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HEPATIC DAMAGE AND OXIDATIVE STRESS INDUCED BY GRISEOFULVIN IN MICE

M. DEL C. MARTINEZ[1, 2], S.G. AFONSO[1], R.P. MEISS[3], A.M. BUZALEH[1, 2] AND A.BATLLE[1]

[1]Centro de Investigaciones sobre Porfirinas y Porfirias (CIPYP), CONICET, Hospital de Clínicas José de San Martín, University of Buenos Aires; [2]Department of Biological Chemistry, Facultad de Ciencias Exactas y Naturales, University of Buenos Aires, Argentina.[3] Department of Pathology, Instituto de Estudios Oncológicos, Academia Nacional de Medicina, Buenos Aires, Argentina.

DR. MARIA DEL CARMEN MARTÍNEZ
Betharram 1720 – Martín Coronado, 1682 - BUENOS AIRES ARGENTINA
Phone 54 11 5950 8346/54 11 4782 7963, Fax 54 11 5950 8347, E-mail: mcmartin@qb.fcen.uba.ar

Dedicated to the memory of our dear colleague and friend Dr. Susana Afonso

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Abstract – Erythropoietic Protoporphyria (EPP) is a disease associated with ferrochelatase deficiency, which produces accumulation of protoporphyrin IX (PROTO IX) in erythrocytes, liver and skin. In some cases, a severe hepatic failure and cholestasis was observed. Griseofulvin (Gris) develops an experimental EPP with hepatic manifestations in animals. The aim of this work was to further characterize this model studying its effect on different metabolisms in mice Gris feeding (0-2.5%, 7 and 14 days). PROTO IX accumulation in liver, blood and feces, induction of ALA-S activity, and a low rate of Holo/Apo tryptophan pyrrolase activity was produced, indicating a reduction of free heme pool. The progressive liver injury was reflected by the aspect and the enlargement of liver and the induction of hepatic damage. Liver redox balance was altered due to porphyrin high concentrations; as a consequence, the antioxidant defense system was disrupted. Heme oxygenase was also induced, however, at higher concentrations of antifungal, the free heme pool would be so depleted that this enzyme would not be necessary. In conclusion, our model of Protoporphyria produced liver alterations similar to those found in EPP patients.

Key words: Griseofulvin, Erythropoietic Protoporphyria, Heme metabolism, Oxidative stress, Liver damage

INTRODUCTION

Erythropoietic protoporphyria (EPP) is a hereditary porphyria caused by a decreased activity of ferrochelatase (Fech) (EC.4.99.1.1), the final enzyme of heme biosynthesis which catalyzes the incorporation of ferrous iron (II) into protoporphyrin IX (PROTO IX) to form heme (4, 41). All symptomatic patients have photosensitivity due to the prescence of PROTO IX in the skin (35). A liver disease is caused due to the toxic effect of porphyrins on the structure and function of this organ (5, 13, 26). There is a correlation between the importance of the liver damage and PROTO IX concentration in erythrocytes and in some cases the injury is so severe that it could require a liver transplantation (10, 17, 47).

Griseofulvin (7-chloro-,4,6-methylspiro trimetoxi-6-[Benzophenone-2(3H),1-(2)cyclohexene]3,4-dione; Gris) has been used by more than 40 years as an antimicotic drug. Studies in animals clearly showed that Gris has a variety of acute and chronic toxic effects, producing liver cancer and thyroid. The triggering of hepatocellular cancer in mice receiving dietary Gris is preceded by several morphological and biochemical hepatic changes, as a result of a marked inhibition of Fech and the reduction of heme regulatory pool that secondarily stimulates 5-aminolevulinic acid

Abbreviations: ALA-S, δ-Aminolevulinic acid synthetase; **ALT**, Alanine aminotransferase; **AP**, Alkaline phosphatase; **AST**, Aspartate aminotransferase; **CYP**, Cytochrome P450; **EPP**, Erythropoietic protoporphyria; **GGT**, gamma Glutamil transpeptidase; **GPx**, Glutathione peroxidase; **GRed**, Glutathione reductase; **Gris**, Griseofulvin; **GSH**, Reduced glutathione; **HO**, Heme oxygenase; **MDA**, Malondialdehyde; **PROTO IX**, Protoporphyrin IX; **ROS**, Reactive Oxygen Species; **S.D.**, Standard Deviation; **SOD**, Superoxide dismutase; **TRP**, Tryptophan pyrrolase

synthetase (ALA-S) enzyme. Porphyrin accumulation is followed by cellular damage, inflammatory and necrotic events (19), resembling human erythropoietic protoporphyria (EPP) associated with liver failure (18, 24, 36, 46).

In an attempt to further characterize the experimental model of EPP produced by Gris, we investigated the effects of different concentrations of Gris, on heme metabolism and cellular damage and oxidative stress markers. For this purpose, the effect of Gris administration on ALA-S, Heme oxygenase (HO) and Tryptophan pyrrolase (TRP) in liver was evaluated. The accumulation of porphyrins was also measured in blood, liver and feces. Plasma activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), and gamma glutamil transpeptidase (GGT) were used as liver damage markers. Oxidative stress development was investigated determining the activities of catalase, superoxide dismutase (SOD), glutathione reductase (GRed), glutathione peroxidase (GPx) and glutathione-S-transferase (GST). The levels of reactive oxygen species (ROS), malondialdehyde (MDA) and reduced glutathione (GSH) in liver were also investigated. Moreover, taking into account that Gris is metabolized throught cytochrome P450 (CYP) producing N-methyl protoporphyrin, a potent inhibitor of Fech enzyme (12, 30), the effect on Phase I drug metabolism system was also investigated measuring total CYP levels and some CYP isoforms.

MATERIALS AND METHODS

Animals

Male mice *CF1* weighing 15-17 g at the starting time of intoxication were used. Animals were maintained in controlled conditions and allowed free access to food (Purina 3, Asociación de Cooperativas Argentinas, San Nicolás, Buenos Aires, Argentina) and water. All experiments were done at the same hour in the day. Animals received human care and were treated in accordance with the guidelines stablished by the Comittee of the Argentine Association of Specialists in Laboratory Animals (AADEALC).

Experimental design

The animals were separated into 4 groups of 8 mice each. One group received control diet (standard diet supplemented with corn oil, 10ml/100g), the other groups received standard diet supplemented with different Gris concentrations (0.5%, 1.5% and 2.5% w/w, in corn oil; 10ml/100g food) and were sacrificed 7 or 14 days later.

Tissue preparation

Mice were killed under ether anaesthesia. Blood was obtained by cardiac puncture and plasma was separated by centrifugation. Liver was removed and immediately processed. A fraction of non-perfused liver was used for measuring ALA-S activity. The remainder liver was perfused with sterile ice saline, removed and used for the other determinations. Experimental conditions for liver homogenate preparations were different depending on the parameter assayed.

