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ARTICLE

Fasting Status and Thermally Oxidized Sunflower Oil Ingestion Affect the Intestinal Antioxidant Enzyme Activity and Gene Expression of Male Wistar Rats

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The effect of thermally oxidized sunflower oil ingestion on antioxidant levels, enzyme activities and expressions in the small intestine of fed and fasted rats was studied. For three consecutive days, 12 male Wistar rats received 0.5 g of unused sunflower oil/100 g of body weight (controls, C) while another 12 were given 0.5 g of thermally oxidized sunflower oil/100 g of body weight (test group, T). On the night of day 3, 6 rats from each group were fasted (FC and FT, respectively) while the other 6 animals from each group were given free access to food (NFC and NFT, respectively). On day 4, FC and NFC rats received 1 g of unused oil/100 g of body weight, while FT and NFT rats were given 1 g of altered oil/100 g of body weight. Small intestines were extracted after 4 h exposure to the oils. Fasting and oil alteration significantly interacted modifying total, Se-GPx (both, $P < 0.001$) and non-Se-GPx ($P < 0.05$) activity, and GPx and Cu,Zn-SOD expressions (both $P < 0.001$). FT rats showed a significant increase in TBARS ($P < 0.05$) and catalase activity ($P < 0.001$) and a decrease in SOD, Se- and non-Se-dependent GPx activities (at least, $P < 0.05$) with respect to FC and NFT animals. SOD and GPx expressions decreased ($p<0.001$) but that of TNFα increased significantly ($P < 0.001$) in FT rats with respect to FC and NFT animals. Lengthy fasting and consumption of food containing oxidized fat should both be avoided to prevent intestinal oxidative stress.

KEYWORDS: Fasting; glutathione; catalase; frying; glutathione peroxidase; glutathione reductase; gene expression; superoxide dismutase; TBARS; TNFα; thermally oxidized oil

INTRODUCTION

Frying is a cooking method used extensively throughout the world that clearly differs from heating because the presence of food in the fryer oil makes the situation rather more complex (*1*, *2*). During frying, the food is submerged in fat which is heated in the presence of air. Therefore, the fat is exposed to the action of moisture from the foodstuff that may induce hydrolytic alteration, thus yielding diacylglycerols, monoacylglycerols, and free fatty acids that are commonly classified as hydrolytic products. Moreover, moisture from food produces oil temperature decreases. Apart from migration of lipids from the food into the frying oil, it is important to take into account that the presence of some compounds from the foods can substantially affect the thermal oxidation reactions. Thus, (a) amphiphilic compounds like phospholipids and emulsifiers can contribute to early foaming; (b) lipid soluble vitamins and trace metals leaching into the frying oil may inhibit or accelerate oil oxidation depending on their antioxidant or prooxidant effects; (c) cholesterol and its oxidation derived compounds from fatty animal foods can be transferred to vegetable frying oils and then incorporated into nonfatty foods during subsequent frying operations; (d) pigments and Maillard browning products can modify the susceptibility against oxidation of frying oils and contribute to darkening; (e) phenolic compounds present in the foods or in added spices can increase the frying oil stability; (f) volatile compounds coming from strongly flavored foods like fish or onions can contribute to specific off-flavors (*2*).

Consumption of industrially prepared fried foods containing a relatively high altered fat content is common in Europe and the United States (*3*, *4*). Studies in animals have demonstrated that consumption of oxidized and nonoxidized polymers and dimers can induce deleterious effects (*1*, *5*). *In vitro* (*6*) and *in vivo* studies (*1*, *7*) have demonstrated that triglyceride polymers and oxidized triacylglycerols are partially hydrolyzed by intestinal enzymes. Moreover, results from *in vivo* studies suggest that these alteration compounds are actively absorbed after four hours (*7*).

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In previous studies, our group found that the liver content of thiobarbituric acid-reactive substances (TBARS) was related to the TBARS content and thermal oxidation of consumed fats, suggesting that this organ actively takes up oxidized lipoprotein components (*8*).

Antioxidant defense against reactive oxygen intermediates is heavily influenced by nutrition (*9*). Oxidized fats produce oxidative stress (*10*), which in turn induces proinflammatory gene expression (*11*). Although the intestinal barrier may limit access of oxidized and polymerized fats to the body and protect against oxygen free radical damage, a relatively low number of studies on the role of the gut in antioxidant defense have been published. Moreover, the values observed in the antioxidant enzyme activity decrease were not as low as expected (*12*). Although some studies have investigated the effect of heated oils on the activity and expression of intestinal antioxidants (*4*), to the best of our knowledge no studies have investigated the effect of consuming oils used in frying, whose alteration levels moderately exceed the cutoff points designated by the legislation of different countries (*2*, *13*), on the activity and expression of antioxidant enzymes.

