Treatment with sodium (*S*)-2-hydroxyglutarate prevents liver injury in an ischemia-reperfusion model in female Wistar rats

Eduardo Cienfuegos-Pecina [1, 2], **Diana P Moreno-Peña** [1], **Liliana Torres-González** [1], **Diana Raquel Rodríguez-Rodríguez** [1], **Diana Garza-Villarreal** [1], **Oscar H Mendoza-Hernández** [1], **Raul Alejandro Flores-Cantú** [1], **Brenda Alejandra Samaniego Sáenz** [1], **Gabriela Alarcon-Galvan** [3], **Linda E Muñoz-Espinosa** [1], **Tannya R Ibarra-Rivera** [4], **Alma L Saucedo** [4], **Paula Cordero-Pérez** [Corresp. 1]

[1] Liver Unit, Department of Internal Medicine, University Hospital "Dr. José E. González", Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico

[2] Blood Bank, Department of Clinical Pathology, University Hospital "Dr. José E. González", Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico

[3] Basic Science Department, School of Medicine, Universidad de Monterrey, Monterrey, Nuevo León, Mexico

[4] Department of Analytical Chemistry, School of Medicine, Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, Mexico

Corresponding Author: Paula Cordero-Pérez
Email address: paula.corderoprz@uanl.edu.mx

Background. Ischemia-reperfusion (IR) injury is one of the leading causes of early graft dysfunction in liver transplantation. Techniques such as ischemic preconditioning protect the graft through the activation of the hypoxia-inducible factors, which are downregulated by the EGLN family of prolyl-4-hydroxylases, a potential biological target for the development of strategies based on pharmacological preconditioning. For that reason, this study aims to evaluate the effect of the EGLN inhibitor sodium (*S*)-2-hydroxyglutarate [(*S*)-2HG] on liver IR injury in Wistar rats.

Methods. Twenty-eight female Wistar rats were divided into the following groups: sham (SH, n = 7), non-toxicity (HGTox, n = 7, 25 mg/kg of (*S*)-2HG, twice per day for two days), IR (n = 7, total liver ischemia: 20 minutes, reperfusion: 60 minutes), and (*S*)-2HG+IR (HGIR, n = 7, 25 mg/kg of (*S*)-2HG, twice per day for two days, total liver ischemia as the IR group). Serum ALT, AST, LDH, ALP, glucose, and total bilirubin were assessed. The concentrations of IL-1β, IL-6, TNF, malondialdehyde, SOD, and glutathione peroxidase were measured in liver tissue, as well as the expression of *Hmox1*, *Vegfa*, and *Pdk1*, determined by RT-qPCR. Sections of liver tissue were evaluated histologically, assessing the severity of necrosis, sinusoidal congestion, and cytoplasmatic vacuolization.

Results. The administration of (*S*)-2HG did not cause any alteration in the assessed biomarkers compared to SH. Preconditioning with (*S*)-2HG significantly ameliorated IR injury in the HGIR group, decreasing the serum activities of ALT, AST, and LDH, and the tissue concentrations of IL-1β and IL-6 compared to the IR group. IR injury decreased serum glucose compared to SH. There were no differences in the other biomarkers assessed. The treatment with (*S*)-2HG tended to decrease the severity of hepatocyte necrosis and sinusoidal congestion compared to the IR group. The administration of (*S*)-2HG did not affect the expression of *Hmox1* but decreased the expression of both *Vegfa* and *Pdk1* compared to the SH group, suggesting that the HIF-1 pathway is not involved in its mechanism of hepatoprotection. In conclusion, (*S*)-2HG showed a hepatoprotective effect, decreasing the levels of liver injury and inflammation biomarkers, without evidence of the involvement of the HIF-1 pathway. No hepatotoxic effect was observed at the tested dose.

Treatment with sodium (*S*)-2-hydroxyglutarate prevents liver injury in an ischemia-reperfusion model in female Wistar rats

Eduardo Cienfuegos-Pecina[1,2], Diana P. Moreno-Peña[1], Liliana Torres-González[1], Diana R. Rodríguez-Rodríguez[1], Diana Garza-Villarreal[1], Oscar H. Mendoza-Hernández[1], Raúl A. Flores-Cantú[1], Brenda A. Samaniego-Sáenz[1], Gabriela Alarcon-Galvan[3], Linda E. Muñoz-Espinosa[1], Tannya R. Ibarra-Rivera[4], Alma L. Saucedo[4], Paula Cordero-Pérez[1].

[1] Universidad Autónoma de Nuevo León. Liver Unit, Department of Internal Medicine, University Hospital "Dr. José E. González", Monterrey, Nuevo León, Mexico.
[2] Universidad Autónoma de Nuevo León. Blood Bank, Department of Clinical Pathology, University Hospital "Dr. José E. González", Monterrey, Nuevo León, Mexico.
[3] Universidad de Monterrey, Basic Science Department, School of Medicine, Monterrey, Nuevo León, Mexico
[4] Universidad Autónoma de Nuevo León. Department of Analytical Chemistry, School of Medicine, Monterrey, Nuevo León, Mexico.

Corresponding Author:
Paula Cordero-Pérez[1]
Av. Gonzalitos #235 Col. Mitras Centro, Monterrey, Nuevo León, 64460, Mexico
Email address: paula.corderoprz@uanl.edu.mx

Abstract

Background. Ischemia-reperfusion (IR) injury is one of the leading causes of early graft dysfunction in liver transplantation. Techniques such as ischemic preconditioning protect the graft through the activation of the hypoxia-inducible factors, which are downregulated by the EGLN family of prolyl-4-hydroxylases, a potential biological target for the development of strategies based on pharmacological preconditioning. For that reason, this study aims to evaluate the effect of the EGLN inhibitor sodium (*S*)-2-hydroxyglutarate [(*S*)-2HG] on liver IR injury in Wistar rats.

Methods. Twenty-eight female Wistar rats were divided into the following groups: sham (SH, n = 7), non-toxicity (HGTox, n = 7, 25 mg/kg of (*S*)-2HG, twice per day for two days), IR (n = 7, total liver ischemia: 20 minutes, reperfusion: 60 minutes), and (*S*)-2HG+IR (HGIR, n = 7, 25 mg/kg of (*S*)-2HG, twice per day for two days, total liver ischemia as the IR group). Serum ALT, AST, LDH, ALP, glucose, and total bilirubin were assessed. The concentrations of IL-1β, IL-6, TNF, malondialdehyde, SOD, and glutathione peroxidase were measured in liver tissue, as well as the expression of *Hmox1*, *Vegfa*, and *Pdk1*, determined by RT-qPCR. Sections of liver tissue

40   were evaluated histologically, assessing the severity of necrosis, sinusoidal congestion, and
41   cytoplasmatic vacuolization.

42

43   **Results.** The administration of (*S*)-2HG did not cause any alteration in the assessed biomarkers
44   compared to SH. Preconditioning with (*S*)-2HG significantly ameliorated IR injury in the HGIR
45   group, decreasing the serum activities of ALT, AST, and LDH, and the tissue concentrations of
46   IL-1β and IL-6 compared to the IR group. IR injury decreased serum glucose compared to SH.
47   There were no differences in the other biomarkers assessed. The treatment with (*S*)-2HG tended
48   to decrease the severity of hepatocyte necrosis and sinusoidal congestion compared to the IR
49   group. The administration of (*S*)-2HG did not affect the expression of *Hmox1* but decreased the
50   expression of both *Vegfa* and *Pdk1* compared to the SH group, suggesting that the HIF-1
51   pathway is not involved in its mechanism of hepatoprotection. In conclusion, (*S*)-2HG showed a
52   hepatoprotective effect, decreasing the levels of liver injury and inflammation biomarkers,
53   without evidence of the involvement of the HIF-1 pathway. No hepatotoxic effect was observed
54   at the tested dose.

55

56   Introduction

57   Orthotopic liver transplantation is the definitive therapy for either end-stage chronic liver disease
58   or severe acute liver failure (Bachir et al. 2012). In Mexico, by the first half of 2020, 317 patients
59   were on a waiting list for liver transplantation and three patients were waiting for combined
60   liver-kidney transplantation. During the same period, only 39 liver transplantations and one
61   combined liver-kidney transplantation were performed in the country (CENATRA 2020).
62   Despite the improvements in the methodologies of HLA-typification and the immunosuppression
63   strategies, there is still a significant incidence of early graft dysfunction, which is defined by a
64   spectrum of clinical signs that end in acute graft rejection and the need for a retransplant
65   (Briceño & Ciria 2010). The leading cause of early graft dysfunction is the ischemia-reperfusion
66   (IR) injury; a process that occurs when blood flow to the organ is impaired, causing long-time
67   ischemia, and then it is suddenly restored, reperfusing the tissue with oxygenated blood.
68   Paradoxically, the oxygen influx enhances the damage mechanisms triggered by hypoxia
69   (Salvadori et al. 2015).

