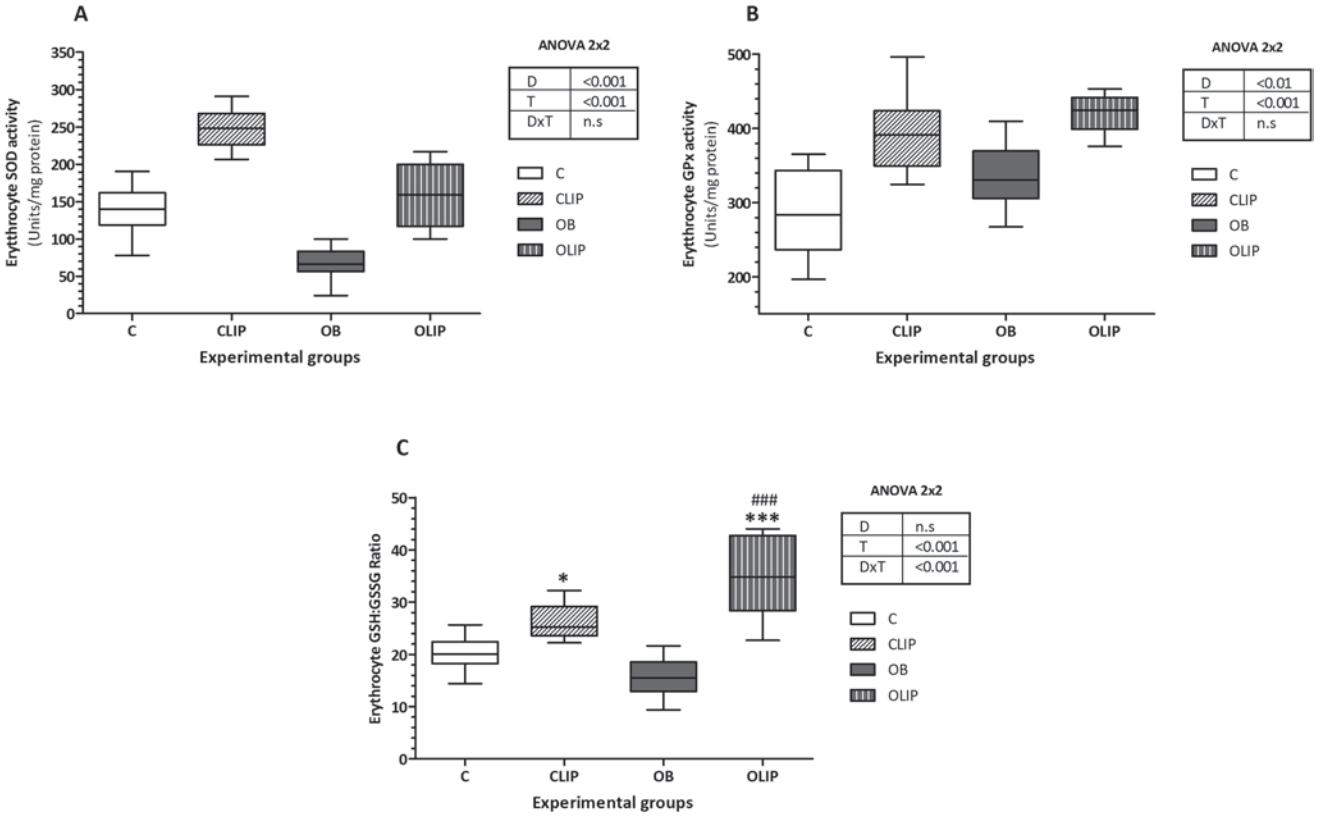

Figure 1. Effects of HFD and LA on erythrocyte antioxidant defences; (A) SOD activity, (B) GPx activity, and (C) GSH:GSSG ratio. Values are mean ± SE; $^{*}p < 0.05$, $^{***}p < 0.001$ *versus* control group; $^{\#\#\#}p < 0.001$ *versus* obese group according to one way ANOVA followed by post-hoc multiple comparisons correction with Bonferroni test.

supplemented in a standard diet (+78.78%). Moreover, LA induced an important increase in GPx activity and also in the GSH:GSSG ratio when it was added to the standard diet and also in the HFD regimen. Interestingly, a important interaction ($p < 0.001$) between diet and treatment was observed in the GSH:GSSG ratio values (Figure 1C). Thus, the stimulatory effects of this molecule are stronger when it is administered with HFD (29.93% in CLIP *versus* 126.63% in OLIP).

