HEME METABOLISM, OXIDATIVE AND NITROSATIVE MARKERS IN A MOUSE MODEL OF HEMOCHROMATOSIS: EFFECT OF ISOFLURANE, ETHANOL AND 5-AMINOLEVULINIC ACID

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Abstract- Hereditary hemochromatosis (HH) is characterized by iron homeostasis alterations. Association between HH and Porphyria Cutanea Tarda has been reported. The aim was to characterize oxidative and nitrosative stress status and its relationship with heme metabolism in a hemochromatosis mouse model ($Hfe^{-/-}$), and to evaluate the effects of Isoflurane, ethanol and 5-aminolevulinic acid (ALA). Male and female $Hfe^{-/-}$ and wild-type C57BL/6J mice received Isoflurane (2 ml/kg); ethanol (30%) or ALA (40 mg/kg). In male $Hfe^{-/-}$, reduced glutathione (GSH) was diminished respect to C57BL/6J mice. Female $Hfe^{-/-}$ showed higher levels of GSH and total antioxidant capacity than male $Hfe^{-/-}$. Catalase activity was lower in male and female $Hfe^{-/-}$ than in controls. 5-Aminolevulinic acid synthetase activity was higher in male and female $Hfe^{-/-}$ than in controls. In male $Hfe^{-/-}$, Porphobilinogen deaminase (PBG-D) activity was augmented and Heme oxygenase (HO) activity was diminished probably to avoid iron increase. Isoflurane and ethanol reduced PBG-D and increased HO activities. HO induction would indicate oxidative stress instauration being more striking due to ethanol that also induced Superoxide dismutase activity. Isoflurane reduced Nitric Oxide Synthase expression. ALA altered antioxidant system. In $Hfe^{-/-}$ mice different metabolisms were altered being more affected by the drugs studied. Findings here described would contribute to increase the knowledge about the association between HH and the Porphyrias and about the effects of volatile anaesthetics on different metabolisms in genetic models of Porphyrias and associated diseases.

Keywords- Heme biosynthesis, heme oxygenase, oxidative stress, volatile anaesthetics, porphyrinogenic drugs

Running Title- Hemochromatosis and porphyrinogenic drugs

Introduction

Hereditary hemochromatosis (HH) is a genetic disorder characterized by increased intestinal absorption of dietary iron and iron release from reticuloendothelial macrophages. The excess of iron entry into the bloodstream results in iron deposition in parenchymal organs such as liver, pancreas, heart and pituitary glands, which subsequently leads to tissue damage and ultimately to cirrhosis, diabetes, cardiomyopathy, hypogonadism, arthropathy and skin pigmentation [1,2]. Although iron is essential for critical cellular functions, the excess may catalyze Haber-Weiss reactions that generate reactive oxygen species (ROS) leading to consequent damage to DNA, proteins and membranes. The increase of lipid peroxidation, catalyzed by iron, has been proposed as an initial step by which excess iron causes cellular injury [3].

The majority of the patients with HH are homozygous for the mutation c.845G>A (p.Cys282Tyr) in the *HFE* gene (HH type 1), being the clinical and biochemical penetrance highly variable [4-6]; the non-HFE hemochromatosis are caused by mutations in different genes involved in iron homeostasis [7].

The hepatic iron accumulation is also a pathological trait of different disorders, and it is often observed in patients with Porphyria Cutanea Tarda (PCT). PCT is the most common type of porphyria. PCT is caused by subnormal activity of uroporphyrinogen decarboxylase (URO-D), the fifth enzyme of heme biosynthetic pathway [8]. The disease is characterized by skin photosensitivity with blistering on sun-exposed areas, skin fragility, hyperpigmentation, and hyperthricosis [9,10]. The clinical manifestations are frequently associated with exposure to precipitating agents, e.g. alcohol, estrogen, polyhalogenated aromatic hydrocarbons, iron overload and virus infection (hepatitis C virus, human immunodeficiency virus, and less frequently, hepatitis B virus [10,12].

Edwards, et al [13] were the first to suggest that mutations in the *HFE* gene were responsible for the hepatic siderosis in many patients with PCT. This was later confirmed by mutational analysis of the *HFE* gene [14].

Isoflurane is a volatile anaesthetic used in clinical medicine to produce general anaesthesia. This drug is metabolized by cytochrome P-450 (CYP) and in particular by the isoform CYP2E1 [15]. We have previously demonstrated that Isoflurane produced significant alterations in heme metabolism and its regulation in a way dependent on sex and strain when it was administered to mice [16,17]. Furthermore, this anesthetic caused changes in the antioxidant defense system and in the expression of Nitric Oxide Synthase (NOS) [18-20]. Moreover, in alcoholized mice, Isoflurane administration also altered Phase I drug metabolizing system [21]. The effects of this anesthetic in mice models of Erythropoietic Protoporphyria and Porphyria Hepatoerythropoietic indicated that administration should be avoided not only in patients with acute porphy-

rias [16] but it should be used with caution in patients with non-acute hepatic porphyrias [22,23].