70

71   IR injury is a complex process, involving a network of damage mechanisms triggered by
72   hypoxia. When intracellular O_2 concentrations plunge, the electron transport chain stops, and the
73   anaerobic glycolysis becomes the main source of ATP for the cell (Dorweiler et al. 2007).
74   Meanwhile, as ATP is depleted, the concentration of ADP increases. ADP is metabolized to
75   hypoxanthine, and its degradation is mediated by the xanthine dehydrogenase, an $NAD^+/NADH$
76   oxidoreductase which activity is impaired during the ischemic period, increasing the
77   hypoxanthine concentration by 10-fold after 2 hours of ischemia (Schoenberg et al. 1985). When
78   the tissue is suddenly reperfused with oxygenated blood, the oxidative environment favors the
79   oxidation of several cysteine residues in the enzyme xanthine dehydrogenase, causing a

80   conformational change that changes the activity of the enzyme to an oxidase (Nishino et al.
81   2005), producing, during the reperfusion, an oxidative burst that is usually lethal to the cells. The
82   fact that reperfusion triggers most of the injury, instead of preventing it, is known as the paradox
83   of IR (Salvadori et al. 2015).
84
85   A diversity of strategies have been developed to ameliorate the IR injury, with a particular
86   emphasis on techniques such as the ischemic preconditioning (IPC) (Murry et al. 1986) and the
87   remote ischemic preconditioning (RIPC) (Przyklenk et al. 1993), which consist in short and
88   intermittent periods of ischemia, either in the target organ or in distal organs. It is known that the
89   primary protective mechanism involved with these techniques is the hypoxia-inducible factor
90   (HIF) pathway (Albrecht et al. 2013).
91
92   HIFs are transcription factors acting as the primary regulators of oxygen homeostasis. These are
93   heterodimers, conformed by an α subunit, oxygen-dependent, and a β subunit constitutively
94   expressed (Semenza 2014). During normoxia, the α subunit is hydroxylated by the EGLN family
95   of prolyl-4-hydroxylases, which are oxygen and α-ketoglutarate dependent dioxygenases, and act
96   as the primary oxygen sensors in the cell. After hydroxylation, α subunits are ubiquitinated by
97   the von Hippel-Lindau E3 ubiquitin ligase and degraded in the proteasome. On the other hand,
98   during hypoxia, EGLN hydroxylases are inactive, and the α subunits get stabilized, translocated
99   to the nucleus, and dimerized with β-subunits, favoring the transcription of their target genes
100 (Dengler et al. 2014). For that reason, this metabolic pathway is a potential therapeutic target for
101 the development of pharmacologic preconditioners.
102
103 The 2-hydroxyglutaric acid is an inhibitor of the EGLN hydroxylases. Its (R) enantiomer was the
104 first oncometabolite described in the literature, found in tumors with high resistance to hypoxia,
105 usually harboring a mutated isocitrate dehydrogenase 1 (Dang et al. 2009). On the other hand, its
106 (S) enantiomer is naturally produced *in vivo* as a product of non-specific reactions of several
107 enzymes, such as the malate dehydrogenase or the lactate dehydrogenase A (Du & Hu 2021;
108 Intlekofer et al. 2017). The (S)-2-hydroxyglutarate [(S)-2HG] has a higher inhibitory effect
109 against EGLN-1 than against EGLN-2 or 3 (Koivunen et al. 2012), and a previous report of our
110 research group showed that the oral administration of its disodium salt has a nephroprotective
111 effect against IR injury in Wistar rats, suggesting the involvement of the HIF-1 pathway in its
112 mechanism (Cienfuegos-Pecina et al. 2020). Given the pharmacologic potential of this
113 compound, we aimed to evaluate whether the administration of (S)-2HG has a hepatoprotective
114 effect against IR injury in Wistar rats.
115

116 ## Materials & Methods
117 **Synthesis of (S)-2HG**
118 (S)-2HG was synthesized using a two-step methodology from L-glutamic acid, by using the
119 optimized synthetic route reported by our laboratory (Cienfuegos-Pecina et al. 2020). Briefly, L-

120   glutamic acid was subjected to a diazotization reaction in the presence of $NaNO_2$ and H_2SO_4 for
121   24 hours. The reaction was stopped with NaCl and extracted three times with ethyl acetate,
122   obtaining the 5-oxotetrahydrofuran-2-carboxylic acid, which was purified by column
123   chromatography, using silica as the stationary phase and ethyl acetate as the mobile phase. This
124   compound was hydrolyzed with NaOH, pH 10 for two hours, obtaining (S)-2HG, which was
125   precipitated with anhydrous methanol.
126
127   The identity of the synthesis product was confirmed by nuclear magnetic resonance (NMR)
128   spectroscopy and polarimetry. NMR data were acquired in a Bruker AVANCE III HD 400 MHz
129   spectrometer (Bruker Corp., Billerica, MA, USA). A 50 mg sample of (S)-2HG was dissolved in
130   double-distilled water and analyzed using sodium 3-(trimethylsilyl)propionate-2,2,3,3-d_4 (TSP)
131   in D_2O (Sigma-Aldrich) as an internal standard. The ^{1}H-NMR and ^{13}C-NMR spectra were
132   acquired using the *noesypr1d* pulse sequence for water signal suppression and compared with
133   those reported in the literature (Bal & Gryff-Keller 2002; Cienfuegos-Pecina et al. 2020).
134
135   The specific optical rotation ($[\alpha]_D^{20°C}$) of the synthetic (S)-2HG was measured in a PerkinElmer
136   341 Polarimeter (PerkinElmer, Waltham, MA, USA). Data were compared with previous reports
137   in the literature (Cienfuegos-Pecina et al. 2020; Ritthausen 1872).
138
139   **Animals**
140   Twenty-eight healthy female Wistar rats, weighing 238 ± 18 g, were used. Every single animal
141   was considered an experimental unit. Rats were bred in-house, and they were kept under
142   standard conditions of light and temperature ($24 \pm 3°C$, 12 hours light-dark cycles), with access
143   to commercial rat pellets (Nutrimix de México, Mexico) and water *ad libitum*. The animals were
144   not genetically engineered, and no previous procedures were performed on the animals before
145   the experiments. All the procedures were performed according to the specifications of the
146   Mexican Official Norm NOM-062-ZOO-1999. This project was submitted to the Ethics and
147   Research Committee of the School of Medicine, Universidad Autónoma de Nuevo León, and
148   approved with the register number HI19-00002.
149
150   **Experimental design**
151   The sample size was decided based on the result of an *a priori* calculation using Equation 1.
152

153
$$n = \frac{(Z\alpha + Z\beta)^2(\sigma_1^2 + \sigma_2^2)}{(\mu_1 - \mu_2)^2}$$

154
155   Equation 1. Estimation of the sample size for comparison of means. $Z\alpha$ = Z-value for α; $Z\beta$ = Z-
156   value for β; σ_1 = expected standard deviation of group 1; σ_2 = expected standard deviation of
157   group 2; μ_1 = expected mean of group 1; μ_2 = expected mean of group 2.
158

159 As a reference, we considered a previously reported serum activity of ALT of 494±84 U/L for
160 rats subjected to the same IR-injury-induction protocol we used (Jiménez Pérez et al. 2016). We
161 calculated the sample size expecting a 40% decreasing of the serum ALT activity after the
162 treatment (with no change in the standard deviation) and considered a two-tail statistical
163 significance (α) of 0.01 and a statistical power (1-β) of 95%, obtaining a total of 7 rats per group.
164
165 Rats were paired with a random number sequence obtained in R v. 4.01. The randomized animals
166 were assigned to the following groups:
167 • Sham group (SH), n = 7: Rats were treated with double distilled water, *p.o.*, twice per
168   day, for two days. Then they were undergone to laparotomy with exposure of the liver
169   hilum, without inducing IR injury. After 1 hour and 20 minutes, rats were sacrificed by
170   exsanguination, obtaining blood and tissue samples.
171 • Nontoxicity group (HGTox), n = 7: Rats were treated with double-distilled-water-
172   dissolved (*S*)-2HG, at a dose of 25 mg/kg, in the same way as the SH group. After
173   treatment, they underwent the same procedure as the SH group. A final n of 6 was
174   considered for the data analysis since one of the rats suddenly died after the anesthesia
175   was administered.
176 • IR group (IR), n = 7: Rats were treated in the same way as the SH group. Then they
177   underwent laparotomy with an exposition of the liver hilum and induction of IR injury
178   (20 minutes of ischemia, one hour of reperfusion).
179 • (*S*)-2HG + IR group (HGIR), n = 7: A dose of 25 mg/kg of (*S*)-2HG, was administered to
180   the rats in the same way as the HGTox group. Then, they underwent the same procedure
181   as the IR group.
182
183 To minimize potential confounders, all rats were housed in the same room during the
184 experiments, under the same conditions of light and temperature. In each surgery, an equal
185 number of rats from each group underwent the surgical procedure. During the analysis of the
186 biological samples, all the involved were blinded to the identity of the samples, except those who
187 then performed the statistical analysis (Eduardo Cienfuegos-Pecina and Paula Cordero-Pérez).
188

189 **Induction of liver injury**
190 Ischemic liver injury was induced following the procedure reported by Jiménez-Pérez *et al*.
191 (Jiménez Pérez et al. 2016). Rats were anesthetized with 100 mg/kg of ketamine (Anesket, PiSA
192 Agropecuaria, S.A. de CV, Mexico), and 10 mg/kg of xylazine (Sedaject, Vedilab S.A. de CV,
193 Mexico), *i. p*. After asepsis, an incision along the midline was performed, exposing the hepatic
194 hilum, which was then occluded by using an atraumatic vascular clamp (Pringle maneuver) for
195 20 minutes. Following the ischemia, the clamp was withdrawn, and the rats were kept under
196 general anesthesia for a reperfusion time of 1 hour, after which rats were sacrificed by
197 exsanguination by a puncture in the aorta. Criteria for early euthanasia was the failure of the
198 anesthesia during the surgery, however, none of the animals needed the use of this protocol.

199   Blood samples were centrifuged at 2000 g for 12 minutes, then the serum was separated. The
200   liver was resected, weighed, and samples of tissue were obtained and frozen at -80 °C until
201   analysis.
202
203   **Biochemical markers, oxidative stress biomarkers, and proinflammatory cytokines**
204   To assess the magnitude of the induced liver injury, the serum activities of alanine
205   aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and
206   alkaline phosphatase (ALP), and the serum concentrations of total bilirubin and glucose were
207   measured. The biochemical determinations were performed in an ILab Aries analyzer
208   (Instrumentation Laboratory, SpA, Milan, Italy), using kinetic and end-point UV-Visible
209   spectrophotometric methodologies.
210
211   Malondialdehyde (MDA) is one of the main products of arachidonic acid peroxidation, and it is a
212   commonly used biomarker to assess oxidative stress (Ayala et al. 2014), alongside the activities
213   of superoxide dismutase (SOD) and glutathione peroxidase (GPx). Since one of the key
214   mechanisms involved in IR injury is the oxidative burst produced by the sudden activation of
215   xanthine oxidase, we compared the tissue levels of MDA, SOD, and GPx among the study
216   groups. To measure the concentration of MDA in liver tissue, 200 mg of liver tissue were
217   mechanically homogenized in 1 mL of RIPA buffer in an ice bath, and then centrifuged at 1600 g
218   for 10 minutes at 4°C. MDA concentration was measured spectrophotometrically in the
219   supernatant, using the thiobarbituric acid colorimetric method with a thiobarbituric acid-reactive
220   substances (TBARS) assay kit (Cayman Chemical Company, Ann Arbor, MI, USA). The
221   product of this reaction was measured spectrophotometrically at a wavelength of 535 nm.
222
223   The total tissue activity of SOD was quantified by measuring the inhibition of the reduction of a
224   tetrazolium salt to formazan by reactive oxygen species using the SOD Assay Kit (Cayman
225   Chemical Company). Briefly, 200 mg of liver tissue were mechanically homogenized in 1 mL of
226   20 mM HEPES buffer, pH 7.2, containing 1 mM EDTA 210 mM mannitol, and 70 mM sucrose.
227   Homogenization was done in an ice bath. Samples were then centrifuged for 15 minutes at
228   10,000 g, at 4°C and the assay was performed using the supernatant, diluted in the buffer. 96-
229   well microplates were read spectrophotometrically at a wavelength of 450 nm and total SOD
230   activity was determined.
231
232   Tissue GPx activity was measured by quantifying the oxidation of NADPH to $NADP^+$ using a
233   GPx Assay Kit (Cayman Chemical Company). To perform the assay, 200 mg of liver tissue were
234   mechanically homogenized in 1 mL of 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM EDTA
235   and 1 mM DTT. Homogenization was performed in an ice bath. Samples were centrifuged at
236   10.000 g for 15 minutes at 4°C, and the supernatants were separated. The supernatants were
237   diluted in the sample buffer provided by the manufacturer before the GPx determination. GPx

238   activity was quantified by a UV kinetic method, measuring the change in the absorbance at a
239   wavelength of 340 nm.