Current concern regarding obesity and excess weight has negatively affected dietary habits. A relatively large percentage of the population does not now eat breakfast (*14*), thus making lunch their first meal of the day. This meal pattern extends the fasting period for several hours and may lower intestinal defenses against oxidative stress.

Taking into account the previous considerations, in the present paper it was hypothesized that the ingestion of oil used in frying containing 33% alteration compounds as polar material may impair the intestinal antioxidant defense system, particularly after a lengthy fasting period. The present study in fed and 15 h fasted growing Wistar rats aimed (a) to compare the activity and gene expression of certain intestinal enzymes engaged in antioxidant defense after ingestion of sunflower oil with high and low alteration levels; (b) to compare intestinal TBARS and glutathione levels and Tumor necrosis factor-alpha (TNFα) expression after ingestion of both oils.

MATERIALS AND METHODS

Frying Performance. Forty domestic deep-fat frying operations of different foods were performed in a 4 week period, at the rate of 10 procedures per week, using 2.5 L steel vessels (Solac, Vitoria, Spain). Refined sunflower oil (Koipesol, Andújar, Jaén, Spain) was employed for frying. Frozen prefried potatoes and battered squid (Pryca hypermarkets, Spain), frozen croquettes (La Cocinera; Torrejón de Ardoz, Madrid, Spain), frozen tuna pastries (Findus-Nestlé España S.A., Esplugues de Llobregat, Barcelona, Spain), frozen spring rolls and frozen breaded veal fillets (Sánchez Romero, Jabugo, Huelva, Spain), frozen fish fingers (breaded hake) (Frudesa, l'Alcudia, Valencia, Spain), sausages (Gran Prix, Getafe, Madrid, Spain), and fresh potatoes (Kennebec variety, Xinzo de Limia, Galicia, Spain) were used. Battered anchovies, battered green peppers, battered sliced aubergines, and meatballs (beef plus wheat flour) were prepared in the laboratory using standard culinary recipes. The following frying conditions were used: thermostat temperature, 180 °C; heating time 9−12 min/frying; frying time 2−6 min/frying; and cooling time ~4 h/frying. Frying oil was replenished with unused oil every ten frying operations to maintain, insofar as possible, a constant food/oil ratio. The frying sequence and the type and amounts of food used in successive frying operations are shown in **Table 1**. The amount of food selected for each frying procedure was based on commercial advertisements (e.g., one piece/person) and the criteria of the cook (standard quantities used at home for four persons).

Animals and Treatments. Twenty-four male Wistar rats weighing approximately 200 g (190−210 g) were obtained from the Animal Research Centre, University of Alcalá (Madrid, Spain), homologated by the Spanish Ministerio de Agricultura, Reference 28005-22A, Real Decreto 233-88. The rats were housed in an animal room under standard conditions of temperature (21 ± 1 °C) and humidity (55 ± 10%), with a 12 h light/12 h dark cycle. All experiments were performed in compliance with Directive 86/609/EEC of November 24, 1986, for the protection of scientific research animals. The animals were divided into four groups of six rats each. All animals had *ad libitum* access to water and a chow diet. For three consecutive days, the rats were administered 0.5 g of oil/100 g of body weight via esophageal probe at 9:00 a.m. Control rats received unused sunflower oil while the test rats received sunflower oil that had been used 40 times to fry food. On the night of day 3, half of the rats were subjected to a 15 h fast while the other half had *ad libitum* access to food and water. On day 4, beginning at 9 a.m., one animal at a time was taken at random from each of the four groups and administered additional oil. All control rats, fasted (FC) and nonfasted (NFC), received 1 g of unused oil/100 g of body weight by means of esophageal probe, while all test rats, fasted (FT) and nonfasted (NFT) were given 1 g of used oil/100 g of body weight). After a 4 h exposure to the fat, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg kg^{-1} of body weight) and euthanized by extracting blood from the descending aorta with a syringe. Following the Sánchez-Muniz et al. (*7*) method, 50 mL of isotonic saline solution was then slowly passed from the proximal esophagus to the distal ileum to eliminate any remaining lumen fat. The whole small intestine was dissected, washed with saline solution, carefully dried, weighed and cut into pieces of approximately 0.5 cm in length. Homogeneous aliquots of the entire small intestine were obtained and stored at −80 °C until analysis.