The aim of this work was to characterize oxidative and nitrosative stress status and its relationship with heme metabolism alterations in a mouse model of hemochromatosis, and to evaluate the changes caused by the administration of Isoflurane. Taking into account the known association between HH and PCT it was of interest to investigate the effect of the porphyrinogenic drug, ethanol, in this model of HH. The results were compared with those produced under the pathological situation of elevated accumulation of the precursor of heme biosynthesis 5-aminolevulinic acid (ALA). A comparative study between male and female mice was performed.

To evaluate the stress oxidative status, the levels of reduced gluthation (GSH), total antioxidant capacity (TAC), and the activities of Catalase and Superoxide dismutase (SOD) were measured. As a marker of nitrosative stress, the expression of NOS was determined. Heme metabolism was studied through the activities of 5-Aminolevulinic acid synthase (ALA-S), the regulatory enzyme; Porphobilinogen deaminase (PBG-D), enzyme reduced in the Acute Intermittent Porphyria (AIP) and Heme oxygenase (HO), involved in heme catabolism and also augmented under oxidative stress [24]. Moreover, it was of interest to determine if the drug metabolizing system was altered, so the activity of the CYP2E1 enzyme responsible of Isoflurane and ethanol Phase I metabolization; and Gluthation S-transferase (GST), involved in Phase II reactions as a marker of hepatic damage [25] were determined.

Materials and Methods

Animals

The hemochromatic mice $Hfe^{-/-}$ (strain B6 129P2-Hfetm1gfn ⁄J from The Jackson Laboratory) and wild-type mice, both on a C57BL/6J background, were bred under specific pathogen-free conditions at the animal facility of the Hospital 12 de Octubre, Madrid, Spain, with unlimited water supply and a maintenance irradiated diet. The animal experimentation was conducted in accordance with "European Council Guidelines" (86/609 CEE, 90/67 CEE, 99/575 CEE) and the Spanish Royal Decree 1201/2005.

Experimental Design

Male and female 8-week-old mice of both genotypes, $Hfe^{-/-}$ and wild-type, were divided in groups of 4-6 animals that received the following treatments: One group received a single dose of 2 ml/kg (0.6:3, v/v. in oil) i.p, of Isoflurane. This was the optimum anaesthetic dose to produce more alterations in the heme pathway, as was previously described [16]. Other group received ethanol (30% in drinking water during a week), and a third group was treated with a single dose of ALA (40 mg ALA/kg in NaCl 0.9%, pH 7; i.p). Control animals received the vehicle and they were sacrificed at the same times corresponding to each treatment.

Tissue Extraction and Assays

All groups of mice were sacrificed at the same time of the day. Blood was obtained by cardiac puncture and collected in heparinised tubes. A liver sample was excised previous to perfusion to assess subsequently the enzymatic activity of ALA-S [26], to quantify ALA [27] and the expression of inducible NOS (iNOS) with a specific antibody (Anti iNOS/NOS II H-174, Santa Cruz Biotechnology, Inc.) [20]. Mice were then perfused through the heart with 20 ml of physiological solution before liver was removed. Liver samples were

excised and immediately homogenized in sucrose 0.25 M and centrifuged at 10,000 g for 20 minutes at 4ºC. The supernatant obtained was used to quantified the levels of GSH [28] and TAC (Innoprot kit P40117, Innovative Technologies in Biological Systems S.L., Bizkaia, Spain), and to assess the enzymatic activities of PBG-D [29], HO [30], Catalase [31] and GST [32]. This first supernatant was then ultracentrifuged at 100,000 g for 60 minutes at 4ºC, the supernatant thus obtained was used to assess SOD activity (Innoprot kit P40118, Innovative Technologies in Biological Systems S.L., Bizkaia, Spain) and the resultant pellet was used for measuring CYP2E1 activity [33]. Protein concentration was measured by Bio-Rad DC Protein Assay (Bio-Rad Laboratories, München, Germany).

Statistical Analysis

The results were expressed as mean and standard deviation (s.d.). The analysis of the variance test (ANOVA) was used to establish the differences between groups and $p < 0.05$ was considered as significant.

Results

Oxidative and Nitrosative Stress Parameters

In [Table-1] are shown the results obtained when basal levels of oxidative stress markers were measured in the mice model of hemochromatosis and compared with that of wild type mice.