240

241   To assess the inflammatory response after the induction of IR injury, we measured the tissue
242   concentrations of the proinflammatory cytokines interleukin 1β (IL-1β), interleukin 6 (IL-6), and
243   tumor necrosis factor (TNF) using a commercial sandwich enzyme-linked immunosorbent assay
244   (ELISA) (R&D Systems, Minneapolis, MN, USA). The same samples from the GPx
245   determination were used, undiluted. ELISA protocols were performed following the
246   manufacturer's instructions. Cytokine concentrations were determined spectrophotometrically, at
247   a wavelength of 450 nm.

248

249   All measurements in tissue were normalized to the protein concentration in the homogenates,
250   measured using Bradford's method. Bradford's reagent was prepared as previously reported in
251   the literature (Kruger 2009). MDA concentration is reported as mmol/mg of protein, SOD
252   activity is reported as IU/mg of protein, GPx activity is reported as nmol/min/mg of protein, and
253   proinflammatory cytokines are reported as pg/mg of protein.

254

255   **Histological evaluation**
256   Samples of liver tissue were fixed in phosphate-buffered 10% formalin solution pH 7.4 and then
257   embedded in paraffin blocks, which were cut using a microtome at a thickness of 4 μm. The
258   tissue sections were deparaffinized and processed using the standard histological technique.
259   Hematoxylin-eosin (H&E) stained sections were blindly evaluated under the microscope,
260   assessing the severity of necrosis, cytoplasmic vacuolization, and sinusoidal congestion by using
261   the damage scale reported by Susuki et al. (1993): 0 = no evident injury; 1 = single-cell necrosis,
262   minimal congestion or vacuolization; 2 = necrosis >30%, mild congestion or vacuolization; 3 =
263   necrosis >60%, moderate congestion or vacuolization; and 4 = necrosis >60%, severe congestion
264   or vacuolization.

265

266   **Quantitative RT-PCR**
267   We used RT-qPCR to measure the expression of *Hmox1*, *Vegfa*, and *Pdk1,* the genes coding for
268   heme-oxygenase 1, vascular endothelial growth factor A, and pyruvate dehydrogenase kinase 1,
269   whose expression is directly regulated through the HIF-1 pathway (Bernhardt et al. 2009;
270   Bujaldon et al. 2019; Forsythe et al. 1996; Zhang et al. 2018). We performed a total RNA
271   extraction from 100 mg of liver tissue using TRIzol reagent (Invitrogen, Thermo Fisher
272   Scientific, Carlsbad, CA, USA) according to the manufacturer's specifications. The RNA was
273   quantified using a Microdrop Multiskan GO (Thermo Fisher Scientific, Carlsbad, CA, USA) and
274   stored at -80°C until analysis.

275

276   We performed all the RT-qPCRs using the GoTaq 1-Step kit (Promega Corporation, Madison,
277   WI, USA). The gene coding for β-actin (*Actb*) was used as the housekeeping gene. The following

278   primers were used: *Hmox1* forward 5'-GCCTGCTAGCCTGGTTCAAGA-3', *Hmox1* reverse 5'-
279   GAGTGTGAGGACCCATCGCA-3', *Vegfa* forward: 5'-CCGTCCTGTGTGCCCCTAAT-3',
280   *Vegfa* reverse: 5'-AAACAAATGCTTTCTCCGCT-3', *Pdk1* forward: 5'-
281   GATTGCCCATATCACGCCTCT-3', *Pdk1* reverse: 5'-CTCGTGGTTGGTTCTGTAATGC-3',
282   *Actb* forward 5'-CCCTGGCTCCTAGCACCAT-3', and *Actb* reverse 5'-
283   GATAGAGCCACCAATCCACACA-3'. We performed every reaction according to the
284   manufacturer's specifications, using 200 ng of RNA for each reaction and the primers at a
285   concentration of 100 nM, to complete a final volume of 20 µL. The following reaction conditions
286   were used: one cycle of reverse transcription at 37 °C for 15 minutes, one cycle of reverse
287   transcription inactivation and Go Taq DNA Polymerase activation at 95 °C for 10 minutes, and
288   40 cycles of denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 30
289   seconds. Fold changes of gene expression from the SH group were calculated using the $2^{-\Delta\Delta Ct}$
290   method.

292   **Statistical analysis**
293   Data were analyzed using a Shapiro-Wilk normality test, followed by either a one-way ANOVA
294   test with a Tukey *post hoc* test or a Kruskal-Wallis test with a Dunn *post hoc* test. For the gene
295   expression assessment, the $-\Delta\Delta C_T$ values were analyzed. All the statistical analyses were
296   performed in R v. 4.0.1, using the packages *tidyverse*, *cowplot*, and *PMCMRplus*. The full
297   datasets are supplied in Supplementary Files 1-4, while the code used for the statistical analysis
298   is supplied in Supplementary File 5. The results are expressed as mean ± standard deviation or
299   median (interquartile range), depending on their distribution. Differences between groups are
300   considered significant at $p < 0.05$.

302   # Results
303   **(*S*)-2HG was successfully synthesized from L-glutamic acid**
304   (*S*)-2HG was produced in a 29.78% yield after alkaline hydrolysis, and it was obtained as a beige
305   powder, highly hygroscopic, and soluble in water but insoluble in organic solvents such as
306   methanol, ethanol, acetone, and ethyl acetate. The ^{1}H-NMR spectrum showed the following
307   signals: 1.82 ppm (1H, m); 1.96 ppm (1H, m); 2.22 ppm (2H, m), and 4.00 ppm (1H, dd, $J' = 7.6$
308   Hz, $J'' = 4.0$ Hz) (Figure S1). In the ^{13}C-NMR spectrum, the following signals were observed:
309   33.91, 36.38, 75.01, 184.10, and 185.71 ppm (Figure S2). Both spectra were consistent with the
310   reported in the literature for this compound (Bal & Gryff-Keller 2002; Cienfuegos-Pecina et al.
311   2020). A value of $[\alpha]_D^{20°C} = 8.4°cm^3g^{-1}dm^{-1}$ was observed, and it was consistent with previously
312   reported data (Cienfuegos-Pecina et al. 2020; Ritthausen 1872).

314   **(*S*)-2HG is hepatoprotective, but not hepatotoxic at the tested dose**
315   To evaluate whether (*S*)-2HG produces an acute hepatotoxic effect, we compared the levels of
316   liver injury biomarkers between the SH and HGTox groups. We did not observe a significant
317   difference between these groups in any of the assessed biochemical markers (Figure 1).

318
319   To assess whether (*S*)-2HG had a hepatoprotective effect, we compared the levels of the liver
320   injury biomarkers among the SH, IR, and HGIR groups. Neither the treatment with (*S*)-2HG nor
321   the IR injury induction affected the liver weight of the animals (Figure S3). The induction of IR
322   injury significantly increased the serum activities of ALT, AST, and LDH compared to the SH
323   group, while the treatment with (*S*)-2HG produced a significant decrease in the serum activities
324   of these enzymes compared to the IR group (Figure 1). A significant decrease in serum glucose
325   concentration was observed in both, the IR and HGIR groups compared to the SH group. No
326   significant differences were observed among groups in the serum activity of ALP and the serum
327   concentration of total bilirubin (Figure 1).
328
329   **The experimental model of total liver IR injury did not affect the levels of oxidative stress**
330   **biomarkers in liver tissue**
331   No significant differences were observed in the levels of oxidative stress biomarkers between the
332   IR and SH groups (Table 1). Their levels were not affected by the administration of (*S*)-2HG in
333   the HGTox group. We observed a decrease in the tissue activity of GPx in the HGIR group
334   compared with both SH and IR groups (Table 1).
335
336   **(*S*)-2HG modulates the concentration of proinflammatory cytokines**
337   Our model of acute liver injury produced a significant increase in the tissue concentration of IL-
338   1β in the IR group compared to the SH group. Treatment with (*S*)-2HG prevented this increase,
339   as we observed a concentration of IL-1β in the HGIR group significantly lower than the
340   concentration in the IR group (Figure 2).
341
342   The induction of liver IR injury did not affect the tissue concentrations of IL-6 and TNF,
343   however, a significant decrease in the tissue concentration of these cytokines was observed in the
344   HGIR group, compared with both IR and SH groups (Figure 2). The administration of (*S*)-2HG
345   to the HGTox group did not cause any alteration in the levels of the assessed cytokines compared
346   to the SH group (Figure 2).
347
348   **(*S*)-2HG produced a trend to decrease the severity of the histological liver injury, but it was**
349   **not significative**
350   The administration of (*S*)-2HG to the HGTox group did not produce a significant change in the
351   scores of histological liver injury compared to the SH group (Table 2, Figure 3). On the other
352   hand, our model of IR injury induced a significant degree of necrosis and sinusoidal congestion
353   in the IR group compared to the SH group, without producing a significant increase in the degree
354   of cytoplasmic vacuolization (Table 2, Figure 3). The histopathological evaluation of the IR
355   group showed cellular eosinophilia and nuclear hyperchromasia. Cellular necrosis was severe at
356   zone 3 of the liver acinus, with disruption of the central vein endothelial border (Figure 3). The
357   administration of (*S*)-2HG tended to decrease the severity of necrosis and sinusoidal congestion