Table 1. Frying Sequence, Type and Amount of Food, and Frying Time

frying operation no.	food	frying time (min)	amount of food (g)
1, 10, 11	fresh potatoes	6	330−500
2, 15, 21, 24, 32, 34, 35	frozen prefried potatoes	6	330−500
3, 7, 12, 18, 22, 29, 33	frozen croquettes	4	280−410
4, 14, 23, 31	tuna pastries	4	305−340
5, 28	spring rolls	6	240−370
6, 16, 26, 36, 38	breaded hake (fish fingers)	4	305−330
8	sausages	2	170
9, 25	frozen breaded veal fillets	4	210−250
13, 30	battered anchovies	4	175−200
17	battered green peppers	4	350−400
19, 27, 39	battered squid	4	260−415
20, 37	battered aubergines	4	310−380
40	meatballs	4	320

Polar Material and Thermal Assessments. Total polar material of the oils was determined by silica column chromatography (*15*). Samples from the unused sunflower oil and from that after being used in 40 fryings were analyzed. To obtain further information about thermally oxidative changes occurring during frying, the polar fractions of used and unused sunflower oils, previously obtained by column chromatography, were analyzed by HPSEC (*15*). Two determinations of each polar fraction of both fresh and used oils were performed.

Fatty Acid Composition. The fatty acid profile of the oils was determined by CG-FID (*16*) using tricosanoic acid as internal standard on a Hewlett-Packard model 5890 series II gas chromatograph (Palo Alto, CA). The instrument was equipped with a fused silica capillary column (0.33 mm i.d. × 50 m in length) BPX70, film thickness 0.25 μm (SGE, Austin, TX).

Vitamin E Determination. Tocopherol content in oil was determined by reversed-phase HPLC analysis on a Varian model 5000 liquid chromatograph, (Palo Alto, CA) equipped with a variable wavelength UV Variant model 50 detector fitted with a Hewlett-Packard model 3394 integrator (Palo Alto, CA). The chromatographic separation was achieved on a 5 μm Spherisorb ODS-2 column (250 × 4.6 mm i.d.) (Bedfordshire, U.K.). The elution conditions were those reported by Tonolo and Marzo (*17*).

Intestinal Sample Preparation. Representative and homogeneous samples of small intestine were homogenized in phosphate buffer (50 mM, pH 7.4), and centrifuged at 3000 rpm (1500*g*) at 4 °C for 15 min. The

resulting supernatant fraction was divided into aliquots and frozen at −80 °C until being used in biochemical assays.

Intestinal Protein. Protein was determined in the small intestine homogenates by the Bradford (*18*) method, using bovine serum albumin as the standard.

Intestinal Thiobarbituric Acid Reactive Substances. Lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) (*19*) in small intestinal homogenates using an Uvicon 930 spectrophotometer (Kontron Instruments, Germany). Concentrations were calculated against a standard curve obtained with malondialdehyde (MDA). TBARS values were expressed as μmol of MDA/protein.

Intestinal Total, Reduced, and Peroxidized Glutathione. The reduced glutathione (GSH) and oxidized glutathione (GSSG) levels were determined in small intestine following the method of Hissin and Hilf (*20*). The amount of GSH and GSSG was calculated from a standard curve and expressed in μg/mg of protein. The redox index was calculated by dividing the concentration of oxidized glutathione by that of total glutathione.

Intestinal Glutathione Peroxidase (GPx) Activity. Total GPx activity in small intestine homogenates was assayed by using H_2O_2 as a substrate for Se dependent-GPx while cumene hydroperoxide as a substrate for total GPx activity (*21*). Values were expressed as nmol of NADPH/min/mg of protein.

Intestinal Glutathione Reductase (GR) Activity. Glutathione reductase (GR) activity in small intestine homogenates was measured by the method of Goldbery and Spooner (*22*). The oxidation of NADPH was followed at 340 nm, and one unit of activity is defined as the oxidation of 1 nmol of NADPH/min/mg of protein.

Superoxide Dismutase (SOD) and Catalase Activities. Total SOD in small intestine homogenates was assayed by monitoring the rate of inhibition of reduction of benzene-1,2,3-triol (pyrogallol) (*23*) by the enzyme. One unit of SOD represents the amount of enzyme required to produce 50% inhibition of pyrogallol reduction per 2−3 min. CAT activity was assayed by monitoring the disappearance of H_2O_2 at 240 nm (*24*). Absorbance at 240 nm was recorded for 1 min at 25 °C. One unit of catalase is defined as half of the peroxide oxygen liberated from the H_2O_2 solution (10 mM) in 100 s at 25 °C.