Biochemical assays

Porphyrins were extracted from liver and blood using the method described by De Matteis and Lim (11), and from feces according to the method of Lockwood *et al.* (21). High-performance liquid chromatography (RP-HPLC) was used for the analysis of porphyrins. Porphyrins were resolved on a Merck LiChrospher 100 RP-18 column (125-mm long and 4 mm internal diameter, 5 mm particle diameter), injection volume 20 ml, and eluted with 1 M ammonium acetate buffer pH 5.16 (solvent A) and 100% methanol (solvent B) with a linear gradient from 10% B (v/v) to 90% B (v/v) in 40 min; followed by a linear gradient from 90% B (v/v) to 10% B (v/v) solvent B for a further 5 min, with a flow rate of 1 ml/min. The porphyrins were positively identified fluorometrically (λ_{ex} 400 nm, λ_{em} 618 nm) using a mixture of uroporphyrin, hepta-, hexa-, penta and meso-carboxylic acid porphyrins and PROTO IX as standard (Frontier– Scientific, USA). Quantitative measurement of PROTO IX was determined fluorometrically (λ_{ex} 400 nm, λ_{em} 632 nm) according to Polo *et al.* (37) with slight modifications.

δ-Aminolevulic acid synthetase (ALA-S) activity was determined following the method of Marver *et al.* (23). The total activity of TRP and the apo and holo enzymes were determined by the method of Badaway and Evans (2).

Reactive oxygen species (ROS) levels were determined in blood using a commercial kit FORT (Callegari, Italy) and expressed as mg/l H_2O_2 (1 unit FORT is equivalent to 0.26 mg/l H_2O_2). Lipid peroxidation was estimated as MDA levels using the method of Ohkawa *et al.* (28). GSH was quantified according to Rossi *et al.* (40). GST activity was determined by the method of Habig *et al.* (15). Catalase activity was measured as described by Chance & Maehly (8), GRed following the method of Pinto and Bartley (34), GPx according to Paglia and Valentine (31), and SOD by the method of Paoletti *et al.* (32).

Plasma activities of AST, ALT, AP, and GGT were determined using a kit from Wiener lab. (Rosario, Argentina).

Total CYP levels were measured according to Omura and Sato (29). CYP2E1 activity was determined using Reinke and Moyer technique (39); CYP2A6, CYP2C9 and CYP1A2 activities following the method of Pearce *et al.* (33).

Protein concentration was determined by the procedure of Lowry *et al.* (22).

Enzyme units were defined as the amount of enzyme that catalyses the formation of 1 nmol of product under the standard incubation conditions. One unit of SOD is defined as the amount of SOD causing 50% inhibition on the rate of NADH oxidation measured in the control (32). Specific activity was expressed as units/mg protein.

Western Blot analysis

Liver tissue was homogenized (1:5, w/v) in 10 mM TRIS-HCl pH 7.4, containing 20% glycerol (v/v), 1.14% KCl (w/v), 0.2 mM EDTA, 0.1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, 10 µg/ml leupeptin and 1 µg/ml pepstatin A. Homogenates were centrifuged with the

same protocol as described above. Proteins were quantified according to Bradford method (6). For the Western Blot analysis of CYP3A4 isoform, 20 µg of protein was separated on a 7.5% SDS-PAGE and transferred to nitrocellulose membranes. After blocking overnight with 5% bovine ***serum albumine*** (BSA) in Tris-buffered saline containing 1% Tween 20 (TBS-T 1%), the blots were incubated for 1 hour at room temperature with the specified primary antibody: anti-CYP3A4 (1:500 v/v in TBS-T 1%; Santa Cruz Biotechnology). After several washings, blots were incubated for 1 h with a secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (1:8,000 v/v in TBS-T 1%, Santa Cruz Biotechnology). Blots were detected by chemiluminescence using ECL detection system (GE Healthcare) and exposed to X-ray film.

RNA extraction and Northern blot analysis

Total RNA was obtained by phenol/chloroform using the method of Chomczynski and Sacchi (9) from the liver and the mRNA expression was determined by Northern blot using specific probes. Total RNA (20 µg) was size fractioned by denaturating agarose gel electrophoresis and then transferred overnight on to a nylon membrane. The crosslinking was done using a UV Stratalinker 1800 (120 mJ UV light). The membrane was pre hybridized with 1 ml ULTRAhybTM (Ambion) each 10 cm^2 of membrane during 4 hr. The cDNA probe for catalase was kindly provided by Dr. Gerez (CIPYP, CONICET, Argentina). To obtain GPx cDNA, RT-PCR was performed using a SUPERSCRIPT^{TM} kit (Invitrogen Life Tech.). GPx cDNA probe was synthesized by RT-PCR using specific primers (FP 5´-TCATTGAGAATGTCGCGTCT-3´; RP: 5´-ATGTCGATGGTACGAAAGCG-3´. A 442 pb PCR amplified product was analyzed on 1% agarose gel and purified using a QIAquick kit (QUIAGEN). The expression of catalase and GPx mRNA was normalized by comparison with the expression of 18S mRNA. The hybridized membrane was exposed to AGF radiographic film to visualize bands.

Histology and immunohistochemistry

Livers were removed and fixed in 10% neutral-buffered formalin. Samples of each hepatic lobules were processed routinely and embedded in paraffin. At least six microtome sections of 3–5 µm were stained with haematoxylin–eosin. Immunohistochemistry was performed using the streptavidin-biotin-peroxidase complex system LSAB (DAKO). In brief, endogenous peroxidase activity was inhibited using 3% H_2O_2 in distilled water. Microwave antigen retrieval (4 cycles of 5 min each in 0.1 M citrate buffer) using 750 W microwave oven was used. Blocking solution (2% normal goat serum) was used before the specific antibody. The sections were incubated overnight at room temperature with anti-HO1 policlonal rabbit antibody diluted 1:500. Control sections without primary antibody served as control. The reaction was developed with 3, 3'-diaminobenzidine (DAB), under microscopic control. Specimens were counterstained with hematoxylin 10%, dehydrated and mounted.

Statistical analysis

Newman-Keuls test was used to assess the degree of significance. Probability levels of 0.01 and 0.05 were used in testing for significant differences between experimental groups.

RESULTS

Macroscopic Alterations

Mice fed with Gris had a marked increase in the size of the abdomen, compared to control group. These mice showed prominent hepatomegaly, with the liver being dark brown in color, and augmented consistence, eventhough at the lowest concentration of the antifungal tested.

When animals received 2.5% of Gris during 14 days, the presence of wounds in the snout, ears and legs at the end of the treatment was observed.

A decrease in body weight was also found reaching a diminution of 35% ($p<0.01$) at day 14. Gris also produced around 50-90% ($p<0.05$) increase of liver weight (Table 1).