TAC values were similar in wild type and $Hfe^{-/-}$ mice of both females and males. Although, if we compare between sex, wild type or $Hfe^{-/-}$, the females showed higher levels than male groups (25%, $p < 0.05$).

Table 1- Basal levels of oxidative stress markers

GROUP	SEX	TAC (nmol/mg)	GSH (nmol/mg)	SOD (U/mg)	CATALASE (nmol/mg)
Control	Males	62 ± 14	25.19 ± 3.91	0.092 ± 0.035	44.57 ± 15.11
Hfe-/-		55 ± 15	18.33 ± 7.57*	0.086 ± 0.028	32.33 ± 8.87*
Control	Females	79 ± 7**	36.48 ± 6.15**	0.080 ± 0.007	53.12 ± 15.70
Hfe-/-		69 ± 11**	31.35 ± 5.23**	0.069 ± 0.011	31.59 ± 5.54*

*$p < 0.05$: significance of differences between Hfe-/- group respect to control group.
**$p < 0.05$: significance of differences between males and females groups.

GSH basal levels were reduced in male $Hfe^{-/-}$ mice (28%, $p < 0.05$) respect to wild type group. This difference was not observed in the groups of females. When we compare the values of males and females, GSH was higher in both female groups: control (45%, $p < 0.05$) and $Hfe^{-/-}$ (70%, $p < 0.05$) than the respective male group.

The differences of basal SOD activity between wild type and $Hfe^{-/-}$ groups were no significant.

Catalase activity was also diminished in the $Hfe^{-/-}$ group, males (27%, $p < 0.05$) or females (40%, $p < 0.05$) mice, without any difference between sex.

The results obtained when animals received Isoflurane, ethanol or ALA are shown in [Fig-1].

In animals treated with Isoflurane no significant differences were found in any of the studied parameters related with antioxidant defence system in both males and females [Fig-1a].

When the effect of ethanol was evaluated an increase of Catalase activity was produced in males (96%, $p < 0.05$) and females (58%, $p < 0.05$) $Hfe^{-/-}$ group. TAC and GSH levels remained unaltered. An increase in SOD activity (65%, $p < 0.05$) was only observed in male $Hfe^{-/-}$ group by the action of this xenobiotic [Fig-1b].

ALA administration reduced 35% (p<0.05) GSH levels in control male group. Moreover, SOD activity was increased in hemochromatic male mice, effect not observed in females [Fig-1c].
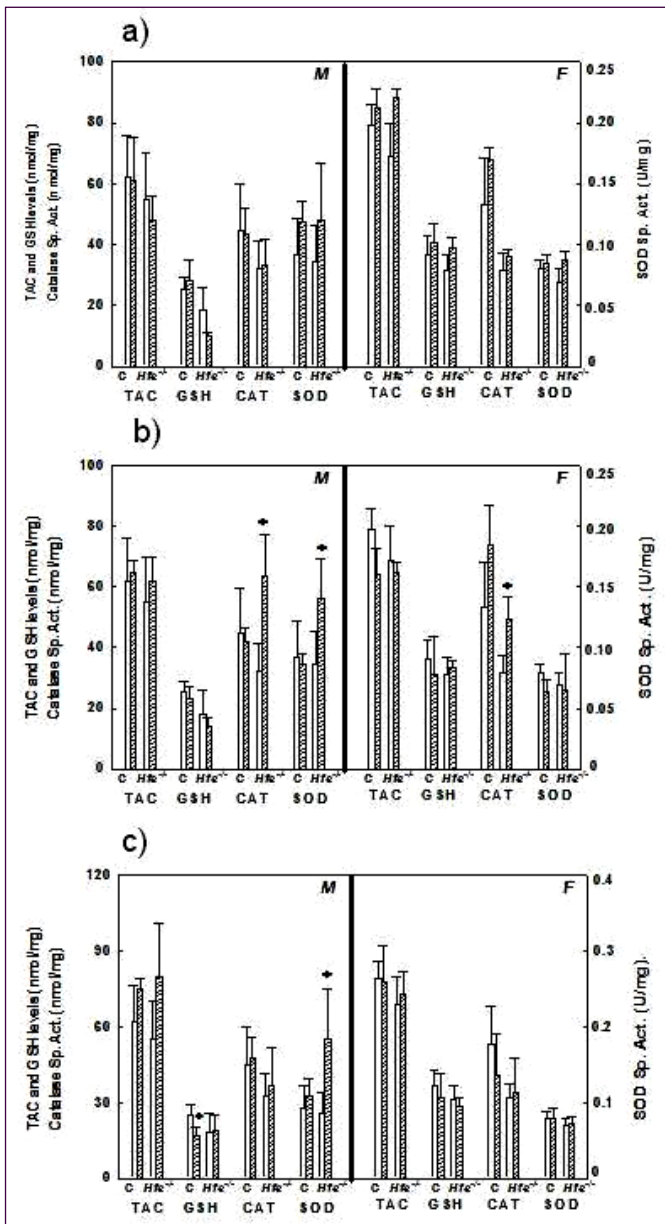

Fig. 1- Oxidative stress markers in animals treated with Isoflurane (a); ethanol (b); and ALA (c).