358 compared to the IR group, although this decrease was not statistically significant (Table 2, Figure
359 3).
360
361 **The treatment with (*S*)-2HG decreased the expression of *Vegfa* and *Pdk1* in liver tissue,**
362 **without affecting the expression of *Hmox1***
363 Contrarily as we expected, the treatment with (*S*)-2HG did not increase the expression of *Hmox1,*
364 *Vegfa,* or *Pdk1* in liver tissue. The expression of *Hmox1* in liver tissue was not affected by the
365 administration of (*S*)-2HG or by the induction of IR injury (Figure 4). On the other hand, we
366 observed a significant decrease in the expression of both, *Vegfa* and *Pdk1* in the rats of the
367 HGTox group compared to the SH group (Figure 4). The induction of IR also decreased the
368 expression of these genes compared to the SH group (Figure 4). These observations suggest that
369 the hepatoprotective effect of (*S*)-2HG is not mediated by the HIF-1 metabolic axis, since we did
370 not observe the genetic footprint of the HIF-1α stabilization, indicating the involvement of a
371 different, currently unidentified mechanism.
372

Discussion

374 Liver transplantation is still the definitive therapy for end-stage liver diseases. However, the lack
375 of donors makes every organ invaluable. Hence, a plethora of strategies has been assessed to
376 improve graft survival. Several strategies have been developed to ameliorate IR injury, aiming to
377 stop the damage cascade at several levels. Pretreatment with antioxidant compounds, such as
378 curcumin or plant extracts with free-radical scavenging activity, has been used to decrease the
379 intensity of the oxidative burst during IR injury (Jiménez Pérez et al. 2016; Shen et al. 2007;
380 Torres-González et al. 2018; Yildiz et al. 2015). The use of anti-inflammatory drugs has also
381 been evaluated, aiming to ameliorate the inflammatory response after IR injury, which is the
382 pathogenic mechanism that drives acute graft rejection (Abdel-Gaber et al. 2015). These
383 strategies are aimed to ameliorate the damage that happens during reperfusion. However, only a
384 few have been designed to prevent during the ischemia the cascade of events that lead to the
385 reperfusion injury. Surgical techniques such as IPC and RIPC have been demonstrated to be
386 promising in pre-clinical studies (Abu-Amara et al. 2011), however, the evidence of their
387 effectiveness in clinical studies is controversial (Zapata-Chavira et al. 2017; Zapata-Chavira et
388 al. 2015). The mechanism behind IPC and RIPC is still not completely understood, but it is well
389 known that the HIF-1 pathway plays an essential role in its protective effect (Cai et al. 2013).
390 The stabilization of HIF-1α through the inhibition of the EGLN family of prolyl-4-hydroxylases
391 has demonstrated a protective effect against IR injury in several tissues (Hill et al. 2008; Vogler
392 et al. 2015). In a previous report of our laboratory, we demonstrated that (*S*)-2HG has a
393 nephroprotective effect against IR injury in Wistar rats (Cienfuegos-Pecina et al. 2020).
394
395 Several animal models have been used to study the mechanisms of IR injury in several organs.
396 Studies relying on relatively big animals, such as pigs (Andria et al. 2013) and dogs (Choi et al.
397 2010) have the advantage of a good similarity to humans, however, these models have been

398   gradually abandoned due to ethical considerations. Experimental models in rodents, such as
399   mice, are considered attractive, given the availability of genetically modified strains. On the
400   other hand, rat models of IR injury have been well characterized (Jiménez Pérez et al. 2016) and
401   have the advantage of larger anatomical structures, allowing the performance of accurate surgical
402   procedures without the need for microsurgery instruments. In addition, rat models are a good
403   starting point for the screening of compounds with potential hepatoprotective activity. Hence, we
404   decided to perform our study using a well-characterized model of liver IR injury in Wistar rats.
405
406   It has been demonstrated that the severity of IR injury is sex-dependent, involving a modulatory
407   effect of the sex hormones. Some studies have shown that female rodents exert a higher
408   resistance to liver IR injury than males, via an estrogen-dependent mechanism (Eckhoff et al.
409   2002; Harada et al. 2003). Estrogen induces the activity of the endothelial cell isoform of NO
410   synthase (eNOS) through the estrogen receptor-α. The mechanism involves either the Akt-
411   mediated activation of eNOS or the upregulation in the transcription of its gene (Harada et al.
412   2003). Since the effect of IR injury is different between females and males, the study of mixed
413   groups is not appropriate. Hence, we choose to assess the effect of our liver IR injury model
414   specifically in female Wistar rats.
415
416   In this study, we observed that the pretreatment with (S)-2HG at a dose of 25 mg/kg protects the
417   liver against IR in a protocol of 20 minutes of ischemia and 1 hour of reperfusion, evidenced by
418   a significant decrease in the serum activities of ALT, AST, and LDH compared to the IR group.
419   These three enzymes are the most used biomarkers to assess hepatocellular necrosis in a clinical
420   context because are highly expressed in the hepatocytes (Giannini et al. 2005; Jiménez Pérez et
421   al. 2016). We also observed a decrease in the serum concentrations of glucose in both, the IR and
422   HGIR groups compared to SH. It has been demonstrated that liver ischemia impairs glucose
423   metabolism, affecting the gluconeogenesis pathway and decreasing the serum glucose
424   concentration during the late ischemia and reperfusion (Bailey & Reinke 2000; Bloechle et al.
425   1994). There was no significant increase, but a trend, in the serum activity of ALP and the serum
426   concentration of total bilirubin after the induction of IR injury, indicating that IR injury induction
427   affected to a lesser extent the bile ducts than the liver parenchyma. On the other hand, (S)-2HG
428   did not affect the levels of the assessed biochemical markers compared in the HGTox group
429   compared to the SH group, evidencing that the compound has no hepatotoxic effect at the tested
430   dose. These results agree with our previous report, in which no acute hepatotoxic or nephrotoxic
431   effect was observed at a dose of 12.5 and 25 mg/kg (Cienfuegos-Pecina et al. 2020), however,
432   additional studies assessing the toxicity of (S)-2HG at higher doses and after chronic exposure
433   are still needed.
434
435   Despite the involvement of oxidative stress in the mechanism of IR injury, we did not observe a
436   significant difference in the assessed oxidative stress biomarkers (MDA, SOD, and GPx) neither
437   in the IR group compared to the SH group, nor in the HGIR group compared to the IR group. It

438   has been reported in the literature that despite the role of oxidative stress in the process of IR
439   injury, its effect on the three assessed biomarkers is detected only after longer periods of
440   ischemia and reperfusion (Gupta et al. 1997). The characteristics of our model (20 minutes of
441   ischemia and 1 hour of reperfusion) allow us to induce a moderated IR injury, however, the
442   assessed biomarkers were not sensitive enough to detect the oxidative stress associated with IR
443   injury. Even when this model is ideal for the screening of new compounds with hepatoprotective
444   activity, additional experiments must be performed to accurately quantify the effect of the
445   evaluated compounds on the redox balance after the induction of IR injury.
446
447   Proinflammatory cytokines play an important role in the physiopathology of IR injury, being
448   involved in the acute rejection of the graft. It has been demonstrated that the intensity of IR
449   injury dictates the magnitude of the inflammatory response, as evidenced by the correlation of
450   the expression of IL-1β and the duration of ischemia and reperfusion (Jiménez-Castro et al.
451   2019). In this study, we observed that only IL-1β was significantly affected by our model of IR
452   injury. This agrees with a previous report showing that a mild ischemic injury is not enough to
453   induce an increase in the tissue concentration of proinflammatory cytokines during the
454   reperfusion period (Jiménez Pérez et al. 2016). We observed a significant decrease in the tissue
455   concentration of IL-1β in the HGIR group compared to the IR group. This indicates that the
456   amelioration of IR injury by the treatment with (S)-2HG is enough to impact the inflammatory
457   response derived from the ischemic insult. Besides, despite our model of IR injury was unable to
458   increase the tissue concentrations of IL-6 and TNF in the IR group compared to the SH group,
459   we observed a decrease in the levels of these cytokines in the HGIR group.
460
461   The induction of IR injury caused a significant disruption of the tissue architecture, evidenced by
462   severe cell necrosis and sinusoidal congestion in zone 3 of the liver acinus. Since zone 3 is the
463   last to be reached by the oxygenated blood coming from the portal triad, it is more prone to be
464   affected after the induction of IR injury (Ali et al. 2015). In our study, our model of mild IR
465   injury produced significant damage in the perivenular zone, without affecting extensively the
466   region around the portal triad. This histopathological finding agrees with the levels of serum
467   ALP and total bilirubin in the IR group, which did not increase significantly compared to the SH
468   group. The administration of (S)-2HG to the HGIR group produced a decrease in the severity of
469   both, cell necrosis and sinusoidal congestion. However, this decrease was not statistically
470   significant in the non-parametric Kruskal-Wallis test. A quantitative morphometric analysis of
471   the tissue sections would be helpful in future studies to measure the effect of (S)-2HG at the
472   tissue level.
473
474   Heme oxygenase 1 is the inducible isoform of heme oxygenase, and it is codified by the gene
475   *Hmox1*. Its expression is directly regulated by the HIF-1 pathway (Lee et al. 1997), just like the
476   genes codifying for the vascular endothelial growth factor A (*Vegfa*) (Forsythe et al. 1996) and
477   the pyruvate dehydrogenase kinase 1 (*Pdk1*) (Majmundar et al. 2010). Together, these genes are