Table 1: Effect of Gris on body and liver weight

Gris (%)	Time of treatment	Body weight (g)	Liver weight (g)
0	7 days	25.31 ± 1.32	1.72 ± 0.53
0.5		24.45 ± 1.23	2.64 ± 0.65
1.0		23.11 ± 0.98	2.75 ± 0.65
2.5		21.93 ± 0.95*	2.87 ± 0.39
0	14 días	25.31 ± 1.32	1.72 ± 0.53
0.5		24.92 ± 1.18	2.74 ± 0.56
1.0		23.34 ± 1.35	3.27 ± 0.71
2.5		16.04 ± 1.16**	2.55 ± 0.44

Mice received normal or Gris containing diet (0.5%, 1.0% or 2.5% w/w) over 7 days or 14 days. Data represent mean values ± S.D. of 8 mice. (*) $p<0.05$ and (**) $p<0.01$ significance of differences between treated and control animals. Other experimental details are described in the Material and Methods

Gris caused a time and concentration progressive increase of liver weight/body weight ratio (Figure 1).

Histology

Histological studies were performed in livers of mice feeding with 0.25%, 0.5%, 1.0% or 2.5% Gris during 14 days (Figure 2). In all the cases, similar alterations were observed. The liver plates tend to become disrupted and distorted. There is an activation of sinusoidal lining cells with prominent Kupffer´s cells. Hepatocellular alterations, predominantly ballooning or feathery degeneration, small foci of

necrosis of isolated hepatocytes and acidophilic bodies, few in number, can be seen (Figure 2 A, B). The portal changes are ductular proliferation, periductal edema and portal inflammation (Fgure 2 A). The ductular proliferation occurs along the margins of the portal triad and the inflammatory infiltrate is predominantly lymphocytic with some mononuclear cells. Cytoplasmic and canalicular cholestasis with the presence of bile plug in interlobular ducts can be seen (Figure 2 B)

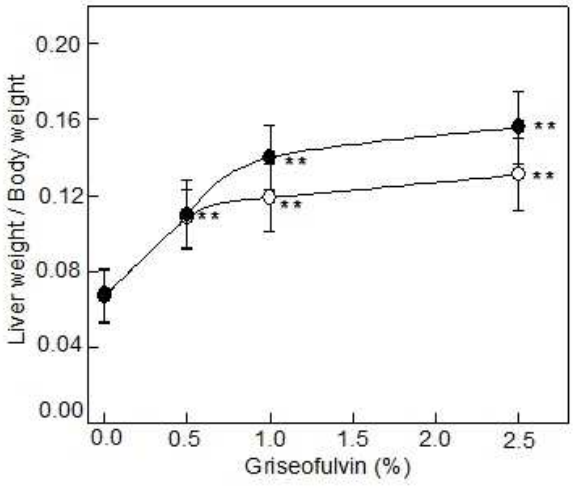

Figure 1. Effect of Gris on liver and body weight ratio
Mice received normal or Gris containing diet (0.5%; 1.0% or 2.5%, w/w) over 7 (○) or 14 (●) days. Data represent mean values ± S.D. of 8 mice. (**) p<0.01, significance of differences between treated and control animals. Experimental details are described in Material and Methods.

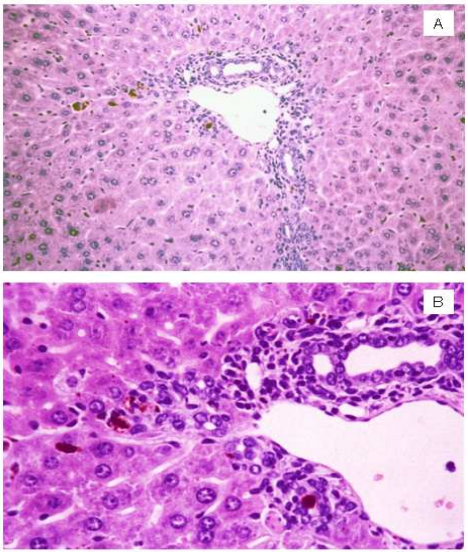

Figure 2. Effect of Gris on liver histology
Mice received 0.5% Gris containing diet over 14 days. A: Liver plates disrupted and distorted with feathery degeneration, foci of necrosis of isolated hepatocytes and acidophilic bodies. Periductal edema and portal inflammation. (H-E, 10x). B: Ductular proliferation in portal triad with cytoplasmic and canalicular cholestasis with the presence of bile plug. (H-E, 25x). Experimental details are described in Material and Methods

Effects of Gris on liver injury enzyme markers

Results are shown in Figure 3. AST (Figure 3 A) and ALT (Figure 3 B) activities augmented in a dose-and time-dependent manner, reaching to a 280% ($p<0.01$) and 730% ($p<0.01$) increase respectively, indicating a severe injury at hepatocyte level. AP (Figure 3 C) and GGT (Figure 3 D) showed a similar pattern, being the activities 350% ($p<0.01$) and 240% ($p<0.01$) increased respectively after 14 days Gris feeding, demonstrating a disturbance in biliary excretion.

Effect of Gris on heme metabolism

HPLC analyses of porphyrins showed an increase of PROTO IX in liver, blood and feces by effect of Gris, without any significant changes in the other types of porphyrins (Figure 4).

The effects of Gris on PROTO IX levels are shown in Table 2. In liver, the accumulation of PROTO IX was time dependent, being increased 47-fold and 65 fold respect to control group after 7 and 14 days of Gris treatment respectively, without no changes with the different Gris concentration assayed. A similar profile was observed in blood. Although, fecal PROTO IX excretion depended on Gris doses and time of intoxication, an increase between 534% and 670% ($p<0.01$) at the end of the treatment was detected.

Gris caused an induction of ALA-S activity as a function of drug concentration. The maximum increase was observed at 2.5% of Gris at day 7 (50%, $p<0.01$) (50%, $p<0.01$) (50%, $p<0.01$) and 80% ($p<0.01$) at day 14 (Figure 5).

HO activity reached a peak of 100% ($p< 0.01$) increase after administration of 1% Gris and then enzyme activity showed an striking decrease leading to below under basal levels; this profile was the same for the two times of intoxication assayed (Figure 6 A). Anti- HO1 immunostain showed negative hepatocytes and positive Kupffer´s cells in control liver (Figure 6 B), while a positive immunostain in isolated hepatocytes and abundant Kupffer´s cells can be also seen in liver of Gris group (Figure 6 C).

To evaluate the effect of Gris on heme regulatory pool, the holo- and apo-TRP activity was measured (Figure 7). The ratio between both protein forms, revealed a reduction only depending on Gris concentration, indicating a depletion of free heme levels.