☐ *No treated animals.* ▨ *Treated animals. TAC: Total antioxidant capacity, GSH: reduced gluthatione, CAT: Catalase, SOD: Superoxide dismutase. Data represent mean value ± s.d. of at least 4-6 animals. p<0.05, significance of differences between treated and non treated mice.*

To evaluate the instauration of nitrosative stress, iNOS expression was measured. No significant differences were observed between control and *Hfe*^{-/-} (data not shown). When Isoflurane was administered to control group, a diminution of 35% (p<0.05) in iNOS was produced; this effect was not detected when the anaesthetic was administered to *Hfe*^{-/-} [Fig-2]. iNOS expression was unchanged by administration of ethanol or ALA in all the groups studied (data not shown).

Heme Metabolism Parameters

[Table-2] shows basal levels of some parameters involved in heme metabolism in control and *Hfe*^{-/-} groups. No significant differences were observed in ALA levels between both groups, in spite that ALA -S activity was 2 fold (p<0.05) higher in *Hfe*^{-/-} mice, male and females, respect to wild type group. PBG-D activity was slightly elevated (27%, p<0.05) in *Hfe*^{-/-}, but this difference was only observed in the males. The basal activity of HO in male *Hfe*^{-/-} was 37% (p<0.05) lower than controls; without no differences between female groups.
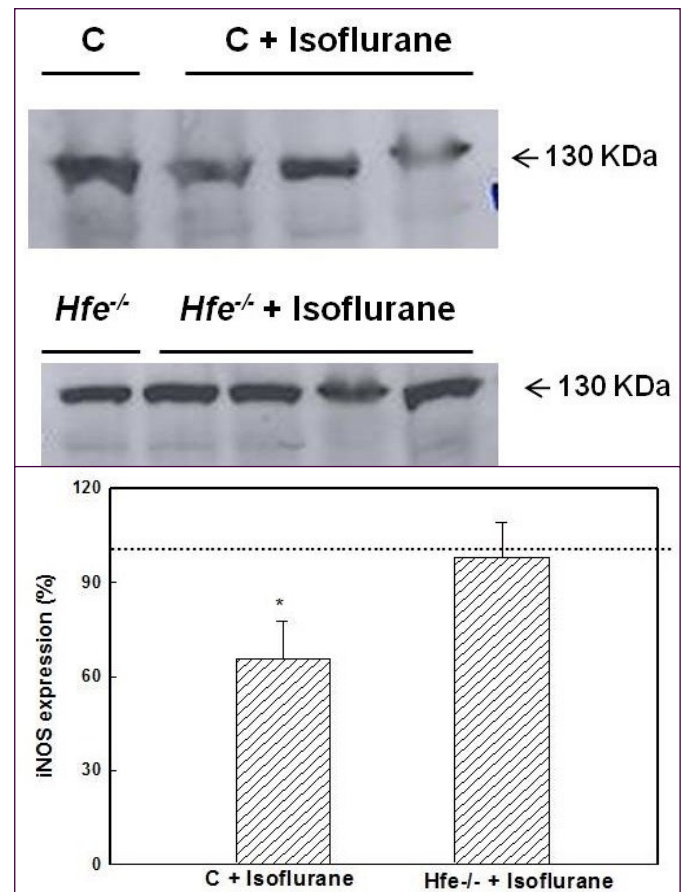

Fig. 2- Expression of NOS-1 in control, *Hfe*^{-/-} group treated or not with Isoflurane.

Western Blot bands were on the top panel. On the bottom panel, columns represent normalized signals in the control and treated animals that were quantified using an Image Analyzer. Values are expressed as mean of at least three determinations run in duplicate and are expressed as a percentage taking the control group as 100%. (*) p<0.05, significance of differences between treated and non treated mice.