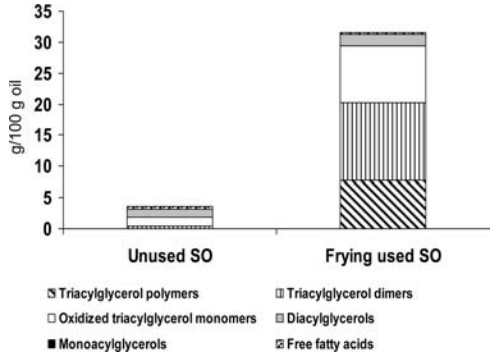

Figure 1. Alteration compound profile (g/100 g of oil) of unused sunflower oil and sunflower oil used in frying. Data correspond to the average figures of two determinations. The polar content of these oils corresponds to the sum of their alteration compounds.

RT-PCR Analysis of SOD, GPx and TNFα. Total RNA was extracted from homogeneous and representative frozen small intestine samples by using the guanidinium thiocyanate/phenol reagent method (*25*). Reverse transcription and amplification using the Titan system involved the preparation of two master-mixes on ice. Mix 1 was composed of dNTPs, primers, dithiothreitol (DTT) and sterile prechilled deionized water. Mix 2 consisted of RT-PCR buffer, enzyme mix (AMV reverse transcriptase, Taq DNA polymerase) and sterile prechilled deionized water. Twenty-four microliters of master mix 1 together with 25 μL of master mix 2 were mixed with 1 μL of extracted RNA (1 μg/μL) in a 0.2 mL PCR tube kept on ice. The working mix was vortexed and centrifuged briefly to collect the sample at the bottom of the tube, and RT was carried out at 50 °C for 30 min. β-Actin cDNA was used as an internal control.

The sequences of the primers were as follows:

Cu, Zn-SOD sense : 5′-GCCGTGTGCGTGCTGAA-3′

Cu, Zn-SOD antisense : 5′-TTTCCACCTTTGCCCAAGTCA-3′

GPx sense : 5′-AGTCCACCGTATATGCCTTC-3′

GPx antisense : 5′-TCTGAGGGGATTTTTCTGGA-3′

TNF ∗ sense : 5′-TGGCCCAGACCCTCACACTC-3′

TNF ∗ antisense : 5′-CTCCTGGTATGAAATGGCAAATC-3′

β-actin sense : 5′-TACAACCTCCTTGCAGCTCC-3′

β-actin antisense : 5′-GGATCTTCATGAGGTAGTCAGTC-3′

The number of PCR cycles was adjusted to avoid saturation of the amplification system. Thus, conditions used were as follow: 95 °C for 30s, 55 °C for 1 min and 72 °C for 30 s (30 cycles) for SOD; 94 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min (30 cycles) for GPx; 94 °C for 1 min, 59 °C for 1 min and 72 °C for 1 min (35 cycles) for TNF; and 94 °C for 30 s, 58 °C for 45 s and 72 °C for 30 s (24 cycles) for β-actin with a final elongation at 72 °C for 10 min. Amplification products were visualized in 1.8% agarose gels containing ethidium bromide (1 μg/mL): SOD product, 383 bp; GPx product, 697 bp; TNFα product, 281 bp; β-actin product

Table 2. Intestinal TBARS and Glutathione Levels and Antioxidant Enzyme Activities of Fasted Control Rats (FC) Fed Unused Sunflower Oil, Fasted Test Rats (FT) Given Sunflower Oil Used in Frying, Nonfasted Control Rats (NFC) Fed Unused Sunflower Oil and Nonfasted Test Rats (NFT) Given Sunflower Oil Used in Frying[a]

	NFC	NFT	FC	FT	fasting factor	oil factor	interaction
TBARS (μmol of MDA/protein)	15.1 ± 1.1	15.2 ± 1.3	15.3 ± 1.5	17.1 ± 1.0* a	ns	<0.05	ns
total glutathione (μg/mg of protein)	9.6 ± 1.7	9.7 ± 1.3	9.5 ± 2.8	7.5 ± 2.6	ns	ns	ns
reduced glutathione (μg/mg of protein)	7.3 ± 1.7	5.9 ± 1.0	6.6 ± 1.9	3.0 ± 1.3** b	<0.01	<0.001	ns
oxidized glutathione (μg/mg of protein)	2.3 ± 0.2	4.8 ± 0.6***	2.9 ± 1.1	4.5 ± 0.9*	ns	<0.001	ns
redox ratio	0.25 ± 0.05	0.50 ± 0.05***	0.30 ± 0.07	0.60 ± 0.04*** b	<0.001	<0.001	ns
Cu,Zn-SOD (U/mg of protein)	4.5 ± 0.3	3.2 ± 1.1*	2.4 ± 0.6 c	1.4 ± 0.4* b	<0.001	<0.001	ns
catalase (U/mg of protein)	4.3 ± 1.1	10.0 ± 1.2***	11.8 ± 0.9 c	16.5 ± 1.1*** c	<0.001	<0.001	ns
Se-GPx (nmol of NADPH/min/mg of protein)	34.8 ± 1.5	36.9 ± 0.6*	36.7 ± 2.2	22.9 ± 0.9*** c	<0.001	<0.001	<0.001
non-Se-GPx (nmol of NADPH/min/mg of protein)	14.7 ± 2.1	13.2 ± 0.5	9.7 ± 0.8 b	5.2 ± 0.8** c	<0.001	<0.001	<0.05
total GPx (nmol of NADPH/min/mg of protein)	49.5 ± 0.9	50.1 ± 0.2	46.4 ± 0.9 c	28.1 ± 0.9*** c	<0.001	<0.001	<0.001
glutathione reductase (nmol of NADPH/min/mg of protein)	149.6 ± 63.5	141.2 ± 30.9	124.6 ± 41.1	123.0 ± 42.0	ns	ns	ns