Effect of Gris on Phase I Drug Metabolizing System

Results are shown in Figure 8. After 7 days Gris feeding, CYP content increased 30%

Table 2: **Effect of Gris on liver, blood and feces PROTO IX levels**

Gris (%)	Time of treatment	PROTO IX levels		
		Liver (ng/mg protein)	Blood (ng/mg protein)	Feces (ng/mg feces)
0	7 days	0.45 ± 0.04	67.3 ± 11.6	2.32 ± 0.05
0.5		11.95 ± 2.26**	178 ± 21**	5.43 ± 0.21**
1.0		15.71 ± 3.32**	573 ± 108**	8.65 ± 0.23**
2.5		21.35 ± 4.28**	857 ± 142**	12.34 ± 0.41**
0	14 days	0.45 ± 0.04	67.3 ± 11.6	2.32 ± 0.05
0.5		22.46 ± 5.24**	753 ± 134**	11.02 ± 0.35**
1.0		29.10 ± 6.34**	1445 ± 206**	15.25 ± 0.51**
2.5		11.02 ± 0.35**	1342 ± 223**	15.56 ± 0.43**

(*) p<0.05 and (**) p<0.01 significance of differences between treated and control animals. Experimental details are described in legend to Table 1.

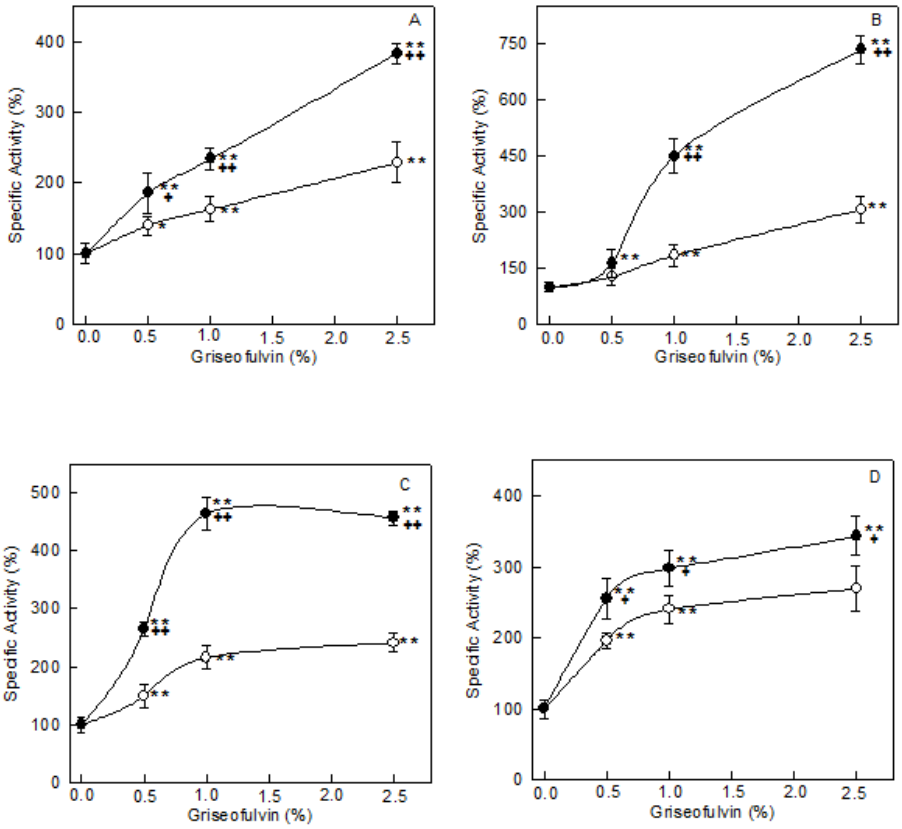

Figure 3. Effect of Gris on liver damage enzyme markers

A: AST, mean control value: 16.60±2.42 U/ml (n-8); B: ALT, mean control value: 27.75±0.45 U/ml (n-8); C: AP mean control value: 469.4±68.3 U/ml (n-8); D: GGT, mean control value: 45.25±7.0 µU/ml (n-8).

Results are expressed as percentage of the corresponding control value taken as 100%.

(*) p<0.05 and (**) p<0.01 significance of differences between treated and control animals; (✚) p<0.05 and (✚✚) p<0.01 significance of differences between animals treated during 7 (○) or 14 (●) days. Experimental details are described in legend to Figure 1.

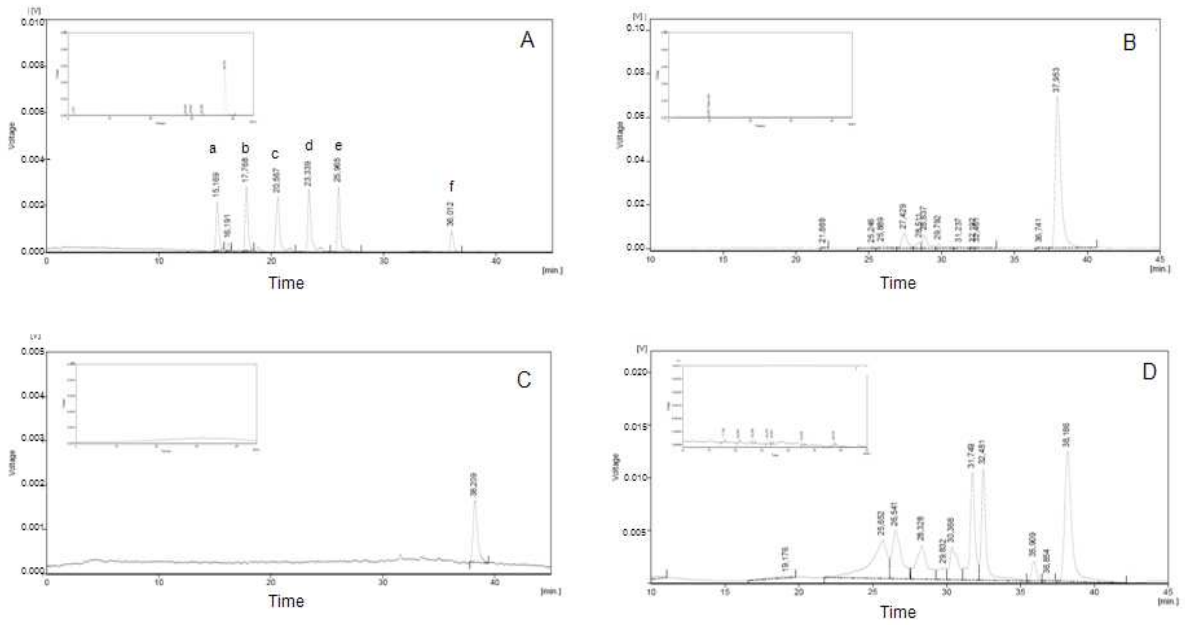

Figure 4. Effect of Gris on liver, blood and fecal porphyrin pattern
A: Standards of porphyrins: a) octa-(uro) carboxyl porphyrin, b) hepta- carboxyl porphyrin, c) hexa- carboxyl porphyrin, d) penta- carboxyl porphyrin, e) tetra-(copro) carboxyl porphyrin, f) di-(meso) carboxyl porphyrin; inset: PROTO IX. B: liver, C: blood, D: feces; insets: control animals. A unique chromatogram is shown as representation of HPLC porphyrin pattern run for each animal because no significant differences were obtained between them. Experimental details are described in Material and Methods.