Effects on liver mitochondrial antioxidant defences in obesity and LA-treated animals: A statistical effect of HFD on SOD2 activity ($p < 0.01$) was found. In fact, the activity of this enzyme was inhibited (–26.29%) in obese animals. Moreover, the effect of LA on this enzyme was higher than the HFD ($p < 0.001$) and was able to activate SOD2 when given with a standard diet (34.35%) and also with a HFD (50.50%). As for the effect of HFD and LA on mtGPx activity and the GSH:GSSG ratio, our data showed an important interaction between HFD and LA ($p < 0.05$; $p < 0.001$, respectively). Therefore, HFD induced a strong reduction in mtGPX (30.91%) and also in the GSH:GSSG ratio (56.19%). Moreover, LA was able to reverse these effects on mtGPX and the GSH:GSSG ratio and increase these values to higher levels (42.82%; 139.88%, respectively, OLIP *versus* C). Although, LA was also able to stimulate significantly mtGPX activity (36.75%; $p < 0.05$) and the GSH:GSSG ratio (114.32%; $p < 0.001$) when included in a standard diet, our data showed that these effects were stronger with HFD suggesting that LA actions could be modulated by the fat content of the diet.

Correlations between erythrocytes and liver mitochondrial antioxidant defences: Erythrocyte antioxidant defences significantly correlated with the hepatic TG content (Figure 2). Additionally, and more importantly, hepatic TBARS levels (Figure 3) and mtDNA damage (Figure 4), a robust markers of oxidative damage, were highly associated with SOD and GPx activities and also with the GSH:GSSG ratio in erythrocytes (Figure 2), which could suggest the potential role of erythrocyte antioxidant defences as biomarkers of liver oxidative damage. Interestingly, all antioxidant defences assessed in erythrocytes and in the hepatic mitochondrial compartment were positively correlated (Figure 5).

Discussion

The spectrum of NAFLD features ranges from asymptomatic steatosis to NASH and cirrhosis (Krawczyk et al. 2010). In general, the prognosis for simple steatosis is very good; however, NASH can progress to cirrhosis and hepatocellular carcinoma in 10% to 15% of patients (Rombouts & Marra 2010). Moreover, noninvasive methods (e.g. abdominal ultrasonography) are safe ways to support a diagnosis of hepatic steatosis, but advanced liver histopathologic findings, including NASH and fibrosis, cannot be identified without performing liver