478   assessed to detect the footprint of the stabilization of HIF-1α, since this protein is not easily
479   detected directly, mainly due to its instability in normoxic conditions (Wang et al. 2017; Zhang
480   et al. 2018). A previous report demonstrates that the upregulation of *Hmox1* by the stabilization
481   of HIF-1α attenuates postischemic myocardial injury in both, *in vitro* and *in vivo* models, with an
482   associated downregulation of the expression of IL-8 (Dawn & Bolli 2005). We previously
483   observed that the administration of (*S*)-2HG at a dose of 25 mg/kg induces a 14.15-fold increase
484   in the expression of *Hmox1* in kidney tissue, with a nephroprotective effect against IR injury
485   (Cienfuegos-Pecina et al. 2020). Surprisingly, in this study, we did not observe any modification
486   in the expression levels of *Hmox1* after the administration of 25 mg/kg of (*S*)-2HG. In addition,
487   instead of observing an increase in the expression of *Vegfa* and *Pdk1* after the treatment with (*S*)-
488   2HG, we observed a significant decrease. Also, the induction of IR injury decreased the
489   expression of both genes.
490
491   The behavior of *Vegfa* in the rats subjected to IR injury agrees with a previous report, showing
492   that during the first stage of the IR injury induction the expression of *Vegfa* is decreased
493   (Bujaldon et al. 2019), however, the decrease of its expression in the HGTox group suggests that
494   the HIF-1 – VEGF axis is not involved in the hepatoprotective effect of (*S*)-2HG since *Vegfa*
495   expression is directly upregulated by HIF-1 (Forsythe et al. 1996). This finding is supported by
496   the behavior of *Pdk1*, which also significantly decreased its expression in the HGTox group.
497   Together, these results suggest that (*S*)-2HG exerts its hepatoprotective effect by a different
498   mechanism than the one observed in kidney tissue (Cienfuegos-Pecina et al. 2020). Further
499   studies are needed to identify the metabolic pathways involved.
500
501   The increased activity of (*S*)-2HG dehydrogenase in liver tissue may play a major role in its
502   pharmacokinetics. In the liver tissue, the (*S*)-2HG dehydrogenase has an activity more than 4
503   times higher than in kidney tissue (Jansen & Wanders 1993). This enzyme catalyzes the
504   oxidation of (*S*)-2HG in a FAD^+-dependent mechanism, producing α-ketoglutarate (Rzem et al.
505   2006), which, according to a previous report, exhibits a hepatoprotective effect by regulating the
506   polarization of Kupffer cells from an M1 to an M2 phenotype, decreasing the severity of the IR
507   injury and the expression of IL-6 (Cheng et al. 2019). The hypothesis of an involvement of an α-
508   ketoglutarate-dependant mechanism would agree with our findings since we observed a
509   downregulation of the tissue concentration of IL-6 in the HGIR group. Because neither SOD nor
510   GPx exhibit FAD^+-dependent mechanisms, an increase in the cellular concentration of $FADH_2$
511   would not affect these biomarkers. Also, it has been reported that α-ketoglutarate inhibits the
512   expression of *Vegfa* in osteosarcoma cell lines (Kałwaj et al. 2020), which agrees with our
513   observation that *Vegfa* was downregulated in the rats treated with (*S*)-2HG. The fact that α-
514   ketoglutarate is the actual substrate of the EGLN hydroxylases could also explain the observed
515   downregulation of *Vegfa* and *Pdk1* by inactivation (instead of stabilization) of HIF-1α.
516   Additional experiments are needed to elucidate the mechanism of action of (*S*)-2HG against IR
517   injury in liver tissue.

518
519 The pharmacokinetics of (*S*)-2HG is still not fully understood. Since the disodium salt of (*S*)-
520 2HG is highly polar, it is very unlikely that it could permeate through cell membranes. However,
521 it has been reported that (*S*)-2HG undergoes protonation and then cyclization at an acidic pH
522 (Bal & Gryff-Keller 2002). This process would form an equilibrium between (*S*)-2HG and its
523 more hydrophilic lactone in the stomach after its administration *p. o.*, increasing the absorption
524 and biodisponibility. Further studies with a diversity of derivatives of (*S*)-2HG are needed to
525 give us a better understanding of its pharmacokinetics and to identify analogs with a higher
526 pharmacologic potential.
527

528 ## Conclusions
529 (*S*)-2HG has a hepatoprotective effect against IR injury, which involved the amelioration of liver
530 injury biomarkers and proinflammatory cytokines. The administration of (*S*)-2HG did not induce
531 an acute hepatotoxic effect at the tested dose. The expression of *Hmox1* in liver tissue was not
532 affected by the pre-treatment with (*S*)-2HG, while the expression of *Vegfa* and *Pdk1* was
533 decreased, indicating that the HIF-1 pathway does not play a role in the hepatoprotective effect
534 of (*S*)-2HG and suggesting the involvement of additional pathways in its mechanism of
535 hepatoprotection.
536

537 ## References

538 Abdel-Gaber SA, Ibrahim MA, Amin EF, Ibrahim SA, Mohammed RK, and Abdelrahman AM.
539   2015. Effect of selective versus non-selective cyclooxygenase inhibitors on ischemia–
540   reperfusion-induced hepatic injury in rats. *Life Sciences* 134:42-48.
541   https://doi.org/10.1016/j.lfs.2015.04.025
542

543 Abu-Amara M, Yang SY, Quaglia A, Rowley P, Tapuria N, Seifalian AM, Fuller BJ, and
544   Davidson BR. 2011. Effect of remote ischemic preconditioning on liver
545   ischemia/reperfusion injury using a new mouse model. *Liver Transplantation* 17:70-82.
546   https://doi.org/10.1002/lt.22204
547

548 Albrecht M, Zitta K, Bein B, Wennemuth G, Broch O, Renner J, Schuett T, Lauer F, Maahs D,
549   Hummitzsch L, Cremer J, Zacharowski K, and Meybohm P. 2013. Remote ischemic
550   preconditioning regulates HIF-1alpha levels, apoptosis and inflammation in heart tissue
551   of cardiosurgical patients: a pilot experimental study. *Basic Research in Cardiology*
552   108:314. https://doi.org/10.1007/s00395-012-0314-0
553

554 Ali JM, Davies SE, Brais RJ, Randle LV, Klinck JR, Allison MED, Chen Y, Pasea L, Harper
555   SFJ, and Pettigrew GJ. 2015. Analysis of ischemia/reperfusion injury in time-zero
556   biopsies predicts liver allograft outcomes. *Liver Transplantation* 21:487-499.
557   https://doi.org/10.1002/lt.24072
558

559 Andria B, Bracco A, Attanasio C, Castaldo S, Cerrito MG, Cozzolino S, Di Napoli D,
560   Giovannoni R, Mancini A, Musumeci A, Mezza E, Nasti M, Scuderi V, Staibano S,

561   Lavitrano M, Otterbein LE, and Calise F. 2013. Biliverdin Protects against Liver
562   Ischemia Reperfusion Injury in Swine. *PLOS ONE* 8:e69972.
563   https://doi.org/10.1371/journal.pone.0069972
564
565 Ayala A, Muñoz MF, and Argüelles S. 2014. Lipid Peroxidation: Production, Metabolism, and
566   Signaling Mechanisms of Malondialdehyde and 4-Hydroxy-2-Nonenal. *Oxidative*
567   *Medicine and Cellular Longevity* 2014:31. https://doi.org/10.1155/2014/360438
568
569 Bachir NM, Larson AM, and Palmer BF. 2012. Adult Liver Transplantation in the United States.
570   *The American Journal of the Medical Sciences* 343:462-469.
571   https://doi.org/10.1097/MAJ.0b013e3182308b66
572
573 Bailey SM, and Reinke LA. 2000. Effect of low flow ischemia-reperfusion injury on liver
574   function. *Life Sciences* 66:1033-1044. https://doi.org/10.1016/S0024-3205(99)00668-2
575
576 Bal D, and Gryff-Keller A. 2002. 1H and 13C NMR study of 2-hydroxyglutaric acid and its
577   lactone. *Magnetic Resonance in Chemistry* 40:533-536. https://doi.org/10.1002/mrc.1053
578
579 Bernhardt WM, Gottmann U, Doyon F, Buchholz B, Campean V, Schödel J, Reisenbuechler A,
580   Klaus S, Arend M, Flippin L, Willam C, Wiesener MS, Yard B, Warnecke C, and
581   Eckardt K-U. 2009. Donor treatment with a PHD-inhibitor activating HIFs prevents graft
582   injury and prolongs survival in an allogenic kidney transplant model. *Proceedings of the*
583   *National Academy of Sciences* 106:21276-21281.
584   https://doi.org/10.1073/pnas.0903978106
585
586 Bloechle C, Kusterer K, Konrad T, Hosch SB, Izbicki JR, Knoefel WT, Broelsch CE, and Usadel
587   KH. 1994. Rat Liver Injury Induced by Hypoxic Ischemia and Reperfusion: Protective
588   Action by Somatostatins is Independent from Changes in Glucose Metabolism. *Hormone*
589   *and Metabolic Research* 26:270-275. https://doi.org/10.1055/s-2007-1001682
590
591 Briceño J, and Ciria R. 2010. Early Graft Dysfunction After Liver Transplantation.
592   *Transplantation Proceedings* 42:631-633.
593   https://doi.org/10.1016/j.transproceed.2010.02.004
594
595 Bujaldon E, Cornide-Petronio ME, Gulfo J, Rotondo F, Ávalos de León C, Negrete-Sánchez E,
596   Gracia-Sancho J, Novials A, Jiménez-Castro MB, and Peralta Uroz C. 2019. Relevance
597   of VEGFA in rat livers subjected to partial hepatectomy under ischemia-reperfusion.
598   *Journal of Molecular Medicine* 97:1299-1314. https://doi.org/10.1007/s00109-019-
599   01811-y
600
601 Cai Z, Luo W, Zhan H, and Semenza GL. 2013. Hypoxia-inducible factor 1 is required for
602   remote ischemic preconditioning of the heart. *Proceedings of the National Academy of*
603   *Sciences* 110:17462-17467. https://doi.org/10.1073/pnas.1317158110
604
605 CENATRA. 2020. Boletín Estadístico Informativo Centro Nacional de Trasplantes. México:
606   Centro Nacional de Trasplantes.