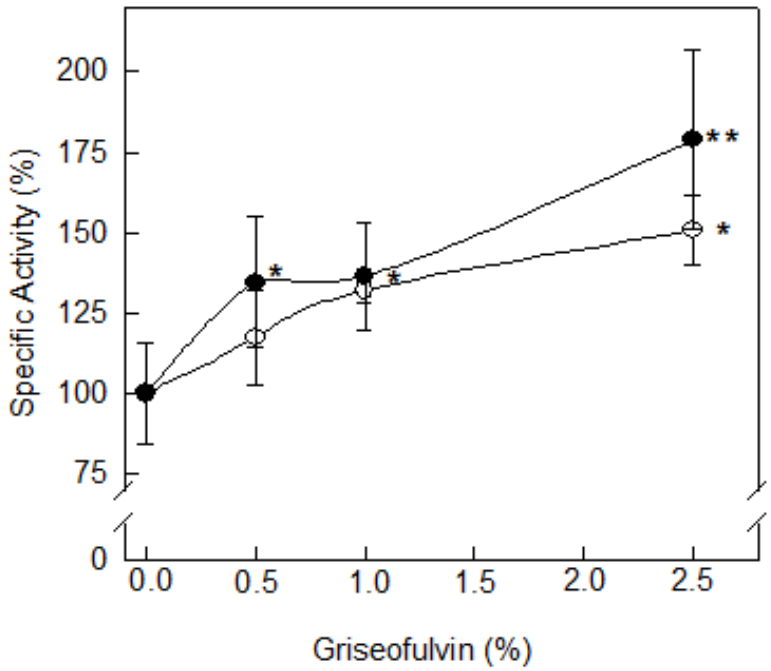

Figure 5. Effect of Gris on ALA-S activity
Mean control value: 0.144±0.021 nmol/mg protein (n-8). Results are expressed as percentage of the corresponding control value taken as 100%. (*) $p<0.05$ and (**) $p<0.01$ significance of differences between treated and control animals. Experimental details are described in legend to Figure 1.

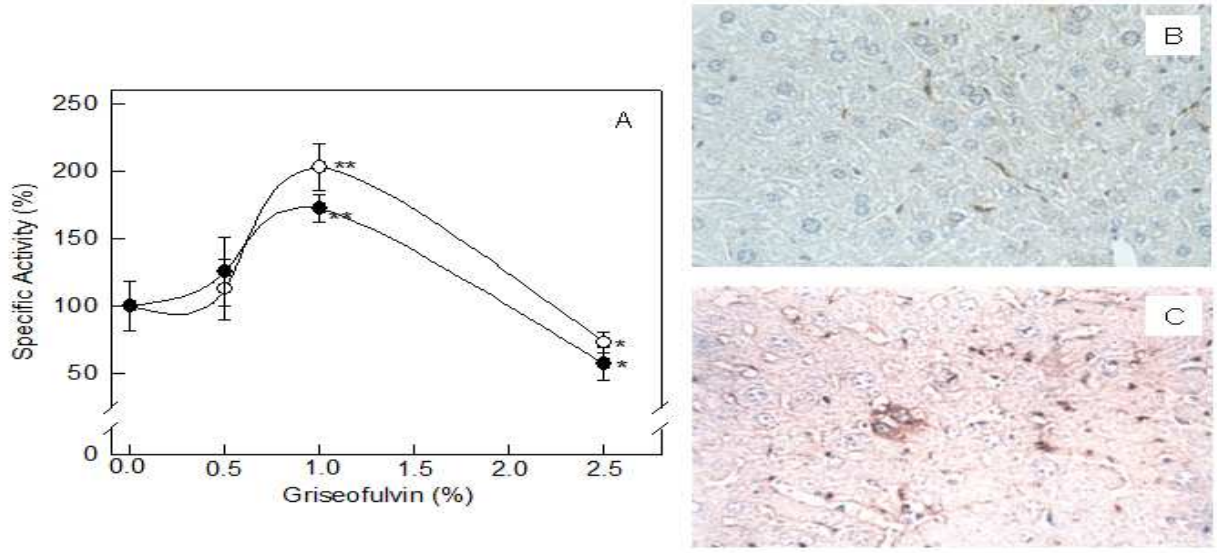

Figure 6. Effect of Gris on HO activity and expression
A: Specific activity. Control value: 1.368±0.257 nmol/mg protein (n-8). Results are expressed as percentage of the corresponding control value taken as 100%. (*) $p<0.05$ and (**) $p<0.01$ significance of differences between treated and control animals. B: Immunohistochemistry HO-1 expression in liver of control animals, Control liver: positive anti-HO-1 Kupffer´s cells with negative hepatocytes. (IHC-H, 25x). C: Immunohistochemistry HO-1 expression in liver of Gris feeding animals. Griseofulvin liver: altered hepatocytes with intense positive anti-HO-1 in isolated cells and numerous positive Kupffer´s cells. (IHC-H, 25x). Experimental details are described in legend to Figure 1 and in Materials and Methods.

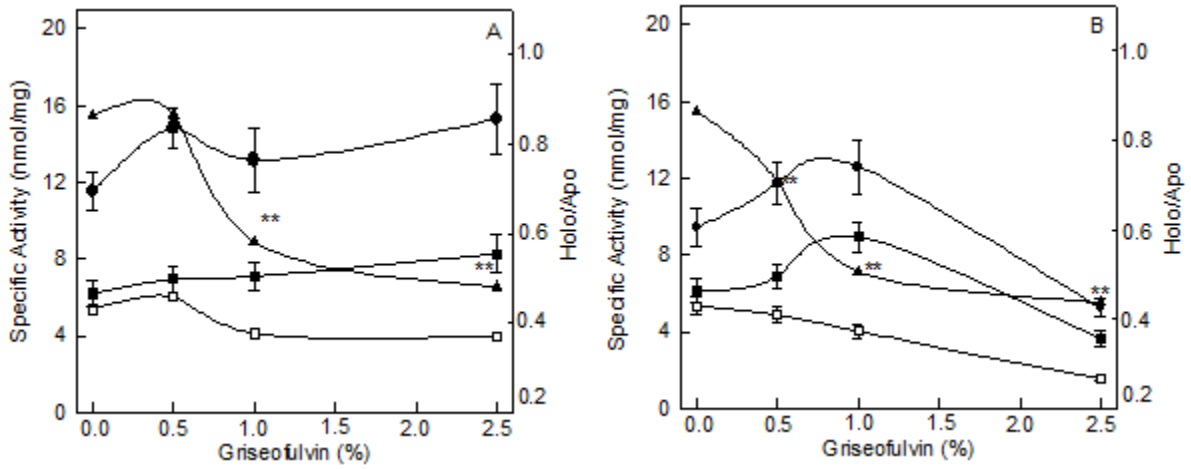

Figure 7. Effect of Gris on TRP activity
A: 7 days, B: 14 days. (●) Total TRP, (■) Holo-enzyme, (□) Apo-enzyme, (▲) Holo-/Apo-enzyme ratio. Mice received normal or Gris containing diet (0.5%; 1.0% or 2.5%, w/w) over 7 or 14 days. Data represent mean values ± S.D. of 8 mice. (**) $p<0.01$ compared to control group. Experimental details are described in Material and Methods.