Table 2- Basal levels of heme metabolism parameters

Group	Sex	ALA (nmol/mg)	ALA-S nmol/mg	PBG-D nmol/mg	HO nmol/mg
Control	Males	0.091 ± 0.017	0.092 ± 0.012	0.329 ± 0.031	0.717 ± 0.374
Hfe^{-/-}		0.086 ± 0.019	0.190 ± 0.042*	0.407 ± 0.044*	0.452 ± 0.138*
Control	Females	0.060 ± 0.007	0.073 ± 0.013	0.381 ± 0.003	0.654 ± 0.085
Hfe^{-/-}		0.087 ± 0.019	0.156 ± 0.036*	0.442 ± 0.071	0.662 ± 0.159
*p<0.05, significance of differences between Hfe^{-/-} group respect to control group					

When we compare these parameters between sex, no significant differences were observed in basal levels of ALA, ALA-S and PBG-D, although HO activity was in females 46% (p<0.05) greater in females than in males.

The results obtained when animals received Isoflurane, ethanol or ALA are shown in [Fig-3].

When the effects of Isoflurane were evaluated, only the activity of PBG-D was 20% (p<0.05) diminished in males and females $Hfe^{-/-}$ mice. Moreover, HO activity was 45% (p<0.05) increased in male mice, without any alteration in the other groups of mice. ALA levels and ALA-S activity were also unchanged after anaesthetic administration [Fig-3a].

Fig. 3- Heme metabolism parameters in animals treated with Isoflurane (a); ethanol (b); and ALA (c).

☐ *No treated animals.* ▨ *Treated animals. ALA-S: 5-Aminolevulinic acid synthetase. ALA: 5-Aminolevulinic acid, PBG-D: Porphobilinogen deaminase, HO: Heme oxygenase. Data represent mean value ± s.d. of at least 4-6 animals. p<0.05, significance of differences between treated and non treated mice.*

When ethanol was administered a 30% (p<0,05) reduction of PBG-D activity was detected in all the studied groups. No significant differences were observed in ALA levels and ALA-S activity, while HO

activity was 200% (p<0.05) induced in $Hfe^{-/-}$ males [Fig-3b].

ALA only induced HO activity in male Hfe$^{-/-}$ while all the other parameters measured were unchanged [Fig-3c].

Drug Metabolism System

In [Table-3] are shown basal levels of CYP2E1 and GST activity of control and $Hfe^{-/-}$ mice.

CYP2E1 activity was 36% (p<0.05) less in female mice of $Hfe^{-/-}$ respect to control group; without differences in male groups or between sex.

Table 3- Basal levels of CYP2E1 and GST activities

GROUP	SEX	CYP2E1 nmol/mg	GST nmol/mg
Control	Males	15.220 ± 4.983	14.027 ± 2.768
Hfe$^{-/-}$		15.773 ± 1.237	19.174 ± 2.801*
Control	Females	18.58 ± 0.624	6.902 ± 2.365
Hfe$^{-/-}$		11.649 ± 2.702*	7.827 ± 1.933
p<0.05, significance of differences between Hfe$^{-/-}$ group respect to control group.			

The group of $Hfe^{-/-}$ male mice had a basal activity of GST 35% (p<0.05) higher than controls. Moreover, in the both groups of females, GST activity was 40-50% (p<0.05) lesser than the respective male mice group.

In animals receiving Isoflurane CYP2E1 activity was 48% (p<0.05) induced in male control mice and in male (28%, p<0.05) and female (54%, p<0.05) $Hfe^{-/-}$ group. GST activity was 68% (p<0.05) increased in female control group, without any alteration in the other studied groups [Fig-4a].

No effect on CYP2E1 and GST activity was produced by ethanol administration [Fig-4b].

CYP2E1 activity was also unchanged after ALA administration. GST activity was increased in female groups, control and $Hfe^{-/-}$ treated with ALA; this response was not observed in male mice [Fig -4c].

Discussion

Considering that the participation of iron in the Fenton reaction will eventually induce the formation of ROS, the aim of this work was first compare basal levels of oxidative stress markers in the mice model of hemochromatosis respect to the wild type mice. To this end GSH, TAC and the activities of Catalase and SOD were measured.

We have found that the levels of GSH and the activities of Catalase in males were reduced indicating that, in this murine model of hemochromatosis, the antioxidant defence system was altered. However in female mice, although Catalase activity was also diminished respect to the wild type group, TAC and GSH levels were higher than controls suggesting an enhancement in the machinery against the oxidative stress.