607 Cheng M-x, Cao D, Chen Y, Li J-z, Tu B, and Gong J-p. 2019. α-ketoglutarate attenuates
608   ischemia-reperfusion injury of liver graft in rats. *Biomedicine & Pharmacotherapy*
609   111:1141-1146. https://doi.org/10.1016/j.biopha.2018.12.149

611 Choi JM, Park KM, Kim SH, Hwang DW, Chon SH, Lee JH, Lee SY, and Lee YJ. 2010. Effect
612   of Necrosis Modulator Necrox-7 on Hepatic Ischemia-Reperfusion Injury in Beagle
613   Dogs. *Transplantation Proceedings* 42:3414-3421.
614   https://doi.org/10.1016/j.transproceed.2010.08.050

616 Cienfuegos-Pecina E, Ibarra-Rivera TR, Saucedo AL, Ramírez-Martínez LA, Esquivel-Figueroa
617   D, Domínguez-Vázquez I, Alcántara-Solano KJ, Moreno-Peña DP, Alarcon-Galvan G,
618   Rodríguez-Rodríguez DR, Torres-González L, Muñoz-Espinosa LE, Pérez-Rodríguez E,
619   and Cordero-Pérez P. 2020. Effect of sodium (S)-2-hydroxyglutarate in male, and
620   succinic acid in female Wistar rats against renal ischemia-reperfusion injury, suggesting a
621   role of the HIF-1 pathway. *PeerJ* 8:e9438. https://doi.org/10.7717/peerj.9438

623 Dang L, White DW, Gross S, Bennett BD, Bittinger MA, Driggers EM, Fantin VR, Jang HG, Jin
624   S, Keenan MC, Marks KM, Prins RM, Ward PS, Yen KE, Liau LM, Rabinowitz JD,
625   Cantley LC, Thompson CB, Vander Heiden MG, and Su SM. 2009. Cancer-associated
626   IDH1 mutations produce 2-hydroxyglutarate. *Nature* 462:739-744.
627   https://doi.org/10.1038/nature08617

629 Dawn B, and Bolli R. 2005. HO-1 induction by HIF-1: a new mechanism for delayed
630   cardioprotection? *American Journal of Physiology-Heart and Circulatory Physiology*
631   289:H522-H524. https://doi.org/10.1152/ajpheart.00274.2005

633 Dorweiler B, Pruefer D, Andrasi TB, Maksan SM, Schmiedt W, Neufang A, and Vahl CF. 2007.
634   Ischemia-Reperfusion Injury. *European Journal of Trauma and Emergency Surgery*
635   33:600-612. https://doi.org/10.1007/s00068-007-7152-z

637 Du X, and Hu H. 2021. The Roles of 2-Hydroxyglutarate. *Frontiers in Cell and Developmental*
638   *Biology* 9. https://doi.org/10.3389/fcell.2021.651317

640 Eckhoff DE, Bilbao G, Frenette L, Thompson JA, and Contreras JL. 2002. 17-Beta-estradiol
641   protects the liver against warm ischemia/reperfusion injury and is associated with
642   increased serum nitric oxide and decreased tumor necrosis factor-alpha. *Surgery* 132:302-
643   309. https://doi.org/10.1067/msy.2002.125718

645 Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, and Semenza GL. 1996.
646   Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible
647   factor 1. *Molecular and Cellular Biology* 16:4604-4613.
648   https://doi.org/10.1128/MCB.16.9.4604

650 Giannini EG, Testa R, and Savarino V. 2005. Liver enzyme alteration: a guide for clinicians.
651   *Canadian Medical Association Journal* 172:367-379.
652   https://doi.org/10.1503/cmaj.1040752

653

654 Gupta M, Dobashi K, Greene EL, Orak JK, and Singh I. 1997. Studies on hepatic injury and
655   antioxidant enzyme activities in rat subcellular organelles following in vivo ischemia and
656   reperfusion. *Molecular and Cellular Biochemistry* 176:337-347.
657   https://doi.org/10.1023/A:1006829902442

658

659 Harada H, Pavlick KP, Hines IN, Lefer DJ, Hoffman JM, Bharwani S, Wolf RE, and Grisham
660   MB. 2003. Sexual dimorphism in reduced-size liver ischemia and reperfusion injury in
661   mice: Role of endothelial cell nitric oxide synthase. *Proceedings of the National*
662   *Academy of Sciences* 100:739-744. https://doi.org/10.1073/pnas.0235680100

663

664 Hill P, Shukla D, Tran MGB, Aragones J, Cook HT, Carmeliet P, and Maxwell PH. 2008.
665   Inhibition of Hypoxia Inducible Factor Hydroxylases Protects Against Renal Ischemia-
666   Reperfusion Injury. *Journal of the American Society of Nephrology* 19:39-46.
667   https://doi.org/10.1681/asn.2006090998

668

669 Intlekofer AM, Wang B, Liu H, Shah H, Carmona-Fontaine C, Rustenburg AS, Salah S, Gunner
670   MR, Chodera JD, Cross JR, and Thompson CB. 2017. L-2-Hydroxyglutarate production
671   arises from noncanonical enzyme function at acidic pH. *Nature Chemical Biology*
672   13:494-500. https://doi.org/10.1038/nchembio.2307

673

674 Jansen GA, and Wanders RJA. 1993. l-2-Hydroxyglutarate dehydrogenase: identification of a
675   novel enzyme activity in rat and human liver. Implications for l-2-hydroxyglutaric
676   acidemia. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease* 1225:53-
677   56. https://doi.org/10.1016/0925-4439(93)90121-G

678

679 Jiménez-Castro MB, Cornide-Petronio ME, Gracia-Sancho J, and Peralta C. 2019.
680   Inflammasome-Mediated Inflammation in Liver Ischemia-Reperfusion Injury. *Cells*
681   8:1131. https://doi.org/10.3390/cells8101131

682

683 Jiménez Pérez JC, Casillas Ramírez A, Torres González L, Muñoz Espinosa LE, Perales
684   Quintana MM, Alarcón Galván G, Zapata Chavira H, de la Garza G, Javier F, and
685   Cámara Lemarroy CR. 2016. Spironolactone effect in hepatic ischemia/reperfusion injury
686   in Wistar rats. *Oxidative Medicine and Cellular Longevity* 2016.
687   https://doi.org/10.1155/2016/3196431

688

689 Kaławaj K, Sławińska-Brych A, Mizerska-Kowalska M, Żurek A, Bojarska-Junak A, Kandefer-
690   Szerszeń M, and Zdzisińska B. 2020. Alpha Ketoglutarate Exerts In Vitro Anti-
691   Osteosarcoma Effects through Inhibition of Cell Proliferation, Induction of Apoptosis via
692   the JNK and Caspase 9-Dependent Mechanism, and Suppression of TGF-β and VEGF
693   Production and Metastatic Potential of Cells. *International Journal of Molecular Sciences*
694   21. https://doi.org/10.3390/ijms21249406

695

696 Koivunen P, Lee S, Duncan CG, Lopez G, Lu G, Ramkissoon S, Losman JA, Joensuu P,
697   Bergmann U, Gross S, Travins J, Weiss S, Looper R, Ligon KL, Verhaak RGW, Yan H,

698   and Kaelin Jr WG. 2012. Transformation by the (R)-enantiomer of 2-hydroxyglutarate
699   linked to EGLN activation. *Nature* 483:484-488. https://doi.org/10.1038/nature10898
700

701   Kruger NJ. 2009. The Bradford Method For Protein Quantitation. In: Walker JM, ed. *The*
702   *Protein Protocols Handbook*. Totowa, NJ: Humana Press, 17-24.
703

704   Lee PJ, Jiang B-H, Chin BY, Iyer NV, Alam J, Semenza GL, and Choi AMK. 1997. Hypoxia-
705   inducible Factor-1 Mediates Transcriptional Activation of the Heme Oxygenase-1 Gene
706   in Response to Hypoxia. *Journal of Biological Chemistry* 272:5375-5381.
707   https://doi.org/10.1074/jbc.272.9.5375
708

709   Majmundar AJ, Wong WJ, and Simon MC. 2010. Hypoxia-Inducible Factors and the Response
710   to Hypoxic Stress. *Molecular Cell* 40:294-309.
711   https://doi.org/10.1016/j.molcel.2010.09.022
712

713   Murry CE, Jennings RB, and Reimer KA. 1986. Preconditioning with ischemia: a delay of lethal
714   cell injury in ischemic myocardium. *Circulation* 74:1124-1136.
715   https://doi.org/10.1161/01.CIR.74.5.1124
716

717   Nishino T, Okamoto K, Kawaguchi Y, Hori H, Matsumura T, Eger BT, Pai EF, and Nishino T.
718   2005. Mechanism of the Conversion of Xanthine Dehydrogenase to Xanthine Oxidase:
719   IDENTIFICATION OF THE TWO CYSTEINE DISULFIDE BONDS AND CRYSTAL
720   STRUCTURE OF A NON-CONVERTIBLE RAT LIVER XANTHINE
721   DEHYDROGENASE MUTANT. *Journal of Biological Chemistry* 280:24888-24894.
722   https://doi.org/10.1074/jbc.M501830200
723

724   Przyklenk K, Bauer B, Ovize M, Kloner RA, and Whittaker P. 1993. Regional ischemic
725   'preconditioning' protects remote virgin myocardium from subsequent sustained coronary
726   occlusion. *Circulation* 87:893-899. https://doi.org/10.1161/01.CIR.87.3.893
727

728   Ritthausen H. 1872. Ueber das Drehungsvermögen von Glutan- und Aepfelsäure. *Journal für*
729   *Praktische Chemie* 5:354-355. https://doi.org/10.1002/prac.18720050132
730

731   Rzem R, Van Schaftingen E, and Veiga-da-Cunha M. 2006. The gene mutated in l-2-
732   hydroxyglutaric aciduria encodes l-2-hydroxyglutarate dehydrogenase. *Biochimie*
733   88:113-116. https://doi.org/10.1016/j.biochi.2005.06.005
734

735   Salvadori M, Rosso G, and Bertoni E. 2015. Update on ischemia-reperfusion injury in kidney
736   transplantation: Pathogenesis and treatment. *World Journal of Transplantation* 5:52.
737   https://doi.org/10.5500%2Fwjt.v5.i2.52
738

739   Schoenberg M, Fredholm B, Haglund U, Jung H, Sellin D, Younes M, and Schildberg F. 1985.
740   Studies on the oxygen radical mechanism involved in the small intestinal reperfusion
741   damage. *Acta Physiologica Scandinavica* 124:581-589. https://doi.org/10.1111/j.1748-
742   1716.1985.tb00051.x
743