[a] TBARS: thiobarbituric acid-reactive substances. SOD: superoxide dismutase. Gpx: glutathione peroxidase. Data are mean ± SD of 6 animals per group. NFT and FT data bearing asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$) were significantly different from NFC and FC, respectively. FC and FT data bearing a letter (a, $P < 0.05$; b, $P < 0.01$; c, $P < 0.001$) were significantly different from NFC and NFT, respectively; ns, not significant.

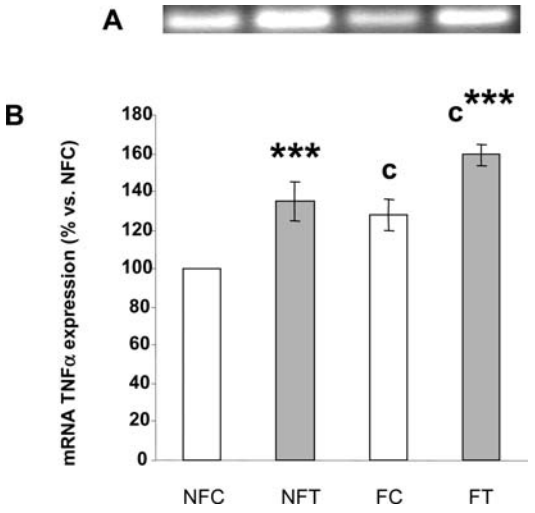

Figure 2. TNFα expression in intestinal homogenates of rats. (**A**) Representative gene expression profile of TNFα product 346 bp assayed by RT-PCR analysis. (**B**) FC: Fasted control rats given unused sunflower oil. FT: Fasted test rats fed sunflower oil used in frying. NFC: Nonfasted control rats given unused sunflower oil. NFT: Nonfasted test rats fed sunflower oil used in frying. The results, expressed as % of the control, are the mean ± SD of determinations in triplicate from 6 rats. Fasting effect, $P < 0.001$; oil effect, $P < 0.001$; interaction, not significant. NFC vs NFT, *** ($P < 0.001$); FC vs FT *** ($P < 0.001$). NFC vs FC (c, $P < 0.001$); NFT vs FT (c, $P < 0.001$).

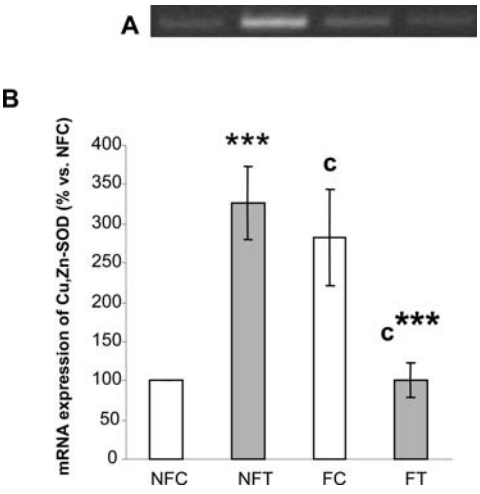

Figure 3. Cu,Zn-SOD expression in intestinal homogenates of rats. (**A**) Representative gene expression profile of Cu,Zn-SOD product 383 bp assayed by RT-PCR analysis. (**B**) FC: Fasted control rats given unused sunflower oil. FT: Fasted test rats fed sunflower oil used in frying. NFC: Nonfasted control rats given unused sunflower oil. NFT: Nonfasted test rats fed sunflower oil used in frying. The results, expressed as % of the control, are the mean ± SD of determinations in triplicate from 6 rats. Fasting effect, not significant; oil effect, not significant; interaction, $P < 0.001$. NFC vs NFT, *** ($P < 0.001$); FC vs FT *** ($P < 0.001$). NFC vs FC (c, $P < 0.001$); NFT vs FT (c, $P < 0.001$).