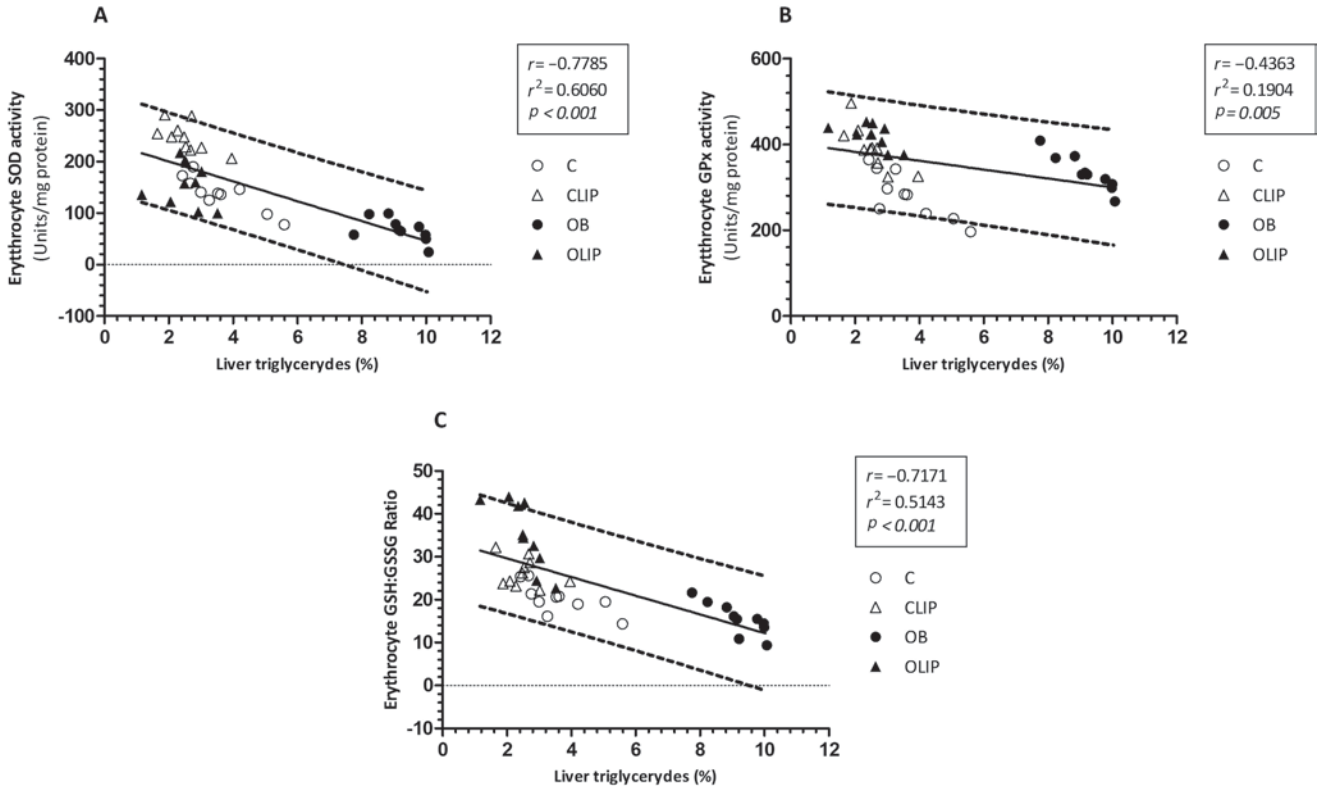

Figure 2. Correlation between erythrocyte antioxidant defences and liver TG content; (A) SOD activity, (B) GPx activity, and (C) GSH:GSSG ratio; r = Pearson correlation coefficient; r^2 = goodness of fit.