744 Shen SQ, Zhang Y, Xiang JJ, and Xiong CL. 2007. Protective effect of curcumin against liver
745   warm ischemia/reperfusion injury in rat model is associated with regulation of heat shock
746   protein and antioxidant enzymes. *World J Gastroenterol* 13:1953-1961.
747   https://doi.org/10.3748/wjg.v13.i13.1953
748
749 Susuki S, Toledo-Pereyra LH, Rodriguez FJ, and Cejalvo D. 1993. NEUTROPHIL
750   INFILTRATION AS AN IMPORTANT FACTOR IN LIVER ISCHEMIA AND
751   REPERFUSION INJURY. *Transplantation* 55:1265-1272.
752   https://doi.org/10.1097/00007890-199306000-00011
753
754 Torres-González L, Cienfuegos-Pecina E, Perales-Quintana MM, Alarcon-Galvan G, Muñoz-
755   Espinosa LE, Pérez-Rodríguez E, and Cordero-Pérez P. 2018. Nephroprotective Effect of
756   Sonchus oleraceus Extract against Kidney Injury Induced by Ischemia-Reperfusion in
757   Wistar Rats. *Oxidative Medicine and Cellular Longevity* 2018.
758   https://doi.org/10.1155/2018/9572803
759
760 Vogler M, Zieseniss A, Hesse AR, Levent E, Tiburcy M, Heinze E, Burzlaff N, Schley G,
761   Eckardt KU, Willam C, and Katschinski DM. 2015. Pre- and post-conditional inhibition
762   of prolyl-4-hydroxylase domain enzymes protects the heart from an ischemic insult.
763   *Pflügers Archiv - European Journal of Physiology* 467:2141-2149.
764   https://doi.org/10.1007/s00424-014-1667-z
765
766 Wang T, Chen H, Lv K, Ji G, Liang F, Zhang Y, Wang Y, Liu X, Cao H, Kan G, Xiong J, Li Y,
767   and Qu L. 2017. Activation of HIF-1α and its downstream targets in rat hippocampus
768   after long-term simulated microgravity exposure. *Biochemical and Biophysical Research
769   Communications* 485:591-597. https://doi.org/10.1016/j.bbrc.2016.12.078
770
771 Yildiz F, Coban S, Terzi A, Aksoy N, and Bitiren M. 2015. Protective Effect of Micronized
772   Purified Flavonoid Fraction on Ischemia/Reperfusion Injury of Rat Liver.
773   *Transplantation Proceedings* 47:1507-1510.
774   https://doi.org/10.1016/j.transproceed.2015.04.062
775
776 Zapata-Chavira H, Hernandez-Guedea M, Jimenez-Perez JC, Perez-Rodriguez E, Munoz-
777   Espinosa L, Munoz-Maldonado G, and Cordero-Perez P. 2017. Modulation of Remote
778   Ischemic Preconditioning by Proinflammatory Cytokines in Renal Transplant Recipients.
779   *Journal of Investigative Surgery*:1-9. https://doi.org/10.1080/08941939.2017.1375052
780
781 Zapata-Chavira HA, Cordero-Perez P, Casillas-Ramirez A, Escobedo-Villarreal MM, Perez-
782   Rodriguez E, Torres-Gonzalez L, Camara-Lemarroy C, Hernandez-Guedea MA,
783   Caballero-Mendoza E, and Munoz-Espinosa LE. 2015. Is Ischemic Preconditioning a
784   Useful Therapeutic Strategy in Liver Transplantation? Results from the First Pilot Study
785   in Mexico. *Archives of Medical Research* 46:296-302.
786   https://doi.org/10.1016/j.arcmed.2015.06.002
787
788 Zhang X, Liu Z, Xiao Q, Zeng C, Lai C-H, Fan X, Ye Q, Wang Y, and Xiong Y. 2018. Donor
789   Treatment With a Hypoxia-Inducible Factor-1 Agonist Prevents Donation After Cardiac

790   Death Liver Graft Injury in a Rat Isolated Perfusion Model. *Artificial Organs* 42:280-
791   289. https://doi.org/doi.org/10.1111/aor.13005

Figure 1

Effect of the administration of sodium (*S*)-2-hydroxyglutarate on the biochemical markers.

a) Effect on the serum activity of ALP; b) effect on the serum activity of ALT, *p < 0.0001 vs SH group, #p = 0.0002 vs IR group; c) effect on the serum activity of AST, *p < 0.0001 vs SH group, #p = 0.0465 vs IR group; d) effect on the serum concentration of glucose, *p = 0.0032 vs SH group, ‡p = 0.0012 vs SH group; e) effect on the serum activity of LDH, *p < 0.0001 vs SH group, #p = 0.0109 vs IR group; f) effect on the serum concentration of total bilirubin. One-way ANOVA test with Tukey *post hoc* test in a – e; Kruskal-Wallis test with Dunn *post hoc* test in f. ALP = alkaline phosphatase; ALT = alanine aminotransferase; AST = aspartate aminotransferase; LDH = lactate dehydrogenase. Values expressed as mean ± standard deviation.

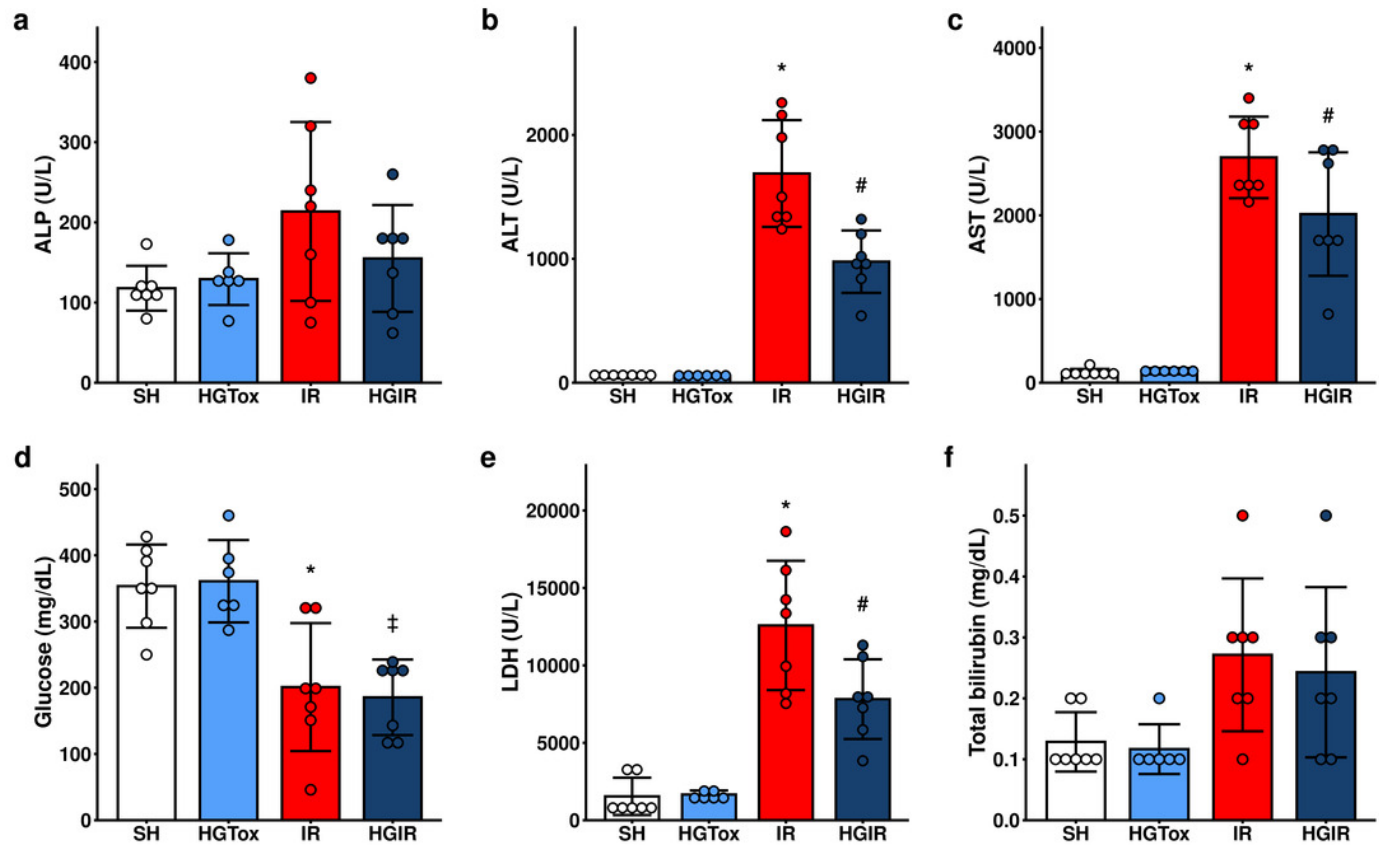



Figure 2

Effect of the administration of sodium (*S*)-2-hydroxyglutarate on the proinflammatory cytokines.

a) Effect on the tissue concentration of IL-1β, *p = 0.0228 vs SH group, #p = 0.0336 vs IR group; b) effect on the tissue concentration of IL-6, #p = 0.0054 vs IR group, ‡p = 0.0002 vs SH group; c) effect on the tissue concentration of TNF, ‡p = 0.0050 vs SH group. One-way ANOVA test with Tukey *post hoc* test. IL-1β = interleukin 1β; IL-6 = interleukin 6; TNF = tumor necrosis factor. Values expressed as mean ± standard deviation.