630 bp. A 100 bp DNA ladder was used as marker. Products were quantified by laser densitometry (Genius Syngene, USA).

Statistical Analysis. All analyses were performed in triplicate. Data were expressed as means ± SD. ANOVA two-way analysis (fasting × oil alteration) was followed by Student *t*-test *post hoc* study. *P* values of < 0.05 were considered statistically significant. Statistical analyses were performed using the SPSS statistical software package (version 15.0).

RESULTS

Oil Alteration. After 40 fryings, linoleic acid markedly decreased (59.5 to 39.5 g/100 g of oil) while minor changes were observed for the linolenic acid (0.08 to 0.07 g/100 g of oil). Oleic and palmitic acids increased (22.7 to 26.8; 6.5 to 9.0 g/100 g of oil, respectively) while stearic acid did not change (4.7 to 4.7 g/100 g of oil). The total tocopherol amount of sunflower oil decreased from 570 to 45 mg/kg of oil.

The total polar content and the alteration compound profile of the unused sunflower oil and that of the sunflower oil used in 40 deep-fat frying operations are shown in **Figure 1**.

Small Intestinal TBARS. The TBARS content was significantly affected by oil alteration. Significant differences were observed between FC and FT rats ($P < 0.05$) and between NFT and FT rats ($P < 0.05$) (**Table 2**).

Small Intestinal Total, Reduced and Peroxidized Glutathione. The concentration of total, reduced and oxidized glutathione is shown in **Table 2**. Total glutathione concentrations were not affected by fasting and oil. Reduced glutathione was affected by fasting ($P < 0.01$) and oil alteration ($P < 0.001$). FT rats show lower reduced glutathione values than NFT ($P < 0.01$) and FC rats ($P < 0.01$). The oxidized glutathione levels were affected by oil alteration ($P < 0.001$). The oxidized glutathione levels of NFT and FT animals were greater than those of NFC and FC animals ($P < 0.001$ and $P < 0.05$, respectively). The redox ratio was significantly affected by fasting ($P < 0.001$) and oil alteration ($P < 0.001$). FT rats displayed a higher redox index than NFT ($P < 0.01$) and FC rats ($P < 0.001$). NFT rats presented a higher redox ratio than NFC rats ($P < 0.001$).

Small Intestinal Cu,Zn-SOD Activity. The Cu,Zn-SOD activity was affected by fasting ($P < 0.001$) and oil alteration ($P < 0.001$). FT and NFT rats had significantly lower SOD activity ($P < 0.05$) than that of FC and NFC animals. FC and FT rats presented lower SOD activity than NFC and NFT animals ($P < 0.001$ and $P < 0.01$, respectively) (**Table 2**).

Small Intestinal Catalase Activity. The catalase activity was affected by fasting ($P < 0.001$) and oil alteration ($P < 0.001$). **Table 2** also shows that catalase activity in FT and NFT groups was higher (both $P < 0.001$) than that of FC and NFC rats. Fasted rats (FC and FC) displayed higher catalase activity than nonfasted animals (NFC and NFT, both $P < 0.001$).

Small Intestinal Total, Se-GPx and Non-Se-GPx Activities. The different GPx activities were affected by fasting ($P < 0.001$), oil alteration ($P < 0.001$) and fasting × oil interaction (at least $P < 0.05$). Total, Se-GPx and non-Se-GPx activities were significantly lower (at least $P < 0.01$) in FT rats than in their FC and NFT counterparts. Se-GPx concentrations were higher ($P < 0.05$) in NFT animals than in NFC ones. FC rats showed significantly lower ($P < 0.001$) total and Se-GPx activities than NFC rats (**Table 2**).

Small Intestinal GR Activity. Table 2 shows that GR values were not significantly affected ($P > 0.1$) by fasting or oil alteration.

Small Intestinal TNFα Expression. Figure 2 shows a significant increase in the expression of TNFα in FT rats with respect to FC ($P < 0.001$) and NFT ($P < 0.001$) animals. TNFα expression was higher in FC ($P < 0.001$) and NFT ($P < 0.001$) rats than in their NFC counterparts.