($p<0.05$) when the dose of Gris was 1%, returning to control levels at 2.5% Gris concentration. In contrast, in animals treated during 14 days, a 43% ($p<0.01$) increase was detected with 0.5% Gris, followed by a strong decrease up to 36% ($p<0.01$) under basal levels at 2.5% Gris (Figure 8 A).

CYP2A6 activity increased between 300-360% ($p<0.01$) after 7 days of treatment and around 446-510% ($p<0.01$) after the day 14, being statistical significant the difference between values at 7 and 14 days (Figure 8 B). No variations were observed in the activity of CYP2E1, CYP1A2 and CYP2C9 isoforms (data not shown).

The expression of CYP3A4 protein revealed an increase at all Gris concentration analyzed (Figure 8 C).

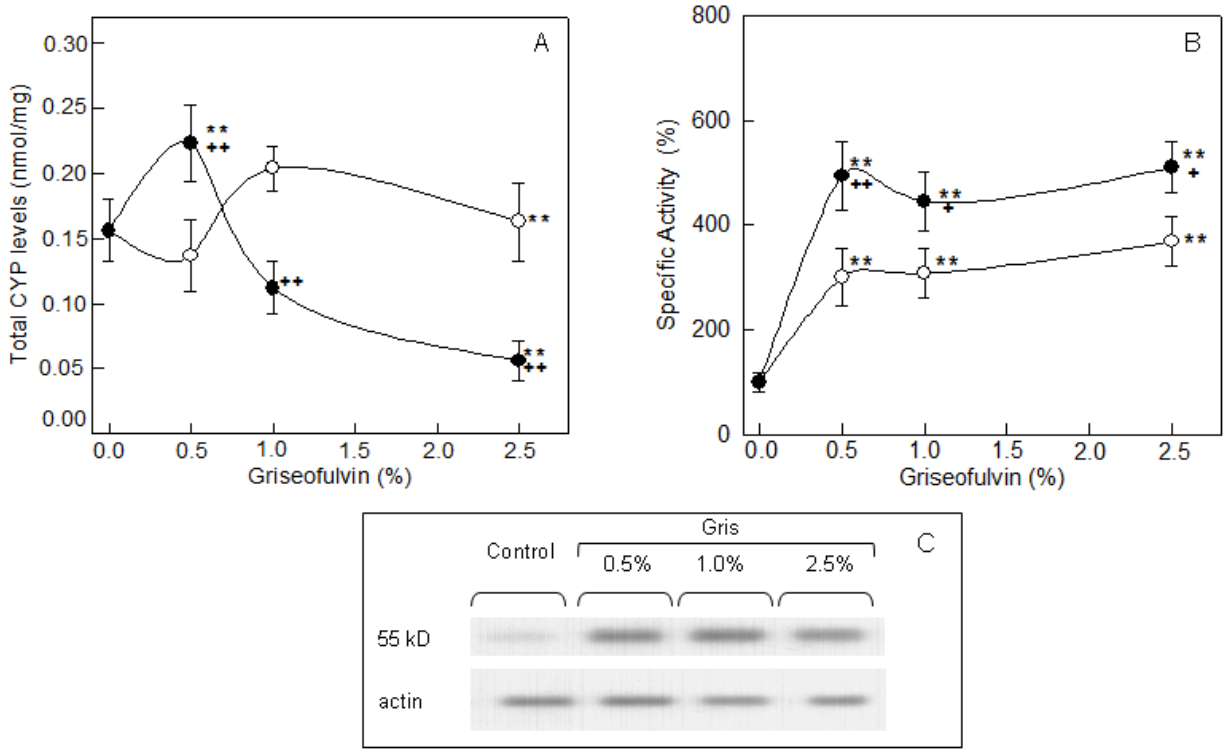

Figure 8. Effect of Gris on total CYP levels, CYP2A6 activity and CYP3A4 expression
Mice were provided with either normal or Gris containing diet (0.5%, 1.0% or 2.5% w/w) over 7 (○) or 14 (●) days.
A: Total CYP levels. Data are expressed as mean values ± S.D.
B: CYP2A6 specific activity. Results are expressed as percentage of the corresponding control values taken as 100%. Control value: 1.46±0.27 nmol/mg protein (n-8).
C: CYP3A4 protein expression. A unique Western blot analysis is shown as representation of at least three determinations run in duplicate.
(**) p<0.01 compared to control group; (+) p<0.05 and (++) p<0.01 significance of differences between animals treated during 7 (○) or 14 (●) days. Experimental details are described in Material and Methods.

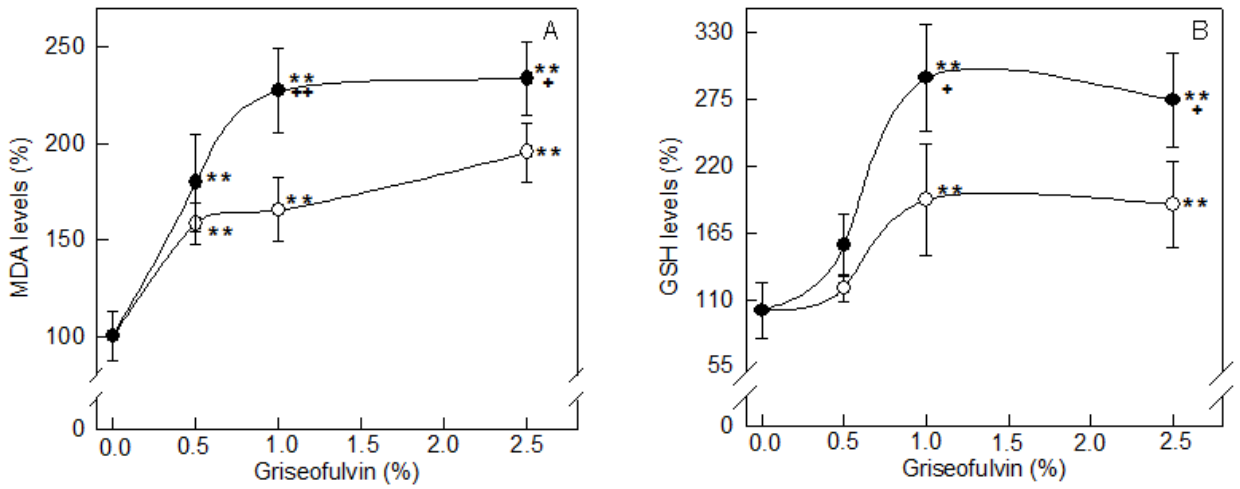

Figure 9. Effect of Gris on MDA and GSH levels
A: MDA levels, mean control value: 3.15±0.40 nmol/mg protein (n-8). B: GSH levels, mean control value: 22.98±5.29 nmol/mg protein (n-8).
(**) p<0.01 compared to control group; (+) p<0.05 and (++) p<0.01 significance of differences between animals treated during 7 (○) or 14 (●) days. Experimental details are described in legend to Figure 3.