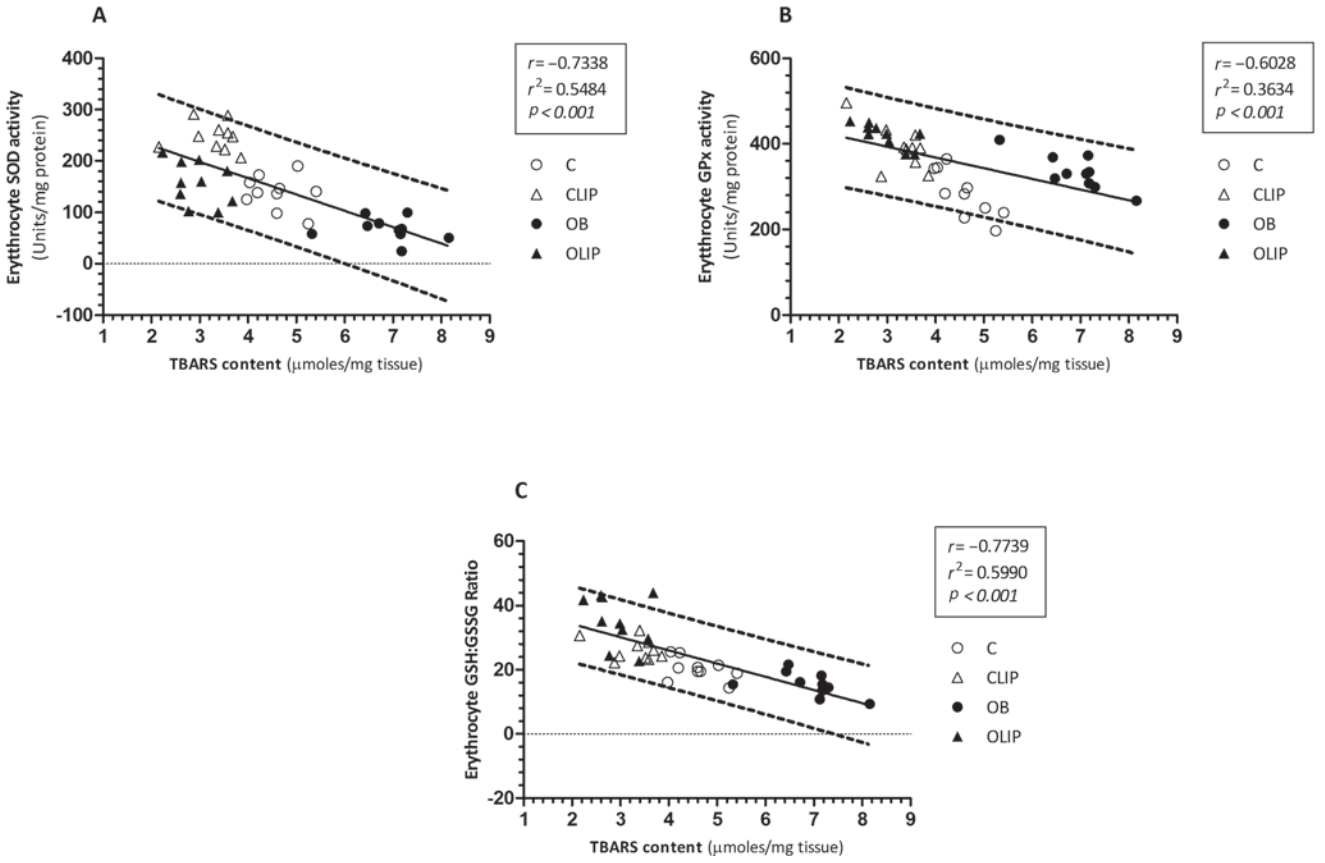

Figure 3. Correlation between erythrocyte antioxidant defences and TBARS content in liver; (A) SOD activity, (B) GPx activity, and (C) GSH:GSSG ratio; r = Pearson correlation coefficient; r^2=goodness of fit.

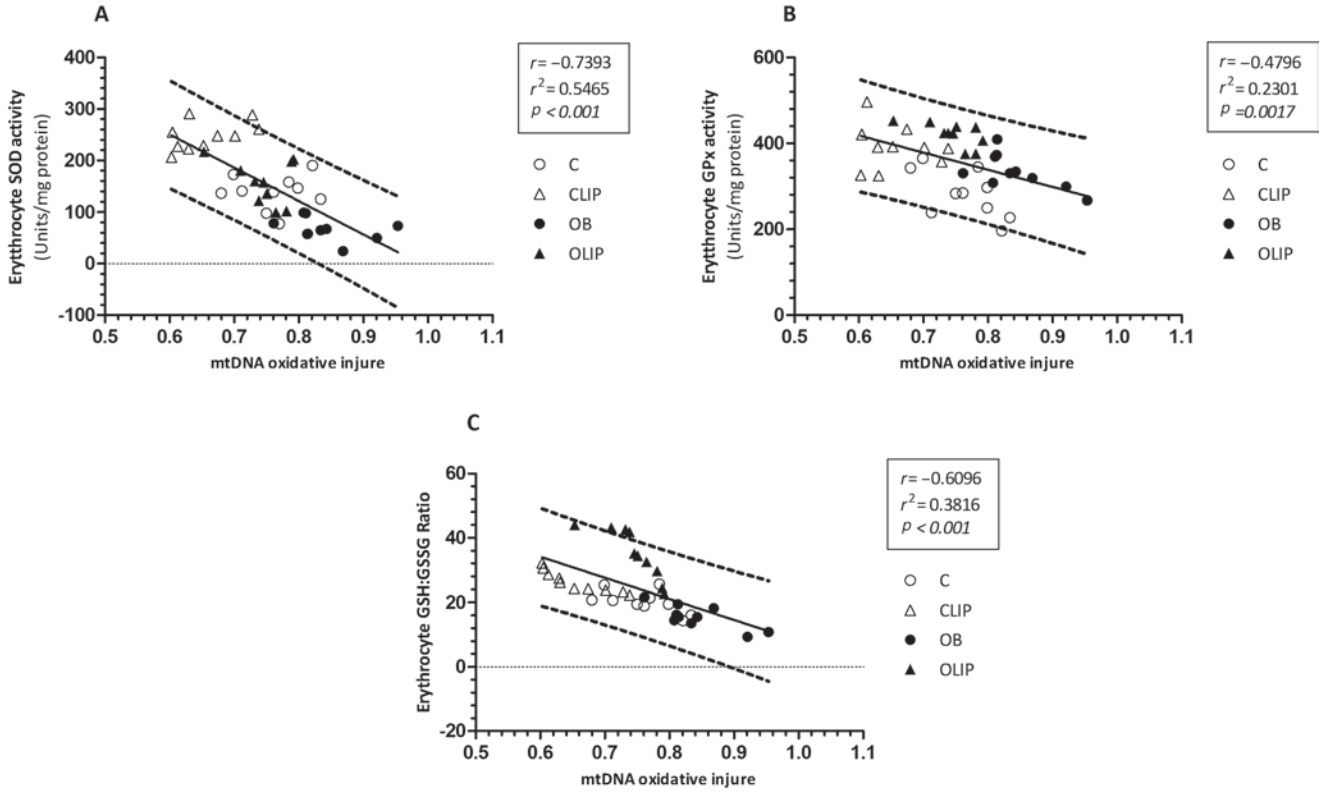

Figure 4. Correlation between erythrocyte antioxidant defences and mtDNA oxidative injury in the liver; (A) SOD activity, (B) GPx activity, and (C) GSH:GSSG ratio; r = Pearson correlation coefficient; r^2 = goodness of fit.