Small Intestinal Expression of Cu,Zn-SOD. Figure 3 shows that Cu,Zn-SOD expression was significantly lower in the FT group than in the FC ($P < 0.001$) and NFT ($P < 0.001$) groups. Cu,Zn-SOD expression was higher in FC ($P < 0.001$) and NFT ($P < 0.001$) rats than in NFC animals. The Cu,Zn-SOD expression was significantly affected by fasting x oil interaction ($P < 0.001$)

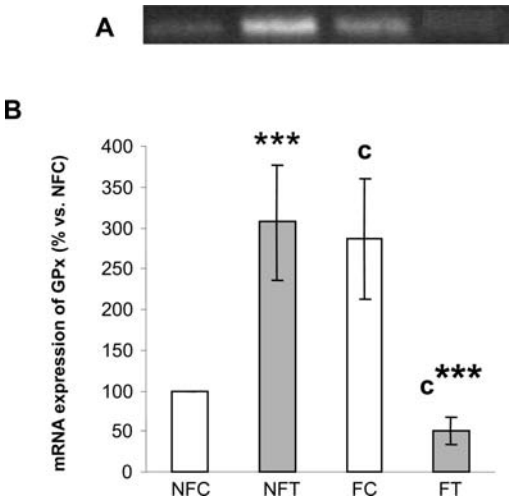

Figure 4. GPx expression in intestinal homogenates of rats. (**A**) Representative gene expression profile of GPx product 260 bp assayed by RT-PCR analysis. (**B**) FC: Fasted control rats given unused sunflower oil. FT: Fasted test rats fed sunflower oil used in frying. NFC: Nonfasted control rats given unused sunflower oil. NFT: Nonfasted test rats fed sunflower oil used in frying. The results, expressed as % of the control, are the mean ± SD of determinations in triplicate from 6 rats. Fasting effect, not significant; oil effect, not significant; interaction, $P < 0.001$. NFC vs NFT, *** ($P < 0.001$); FC vs FT *** ($P < 0.001$). NFC vs FC (c, $P < 0.001$); NFT vs FT (c, $P < 0.001$).

Small Intestinal Expression of GPx. Figure 4 shows the expression of GPx in the different groups. The GPx expression was significantly affected by fasting x oil interaction ($P < 0.001$). GPx expression in FT rats was significantly lower ($P < 0.001$) than that observed in FC ($P < 0.001$) and NFT ($P < 0.001$) animals. FC and NFT rats displayed significantly higher (both $P < 0.001$) GPx expression than NFC animals.

DISCUSSION

To the best of our knowledge, this is the first study testing the interaction between the fed/15 h fasting status and the alteration levels of sunflower oil used in frying of different foodstuffs on the level and expression of some intestinal antioxidant enzymes. As previously indicated, frying clearly differs from heating because of the presence of food in the oil (*2*). Small intestines were used because intestinal cells (i) are presumably exposed to abundant levels of thermal oxidized fatty acids during intestinal passage after ingestion of the frying abused sunflower oil and (ii) efficiently absorb oxidized fatty acids as readily as unoxidized fatty acids (*1*, *7*, *26*).

In recent years, the contribution of dietary oxidized fats to total energy intake has increased in Westernized countries, primarily due to the increasing consumption of fast foods that contain heated and processed dietary fats (*3*, *4*). Fasting, which represents an extreme form of food restriction, has been related to greater oxidative damage as a consequence of increased free radical production and impaired antioxidant protection (*27*). However, other authors associate intermittent fasting with decreased production of free radicals and improved activity of mechanisms that protect from damaging agents (*28*). Rats present two peaks of food consumption (one at night and the other early in the morning) (*29*). Thus, the 15 h fasting conditions of the present study were slightly more severe than those normally used in experiments with rats (12 h) and may contribute to increasing free radical production.

The polar material content and alteration compound profile of the unused sunflower oil employed in this study correspond with those of good quality oil (*2*). Repeated use of unsaturated oils for frying increases the formation of thermal oxidation compounds (dimers and polymers of triacylglycerols and oxidized triacylglycerol monomers) whose consumption may contribute to the pathogenesis and evolution of some diseases (*1*, *30*). Several studies show that lipid peroxidation contributes to a number of cellular alterations, including increased membrane rigidity, osmotic fragility, decreased cellular deformation, reduced erythrocyte survival and membrane fluidity (*30*).

Antioxidant defense is integrated and effective, but not infallible (*9*). TBARS levels are extensively employed as a marker of tissue lipid peroxidation (*8*, *30*). An increase in TBARS and/or MDA values indicates increased lipid peroxidation, leading to tissue injury and the failure of antioxidant defense mechanisms to prevent excess ROS formation (*30*).