Effect of Gris on the development of Oxidative Stress

The effects of Gris on stress oxidative markers are shown in Table 3 and Figure 9.

Free radicals levels increased significantly in a dose-dependent manner up to a concentration of 1% Gris, remaining at these high levels when the dose was 2.5% (Table 3).

MDA levels increased from 60% ($p<0.01$) to 95% ($p<0.01$) after 7 days and from 80% ($p<0.01$) to 130% ($p<0.01$) after 14 days of treatment, being significant the differences between both times of intoxication (Figure 9 A). GSH levels augmented 90% ($p<0.01$) in animals receiving 1.0% and 2.5% Gris during 7 days, while at day 14, the increase was significantly greater than that found after 7 days (170-190%, $p<0.01$) (Figure 9 B).

The effects of Gris on antioxidant defense system are shown in Figures 10 and 11.

At all the doses of Gris tested, an increase of GRed activity of 65% ($p<0.01$) at day 7 and 150% ($p<0.01$) at day 14 was found with a significant difference between the two times treatment (Figure 10 A). SOD activity showed a significant induction with doses, reaching 100% ($p<0.01$) increase at the highest concentration, without differences between time of treatment (Figure 10 B). The variations of GST activity were independent of Gris concentrations and times of intoxication; the activity was increased about 50% ($p<0.01$) (Figure 10 C). A 30% ($p<0.01$) decrease of GPx activity in mice treated with 1% Gris at 7 and 14 days of treatment was found, while in the group receiving 2.5%, a diminution up to 60% ($p<0.01$) was observed after 2 weeks of treatment (Figure 11 A). Catalase activity showed a progressive decrease depending on Gris concentration, reaching 80% ($p<0.01$) decrease when the dose assayed was 2.5% during 14 days, being significantly lower than the activity measured at day 7 (Figure 11 B). Taking into account these last results that revealed a non expected diminution in Catalase and GPx activities, it was of interest to evaluate the effect on mRNA expression of these enzymes. No changes in mRNA expression were observed indicating no effect at transcriptional level (Figure 11 C, D).

To evaluate if the Catalase and GPx diminished activities were a consequence of the reduced heme pool, Gris feeding mice were treated with hemin (15 mg/kg) on days 7, 9, 11 and 13 of treatment. In all the cases, activities return to basal levels demonstrating a direct relationship between these enzymes activities and heme levels (data not shown).

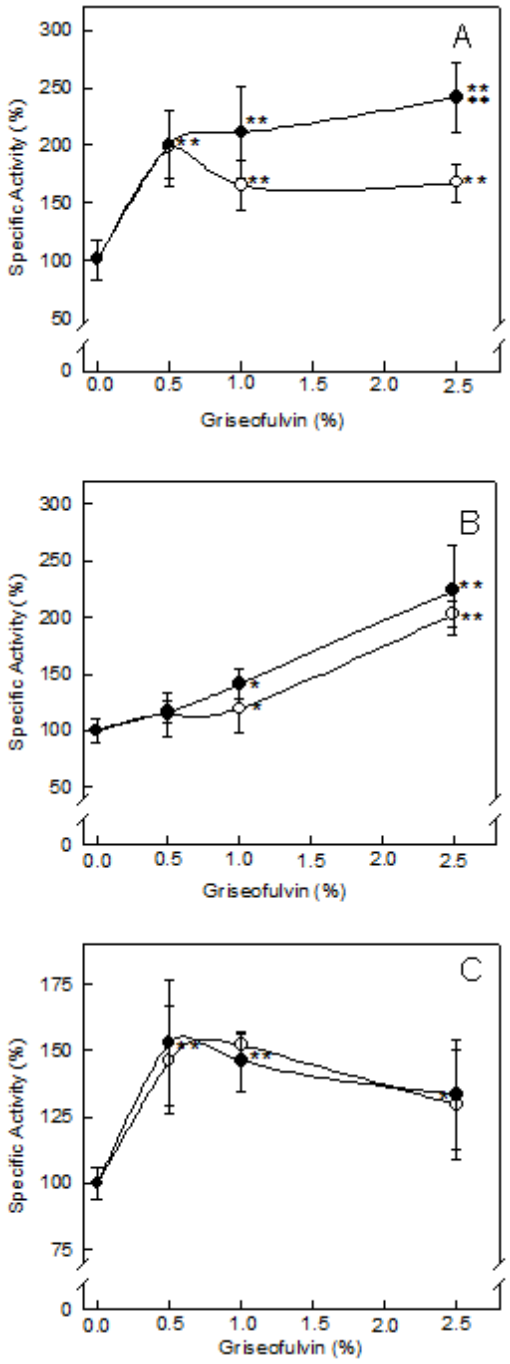

Figure 10. Effect of Gris on liver antioxidant defense system

A: GRed, mean control value: 43.52±7.40 nmol/mg protein (n-8); B: SOD, mean control value: 83.90±8.66 nmol/mg protein (n-8); C: GST, mean control value: 22.77±1.20 µnmol/mg protein (n-8). Results are expressed as percentage of the corresponding control value taken as 100%. (*) $p<0.05$ and (**) $p<0.01$ significance of differences between treated and control animals; (++) $p<0.01$ significance of differences between animals treated during 7 (○) or 14 (●) days. Experimental details are described in legend to Figure 3.

Table 3: **Effect of Gris on free radicals levels**

Gris (%)	Time of treatment	mg/l H_2O_2.
0	7 days	< 41.60
0.5		52.78
1.0		74.36
2.5		78.26
0	14 days	< 41.60
0.5		54.08
1.0		81.64
2.5		75.40

Experimental details are described in Material and Methods and in legend to Table I

DISCUSSION

In this work, the protoporphyria animal model produced by Gris administration was further characterized, studying the effect of this xenobiotic on different metabolisms.

The early administration of Gris and at the lower dose assayed produced a significant liver injury, increase of ALA-S activity and PROTO IX accumulation in liver, blood and feces. These results indicated an alteration in the heme synthesis pathway, accordingly with the control that heme plays, through a feedback mechanism, on ALA-S transcription, translation and activity (4).

As a result of Gris metabolization, at the lowest dose of Gris assayed, an increase of CYP levels and particularly in CYP2A6 activity and CYP3A4 expression was observed. Moreover, as Gris acts as suicide substrate (12, 16), a diminution of CYP was produced at the highest Gris concentration. At this time, synthesis of heme would increase to maintain the free heme pool levels which are necessary for new CYP and other hemeproteins synthesis. The reduction of this reservoir was corroborated by a diminished holo/apo TRP ratio similar to Badaway and Evans results (4).