Because ALA-S is the regulatory enzyme of heme biosynthesis and Fe is also involved in its regulation in erythroid cells, it was also of interest to measure this enzyme activity. Moreover, recalling that Catalase and NOS are hemeproteins, we wished to determine if there was any alteration in the regulatory heme pool that could affect the synthesis of these hemeproteins. The activity of PBG-D, enzyme which is diminished in AIP patients in whom the levels of ALA and porphobilinogen are elevated during the acute attack, was determined. And to further characterized the instauration of oxidative stress, the activity of HO, the rate-limiting enzyme in heme
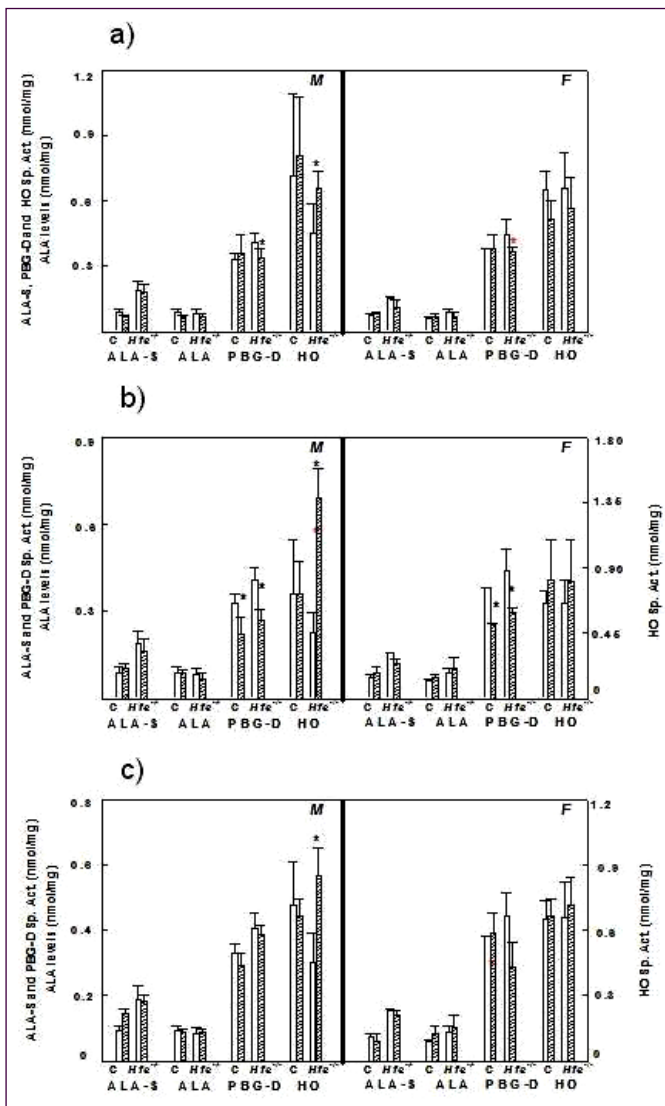
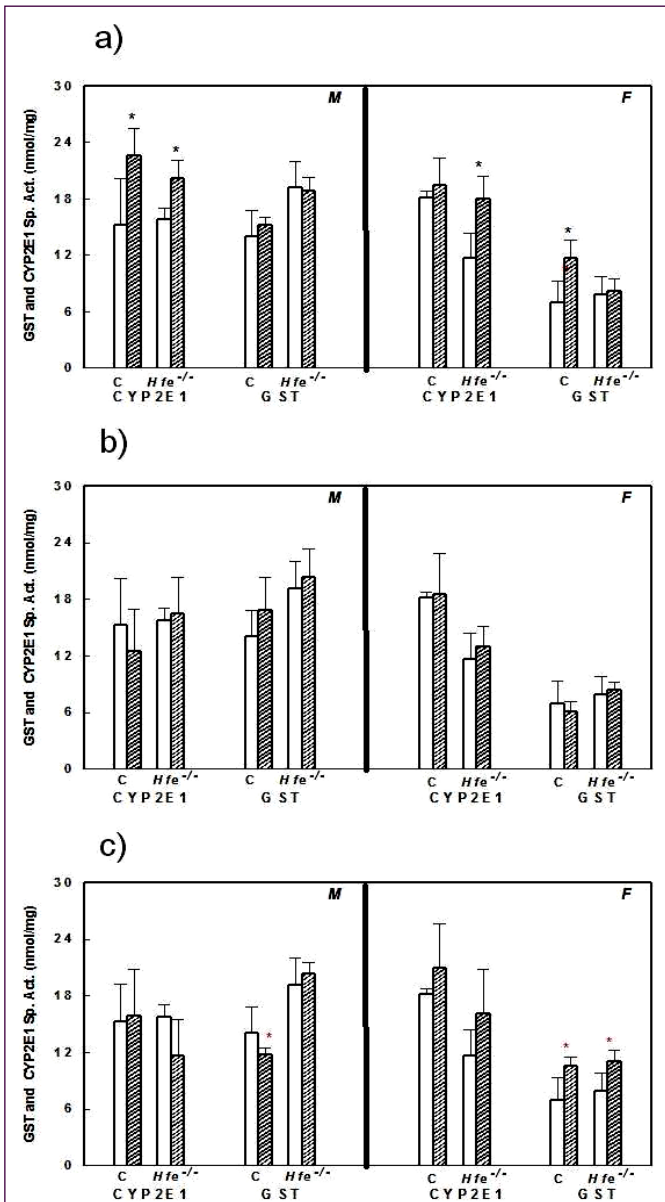degradation and frequently induced under stress status, was measured.