Unexpectedly, only fasted animals (FT group) in the present study displayed a slight increase (~10%) in small intestine TBARS levels after consumption of thermally oxidized oil. According to Yu (*31*) the combination of dietary antioxidants and digestive mucosa antioxidant defense may decrease the free radical damage in the intestinal cells. Thus, it can be suggested that, although total tocopherol content was decreased in the sunflower oil after frying, the presence of antioxidants in diet counterbalanced the prooxidant effect of thermal oxidized fatty acids in intestinal mucosa. Thòmas-Moyá et al. (*32*) found that TBARS levels displayed a nonsignificant tendency to increase (2.25 vs 1.82 nmol/g of tissue) after a 12 h fasting period. Autooxidized oil ingestion may negatively affect antioxidant defense. Ringseis et al. (*4*) reported significantly higher intestinal epithelial cell-TBARS levels in pigs given oxidized sunflower oil heated at 200 °C for 24 h than in pigs fed a blend of unused sunflower and palm oils (93:7, w/w) for the same period of time.

The glutathione antioxidant system is considered very efficient at decreasing the presence of free radicals (*31*). Consumption of used frying sunflower oil decreased total and reduced glutathione levels, increasing oxidized glutathione concentrations and the redox ratio, suggesting losses in the efficacy of such an antioxidant system. This effect was most evident under fasting conditions, because FT animals presented a higher redox index than their NFT counterparts.

Intestinal oxidative stress is accompanied by a reduction of enzymatic antioxidant activity in enterocytes (*33*). The reduced activities of SOD and total, Se- and non-Se-GPx observed in FT rats in the present study may provide further evidence for this assumption. However, the thermally oxidized oil consumed by nonfasted rats did not decrease SOD and GPx activities. Thus, the presence of food in the gastrointestinal tract appears to partially block the negative effect of thermally oxidized oil on the glutathione system, SOD and GPx activities.

GR activity was not affected by oil alteration or fasting conditions. This finding suggests that GR acted effectively to maintain the level of reduced glutathione as high as possible under the existing conditions. Present data suggest that fasting and altered oil consumption induce high generation of hydrogen peroxide. During fasting, low insulin levels increase fatty acyl-CoA oxidase activity, triggering β-oxidation of fatty acids and the production of a large amount of H_2O_2 (*34*). Moreover, the FT animals in our study displayed the greatest increase in catalase activity, suggesting an increase in their peroxide levels, including those of H_2O_2.

Thòmas-Moyá et al. (*32*) described that, after a 15 h fast, GPx activity in male Wistar rats was significantly lower than that observed in fed animals. In control animals, fasting affected non-Se-GPx activity, while the joint effect of fasting and altered oil consumption modified total, Se- and non-Se-GPx activities. Reduction in SOD activity produces an accumulation of O_2

radicals that are available to react with Fe^{3+} and form Fe^{2+}, contributing to the oxidative damage of DNA, proteins and lipids (*30*). Fed status contributes to improve the antioxidant status, with more intestinal enzyme activity and higher SOD and GPx expressions than fasted animals. In addition to the most efficient enterocyte antioxidant barrier of fed rats, the role of some food antioxidants ingested cannot be ruled out (*26*, *35*). Moreover, antioxidant mechanisms induced in enterocytes of fed animals may produce mRNA-dependent SOD and GPx to combat the oxidative burden of the lipid peroxides consumed, contributing to maintain intestinal levels of antioxidant substrates and enzymes as high as possible and those of oxidized compounds as low as possible. Enzyme activities do not always change in parallel to the enzyme expressions (*36*). There will be different explanations. Thus, an excess of enzymes would induce enzyme downregulation and vice versa insufficient enzyme activity would produce increases in enzyme expression. Furthermore, the present situation found would be the resultant of several adjustments or feedbacks.

TNFα cytokine plays a key role in inflammation (*37*) and intestinal degenerative diseases (*38*). Results of the present study suggest that the oxidized and polymerized compounds of the used oil increased TNFα expression by 30%, a fact that would negatively affect the antioxidant capacity of the intestinal barrier.

TNFα acts as a potent activator of NADPH-based oxidase in colon epithelial cells (*37*), suggesting a potential role of this oxidase in the colon inflammation. The fact that TNFα expression was lowest in fed animals suggests that free radical damage to enterocytes may be partially blocked by the presence of food compounds.

In conclusion, results of the present study demonstrate that the ingestion of thermally oxidized sunflower oil by rats fasted for 15 h decreased intestinal glutathione levels, SOD and GPx activities and SOD, GR and GPx expression, but increased catalase activity and TNFα expression. These effects were less evident in nonfasted rats. Thus, prolonged overnight fasting should be avoided, especially after ingestion of potentially fat-rich processed food, with high oxidized fatty acid content. More studies are needed to understand the complex mechanisms involved in intestinal metabolism of altered fats.

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