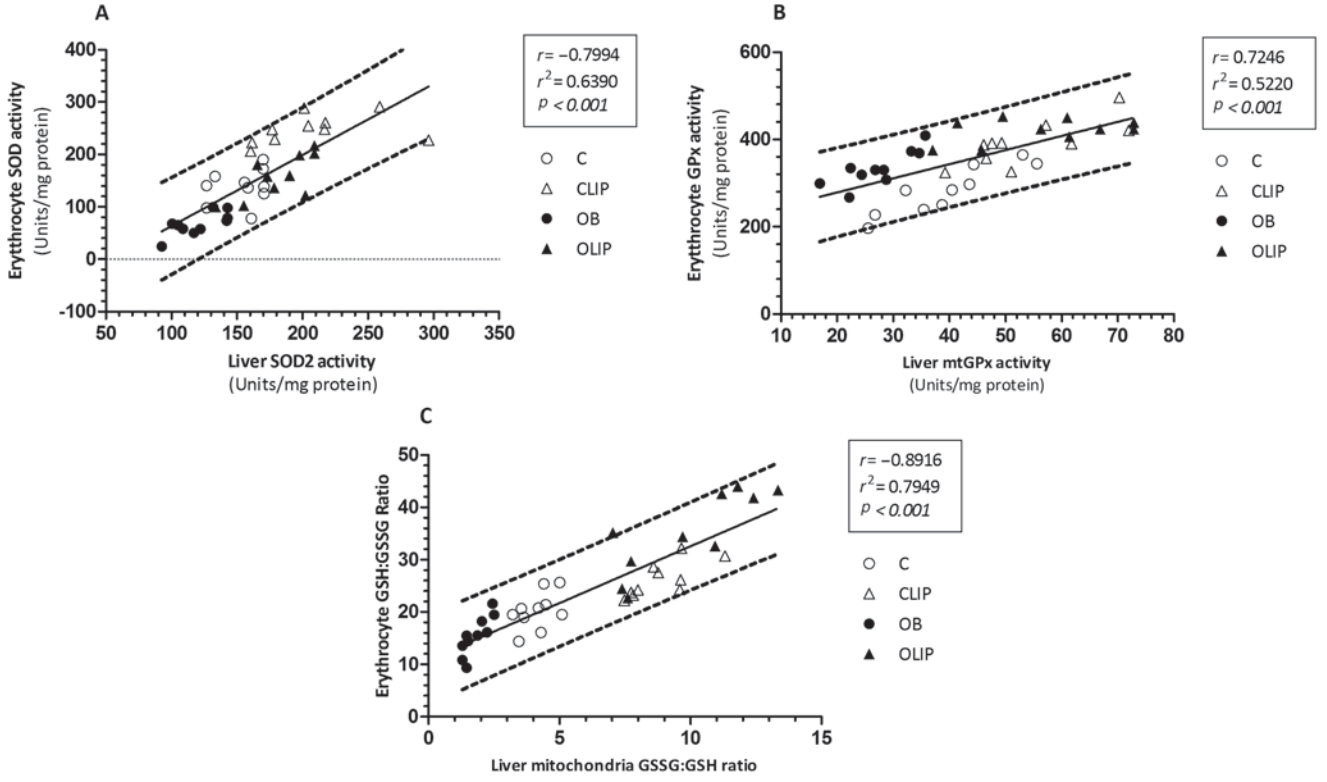

Figure 5. Correlation between the main antioxidant defences analyzed in erythrocytes and in the hepatic mitochondrial compartment; (A) SOD activity, (B) GPx activity, and (C) GSH:GSSG ratio; r = Pearson correlation coefficient; r^2 = goodness of fit.

biopsies (Cortez-Pinto & Camilo 2004; Adams & Feldstein 2011). Given the positive prognosis of steatosis and the difficulty in treating NASH, it is important to find noninvasive methods to predict the progression of NAFLD to NASH based on the pathogenesis of NAFLD/NASH. From this point of view, our study reports, for the first time, that erythrocyte antioxidant defences are highly correlated with antioxidant status in the hepatic mitochondrial compartment and liver oxidative damage under different antioxidant conditions. These data highlight the possibility of evaluating the oxidative balance in the hepatic mitochondrial compartment just through blood samples instead of using liver biopsies.