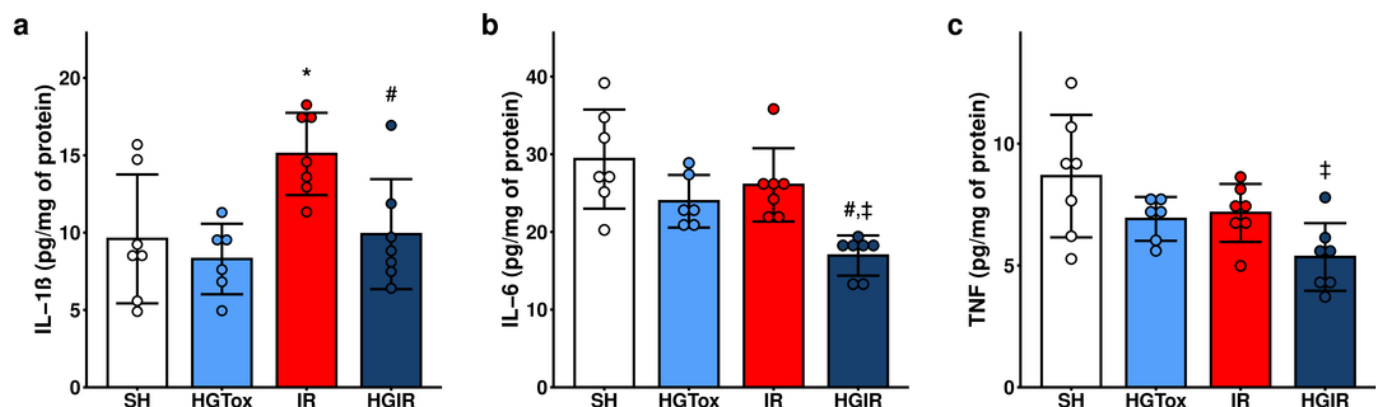



Figure 3

Representative liver micrographs of the experimental groups.

Hematoxylin and eosin staining (original magnification: 400×). a) SH group, b) HGTox group. Tissue architecture was conserved in a and b, without significant cell necrosis or sinusoidal congestion. c) IR group, d) HGIR group. Severe cellular necrosis is observed in zone 3 of the liver acinus in c, while the severity of the damage was decreased in d.

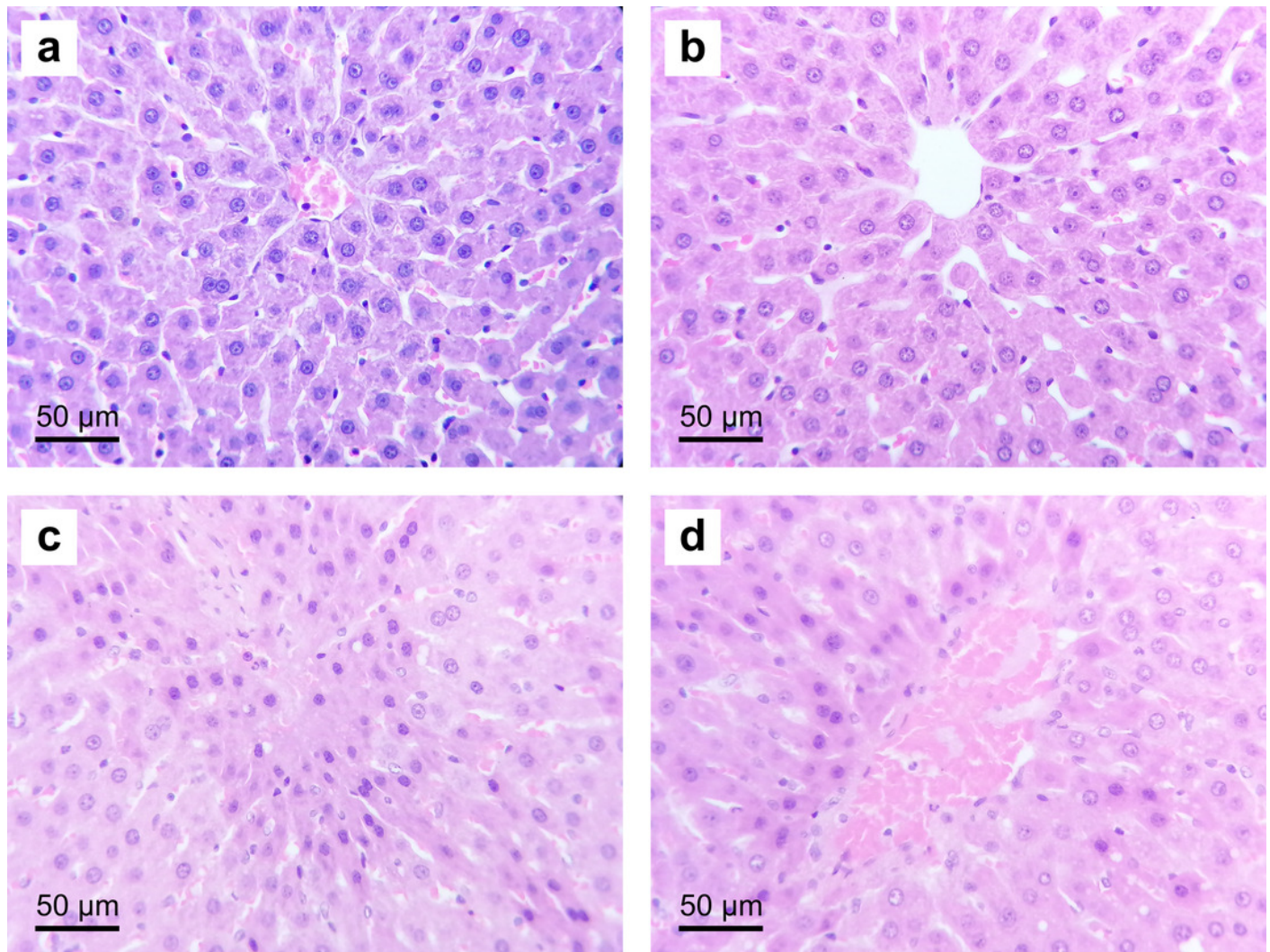
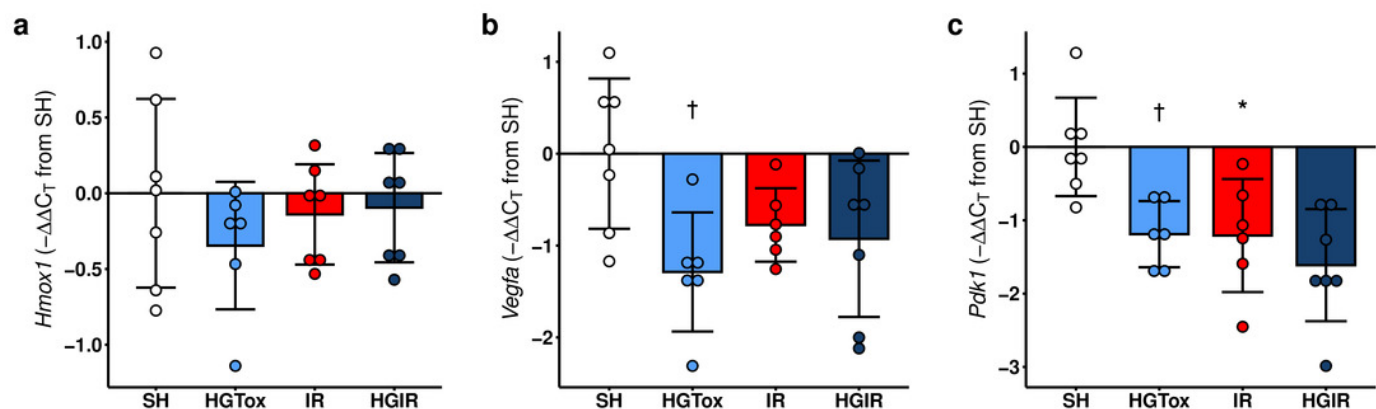



Figure 4

Effect of the administration of sodium (*S*)-2-hydroxyglutarate on the expression of genes regulated by the HIF-1 pathway in liver tissue.

a) Effect on the tissue expression of *Hmox1*; b) effect on the tissue expression of *Vegfa*, †*p* = 0.0185 vs SH group; c) effect on the tissue expression of *Pdk1*, †*p* = 0.0227 vs SH group, **p* = 0.0204 vs SH group. One-way ANOVA test with Tukey *post hoc* test. Values expressed as mean ± standard deviation.

Effect of the administration of sodium (*S*)-2-hydroxyglutarate on the oxidative stress biomarkers.

One-way ANOVA test, Tukey post hoc test. MDA = malondialdehyde; SOD = superoxide dismutase; GPx = glutathione peroxidase. *p = 0.0304 vs SH group.

1   Table 1: Effect of the administration of sodium (*S*)-2-hydroxyglutarate on the oxidative stress
2   biomarkers.

Biomarker	SH	HGTox	IR	HGIR
MDA (nmol/mg of protein)	651.8±89.8	745.5±102.8	594.8±46.6	615.7±61.8
SOD (IU/mg of protein)	23.19±3.15	27.94±7.33	26.28±4.98	24.58±9.53
GPx (nmol/min/mg of protein)	831.0±91.3	840.2±95.4	783.3±32.6	722.9±44.2*

3   One-way ANOVA test, Tukey *post hoc* test. MDA = malondialdehyde; SOD = superoxide dismutase; GPx = glutathione
4   peroxidase. *p = 0.0304 vs SH group.

Histological evaluation after administration of sodium (*S*)-2-hydroxyglutarate.

Kruskal-Wallis test, Dunn post hoc test. *p = 0.0002 vs SH group; **p = 0021 vs SH group.
The whole dataset is shown in the table.

1   Table 2: Histological evaluation after administration of sodium (*S*)-2-hydroxyglutarate.

Parameter	SH	HGTox	IR	HGIR
Cell necrosis	0	1	3	3
	0	1	2	3
	0	0	3	1
	0	0	3	3
	0	1	3	2
	0	2	3	1
	0	ND	3	2
Median (Interquartile range)	0.00 (0.00 – 0.00)	1.00 (0.25 – 1.00)	3.00 (3.00 – 3.00)*	2.00 (1.50 – 3.00)
Sinusoidal congestion	1	1	3	1
	2	2	3	2
	1	0	2	2
	0	1	2	2
	0	2	3	2
	0	0	3	1
	1	ND	3	0
Median (Interquartile range)	1.00 (0.00 – 1.00)	1.00 (0.25 – 1.75)	3.00 (2.50 – 3.00)**	2.00 (1.00 -2.00)
Cytoplasmic vacuolization	0	0	0	1
	0	0	1	1
	0	0	0	0
	0	1	0	0
	0	1	1	2
	0	0	3	1
	0	ND	0	0
Median (Interquartile range)	0.00 (0.00 – 0.00)	0.00 (0.00 – 0.75)	0.00 (0.00 – 1.00)	1.00 (0.00 – 1.00)

2   Kruskal-Wallis test, Dunn *post hoc* test. *p = 0.0002 vs SH group; **p = 0021 vs SH group. The whole dataset is shown in the
3   table.