Fig. 4- CYP2E1 and GST activities in animals treated with Isoflurane (a), ethanol (b) and ALA (c)

☐ *No treated animals.* ▨ *Treated animals. GST: Gluthatione S-transferase. Data represent mean value ± s.d. of at least 4-6 animals. p<0.05, significance of differences between treated and non treated mice.*

In [Fig-4] are shown the results obtained when animals received Isoflurane, ethanol or ALA.

Male *Hfe*^{-/-} mice showed a high activity of ALA-S and PBG-D which would lead to increased heme synthesis, more available for the synthesis of hemeproteins such as Catalase which instead we have found, diminished. In the female group, ALA-S was also augmented nevertheless no differences were observed in PBG-D activity. Interestingly, in female mice, the activity of HO was lower than in male group. We can propose two hypothesis to explain these findings, one would indicate that a low activity of HO could not be sufficient to degrade the heme necessary for the synthesis of the hemepro-

teins; other possibility is that liberation of iron, product of heme degradation by HO would not occur.

The heme metabolism and the drug metabolizing system are related by means of the hemeprotein CYP, being the isoform CYP2E1 involved in the metabolization of Isoflurane and ethanol. So to complete this work, the activity of this CYP2E1 was also measured.

Moreover to evaluate if there were any hepatic damage, the activity of GST, enzyme of Phase II metabolism, was quantified. We have found that GST activity increased only in *Hfe*^{-/-} male mice. In a Proteomic study, Moran-Jimenez et al demonstrated an enhancement in the levels of this protein (unpublished data).

When we evaluated the effect of porphyrinogenic drugs, Isoflurane and ethanol, results were different depending on the drug and the sex. Both xenobiotics altered heme metabolism showing a diminution on PBG-D activity and an increase in HO activity. The induction of HO would indicate a potentiation of oxidative stress that was more drastic due to ethanol administration because this drug also causes an induction of SOD activity.

The alterations produced by Isoflurane on heme synthesis in the murine model of HH were similar to those observed when this anaesthetic was administered to *CF1* mice [16] and added to a culture of a cell line established from hepatoerythropoietic porphyria patients [22] although, unexpectedly, induction of ALA-S did not occur. Instead, more recently we have found that giving Isoflurane to a genetic model of Erythropoietic Protoporphyria, ALA-S activity was strikingly induced, but reduction of PBG-D activity was not detected. Yet, we can not offer any plausible explanation for these differences in the response of ALA-S. We could speculate that due to the fact that in Hfe-/- mice, ALA-S basal activity was greatly increased with respect to the wild type group, HO was induced in CF1 mice liver after Isoflurane administration but not in the non acute porphyria models.

The antioxidant defense system was altered too in animals receiving ALA. It was suggested that ALA produce ROS through oxidation catalyzed by iron [34, 35].

Findings here described would contribute to increase the knowledge about the association between HH and the Porphyrias and about the effects of volatile anaesthetics on different metabolisms in genetic models of Porphyrias and associated diseases.

Abbreviations

AIP: Acute Intermittent Porphyria,

ALA: 5-Aminolevulinic acid,

ALA-S: 5-Aminolevulinic acid synthase,

CYP: Cytochrome P-450,

GSH: Reduced Gluthation,

GST: Gluthation S-transferase,

HH: Hereditary Hemochromatosis,

HO: Heme Oxygenase,

iNOS: inducible Nitric Oxide Synthase,

NOS: Nitric Oxide Synthase,

PBG-D: Porphobilinogen Deaminase,

PCT: Porphyria Cutanea Tarda,

ROS: Reactive Oxygen Species,

SOD: Superoxide Dismutase,

TAC: Total Antioxidant Capacity.

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Conflicts of Interest: The authors have no conflicts of interest to declare.

References

[1] Babitt J.L. and Lin H.Y. (2011) *Semin Liver Dis.*, 31, 280-292.

[2] Deugnier Y. and Turlin B. (2011) *Semin Liver Dis.*, 31, 260-271.

[3] Houglum K., Ramm G.A., Crawford D.H., Witztum J.L., Powell L.W. and Chojkier M. (1997) *Hepatology*, 26, 605-610.

[4] Rossetti M.V. Méndez M., Afonso S., Gerez E., Batlle A., Muñoz A. and Parera V. (2009) *Cell. Mol. Biol. (Noisy-le-grand)*, 55, 31-35.