The NAFLD is now considered the main hepatic manifestation of obesity and metabolic syndrome (Carmiel-Haggai et al. 2005; Lewis & Mohanty 2010). In fact, our data demonstrate that high fat feeding increased body weight gain and subsequently induced liver steatosis. Further events in the liver include oxidative stress and the decrease in antioxidant defenses induced by early mitochondrial dysfunction (Abdel-Wahab et al. 2002; Gambino et al. 2011). Thus, oxidative stress represents an imbalance between the production and manifestation of ROS and the antioxidant defenses that are able to readily detoxify the reactive intermediates or to repair the resulting damage (Furukawa et al. 2004; Vincent & Taylor 2006; Valdecantos et al. 2009).

Disturbances in the normal redox state can cause injury in other cell components, including lipids. Indeed, several studies have described an increase in TBARS content, a good marker of lipid oxidative damage, in steatotic livers (Caito et al. 2010; Park et al. 2011). Accordingly, in this study obese animals presented an increase in TBARS levels that was prevented by LA treatment, suggesting that the HFD produced an increase in hepatic oxidative stress, inducing oxidative damage in lipids.

Furthermore, oxidative damage of mtDNA has been correlated with several diseases, such as neurodegenerative disease (Cortopassi et al. 1992; Michikawa et al. 1999), cancer (Brunet et al. 2004; Rohan et al. 2010), or diabetes (Madsen-Bouterse et al. 2010). Moreover, mtDNA is more susceptible to oxidative injury than nuclear DNA due to its localization near to the site of free radical generation (Ballinger et al. 1999). Accordingly, our data showed that a decrease in antioxidant defenses in the hepatic mitochondrial compartment of obese animals was associated with enhanced mtDNA oxidative generated damage and LA treatment is able to reverse the deleterious effect in mtDNA, corroborating its beneficial effects on oxidative stress in liver mitochondria after the ingestion of a HFD.

There is no established treatment for NAFLD, except for weight loss and treatments that prevent obesity and metabolic syndrome. In this context, LA exerts beneficial physiological effects, such as attenuation of oxidative stress (Maritim et al. 2003), modulation of glucose metabolism (Sadi et al. 2008), prevention of body weight gain induced by HFD (Wollin et al. 2004) as well as a reduction of energy efficiency (Prieto-Hontoria et al. 2009). Our results confirm that LA treatment can prevent body weight gain and attenuate oxidative stress through the stimulation of antioxidant defences, both systemic and in the hepatic mitochondrial compartment, specially the GSH:GSSG ratio, whose role as a regulator of antioxidant status in mitochondria has been described (Muyderman et al. 2007; Kimura et al. 2010).

Furthermore, previous experiments have revealed that liver oxidative stress plays a central role in several hepatic disorders (Copple et al. 2010). In addition, other studies have demonstrated an association between liver disease and systemic oxidative stress (Ljubuncic et al. 2000; Powell et al. 2010). Moreover, the central role of mitochondrial changes and oxidative stress in the evolution of fatty liver linked to obesity (Perez-Carreras et al. 2003; Rector et al. 2010) suggest that mitochondrial antioxidant status is a fine predictor of the pathological development of an important liver complication of obesity. However, liver biopsy is necessary to assess mitochondrial antioxidant status and consequently to predict progression of NAFLD to NASH. Interestingly, our data showed a strong correlation between antioxidant biomarkers in erythrocytes and the liver mitochondrial compartment under different antioxidant status conditions and highlight the possibility of using erythrocytes as noninvasive biomarker of the progression of NAFL to NASH instead of liver biopsy.

Conclusions

In conclusion, our results show that the assessment of the activity of antioxidant defences (such as SOD and GPx) and the GSH:GSSG ratio in erythrocytes could be used as good markers of the antioxidant status of the hepatic mitochondrial compartment and, consequently, they could be good predictors of the development of NAFLD after a HFD. In addition, LA treatment is able to prevent the impairment of systemic and hepatic mitochondrial antioxidant defences induced by a HFD intake.

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Declaration of interest

The authors declare that they have no competing interest. This work has been supported by Línea especial "Nutrición, Obesidad y Salud" (University of Navarra

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