The increase in liver weight/body weight after treatment with Gris could be attributed to an increase in the mitotic activity in the hepatocytes of these mice (19, 43). Hepatic histological lesions and the alterations of serum AST and ALT would indicate a functional degeneration and necrosis of the liver. Moreover, AP and GGT activities changes would also reflect an obstructive intrahepatic bile ducts damage.

These results are consistent with previous studies carried out in a similar model of intoxication with Gris in control animals (18, 24, 36, 42, 45), and also when Gris was administered to a knock out mice which is deficient for Fech (48).

We observed changes in the redox balance of the liver due to high concentrations of porphyrins, so, the antioxidant defense system was disrupted. The accumulation of PROTO IX and iron, primarily in the mitochondria, and then in other organelles and cytosol (1, 20, 27, 38), could be the cause of oxidative damage. In our study, the oxidative stress was demonstrated through the increase of ROS and MDA levels after Gris treatment. As a consequence of oxidative injury, we also found induction of GSH levels and in the activities of SOD, GRed, GST and HO.

At physiological concentrations of GSH, a low activity of GRed is enough to reduce the oxidized glutathione from GPx activity; under conditions of oxidative stress, the increase of the activity of both enzymes would provide cell antioxidant defense mechanisms (25).

The activity of SOD was increased; this enzyme is also induced by oxidative stress (25). Due to the high substrate specificity of the SOD, the increase of its activity would indicate the generation of superoxide radical above physiological values (14). The dismutation of superoxide anion leads to the formation of H_2O_2, which cannot be consume due to the low activities of catalase and GPx observed, and then to the development of lipid peroxidation (14).

The increase of GST activity in mice that received Gris is indicative of liver damage (7). In addition, its activity is also increased in rats receiving pro-oxidants (44).

As was expected, HO was also induced as a consequense of oxidative stress generated by Gris and PROTO IX accumulation. However, at higher concentrations of antifungal, the free heme pool is so depleted that HO would not be necessary.

Inafuku et al. (18) reported that in the model of Protoporphyria induced by Gris, levels of HO-1 mRNA increased very quickly due to chemically stressed hepatocytes and accumulation of heme precursors, or N-methyl porphyrin generated by Gris. Gris by itself can

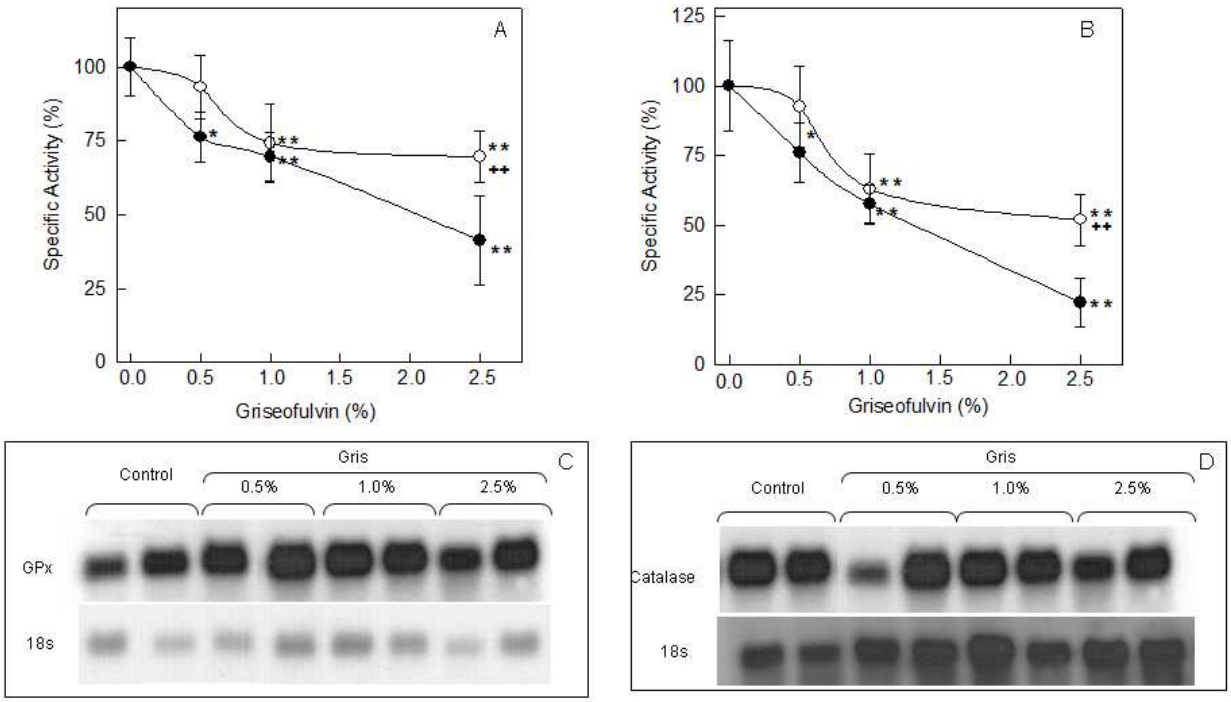

Figure 11. Effect of Gris on GPx and Catalase enzyme activities and mRNA expression
Mice received normal or Gris containing diet (0.5%, 1.0% or 2.5% w/w) over 7 (○) or 14 (●) days.
A: GPx activity, mean control value: 646.2±64.5 nmol/mg protein (n-8). B: Catalase specific activity, mean control value: 68.18±11.07 nmol/mg protein (n-8). Results are expressed as percentage of the corresponding control value taken as 100%. (*) $p<0.05$ and (**) $p<0.01$ compared to control group; (++) $p<0.01$ significance of differences between animals treated during 7 (○) or 14 (●) days.
C: GPx mRNA expression profile. D: Catalase mRNA expression profile. Autoradiogram shows Northern blot analysis of mRNA enzymes and 18S mRNA. A unique Northern blot analysis is shown as representation of at least three determinations run in duplicate. Experimental details are described in Material and Methods.

potentially induce the expression of HO-1. In addition, these authors argued that this augmented expression may be due to free iron increase, which could not be incorporated into PROTO IX.

In conclusion, in our experimental model of Protoporphyria, the alterations found in the liver are similar to those observed in patients with EPP. Although the pathogenesis of cellular damage in EPP cannot be associated only to deposits of PROTO IX in hepatocytes and bile canalicular, it could contribute to liver failure especially in late stages of disease when ultrastructural abnormalities are present (38). So, it is of interest to investigate the effect of drugs, such as bile acids or antioxidants, which act eliminating the porphyrins accumulated and also diminishing ROS production. For this purpose, we think that, according to these results, a dose of 0.5 % of Gris, during 14 days, is the more adequate model of EPP, because the liver is not so deteriorated even if the effects of Gris are evident.

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