[5] European Association for the Study of the Liver (2010) *J. Hepatol.*, 53, 3-22.

[6] Gan E.K., Powell L.W. and Olynyk J.K. (2011) *Semin Liver Dis.*, 31, 293-301.

[7] Pietrangelo A., Caleffi A. and Corradini E. (2011) *Semin Liver Dis.*, 31, 302-318.

[8] Mendez M., Granata B.X., Jiménez M.J., Parera V.E., Batlle A., de Salamanca R.E. and Rossetti M.V. (2012) *Mol. Genet. Metab.*, 105, 629-633.

[9] Batlle A. (1997) *Acta Bioquímica Clínica Latinoamericana,* 37-69.

[10] Drobacheff C., Derancourt C., Van Landuyt H., Devred D., de Wazieres B., Cribier B., Rey D., Lang J.M., Grosieux C., Kalis B. and Laurent R. (1998) *Eur. J. Dermatol.,* 8, 492-496.

[11] Mendez M., Rossetti M.V., Batlle A. and Parera V.E. (2005) *J. Am. Acad. Dermatol.,* 52, 417-424.

[12] Bonkovsky H.L. (2013) *Liver Int.,* 33(1), 162.

[13] Edwards C.Q., Griffen L.M. and Kushner J.P. (1988) *Tissue Antigens,* 31, 250-253.

[14] Beutler E., Felitti V., Gelbart T. and Ho N. (2000) *Ann. Intern. Med.*, 133, 329-337.

[15] Kharasch E.D. and Thummel K.E. (1993) *Anesthesiology*, 79, 795-807.

[16] Buzaleh A.M., Enriquez de Salamanca R. and Batlle A. (1992) *J. Pharmacol. Toxicol. Methods.*, 28, 191-197.

[17] Buzaleh A.M. and Batlle A.M. (1996) *Gen. Pharmacol.,* 27, 1009-1012.

[18] Buzaleh A.M., Afonso S.G., Martínez M. del C. and Batlle A. (2002) *Medicina*, 62(5), 111, 419.

[19] Rodriguez J.A., Martínez M., Del C., Gerez E., Batlle A. and

Buzaleh A.M. (2005) *Cell. Mol. Biol.,* 51, 487-494.

[20] Lavandera J., Batlle A. and Buzaleh A.M. (2011) *Drug Metab. Lett.,* 5, 25-29.

[21] Buzaleh A.M., Vazquez E. and Batlle A.M. (1996) *Pharmacology Communications,* 7, 101-106.

[22] Buzaleh A.M., García Bravo M., Navarro S., Moran Jiménez M.J., Méndez M., Batlle A., Fontanellas A. and Enríquez de Salamanca R. (2004) *Int. J. Biochem. Cell. Biol.,* 36, 216-222.

[23] Buzaleh A.M., Morán-Jiménez M.J., García-Bravo M., Sampedro A., Batlle A., Enríquez de Salamanca R. and Fontanellas A. (2009) *Cell. Mol. Biol.,* 55, 38-44.

[24] Maines M.D. and Gibbs P.E. (2005) *Biochem. Biophys. Res. Commun.,* 338, 568-577.

[25] Hayes J.D. and Strange R.C. (2000) *Pharmacology,* 61, 154-166.

[26] Marver H.S., Tschudy D.P., Perlroth M.G. and Collins A. (1966) *J. Biol. Chem.,* 241, 2803-2809.

[27] Mauzerall D. and Granick S. (1956) *J. Biol. Chem.,* 219, 435-4

[28] Rossi R., Cardaioli E., Scaloni A., Amiconi G., Di Simplicio P. (1995) *Biochim. Biophys. Acta.,* 1243, 230-238.

[29] Anderson P.M. and Desnick R.J. (1982) *Enzyme,* 28, 146-157.

[30] Tenhunen R., Marver H.S. and Schmid R. (1970) *J. Lab. Clin. Med.,* 75, 410-421.

[31] Chance B. and Maehly A. (1955) *Methods Enzymol.,* 2, 764-768.

[32] Habig W.H., Pabst M.J. and Jakoby W.B. (1974) *Biol. Chem.,* 249, 7130-7139.

[33] Reinke L.A. and Moyer M.J. (1985) *Drug Metab. Dispos.,* 13, 548-552.

[34] Monteiro H.P., Abdalla D.S., Faljoni-Alario A. and Bechara E.J. (1986) *Biochim. Biophys. Acta.,* 881, 100-106.

[35] Hermes-Lima M., Valle V.G., Vercesi A.E. and Bechara E.J. (1991) *Biochim. Biophys. Acta.,* 1056, 57-63.