Free Radical Biology and Medicine 96 (2016) 323–333

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original article

Propofol but not sevoflurane prevents mitochondrial dysfunction and oxidative stress by limiting HIF-1α activation in hepatic ischemia/reperfusion injury

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ARTICLE INFO

Article history:
Received 20 February 2016
Received in revised form
16 April 2016
Accepted 1 May 2016
Available online 3 May 2016

Keywords:
Liver mitochondria
Anesthetics
Ischemia/reperfusion
Apoptosis

ABSTRACT

Mitochondrial dysfunction, reactive oxygen species (ROS) production and oxidative stress during reperfusion are determinant in hepatic ischemia/reperfusion (I/R) injury but may be impacted by different anesthetic agents. Thus, we aimed at comparing the effects of inhaled sevoflurane or intravenous propofol anesthesia on liver mitochondria in a rodent model of hepatic I/R injury. To this, male Wistar rats underwent I/R surgery using sevoflurane or propofol. In the I/R model, propofol limited the raise in serum aminotransferase levels as compared to sevoflurane. Mitochondrial oxygen uptake, respiratory activity, membrane potential and proton leak were altered in I/R; however, this impairment was significantly prevented by propofol but not sevoflurane. In addition, differently from sevoflurane, propofol limited hepatic I/R-induced mitochondria H_2O_2 production rate, free radical leak and hydroxynonenal-protein adducts levels. The I/R group anesthetized with propofol also showed a better recovery of hepatic ATP homeostasis and conserved integrity of mitochondrial PTP. Moreover, hypoxia-inducible factor 1 alpha (HIF-1α) expression was limited in such group. By using a cell model of desferoxamine-dependent HIF activation, we demonstrated that propofol was able to inhibit apoptosis and mitochondrial depolarization associated to HIF-1α action. In conclusion, hepatic I/R injury induces mitochondrial dysfunction that is not prevented by inhaled sevoflurane. On the contrary, propofol reduces liver damage and mitochondrial dysfunction by preserving respiratory activity, membrane potential and energy homeostasis, and limiting free radicals production as well as PTP opening. These hepatoprotective effects may involve the inhibition of HIF-1α.

1. Introduction

In several surgery procedures (tumors resection, vascular reconstructions, transplantation) or other conditions such as trauma or hypovolemic shock, an interruption of the hepatic blood flow followed by reperfusion causes a severe cell damage which compromises the function of the liver [1]. In all these conditions, liver injury and consequent postoperative dysfunction may be caused by hepatic inflow occlusion which results in a transient ischemia period followed by reperfusion (I/R) [2]. Mitochondrial dysfunction, reactive oxygen species (ROS) production and oxidative stress during reperfusion are the most quoted pathogenic mechanism in hepatic I/R injury [3]. In particular, mitochondria are considered as both targets and producers of ROS, contributing to liver damage [4]. Dysfunctional mitochondria in hepatic I/R injury show

Abbreviations: I/R, ischemia/reperfusion; ROS, reactive oxygen species; SEVO, sevoflurane; PROP, propofol; PTP, permeability transition pore; HIF-1α, hypoxia-inducible factor 1 alpha; DFX, deferoxamine; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GSH, reduced glutathione; GSSG, oxidized glutathione; Δψ, mitochondrial membrane potential; HNE, 4-hydroxy-2-nonenal; CsA, cyclosporin A; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-(4,5-Dimethylthiazoly-2-yl)-2,5-diphenyl-tetrazolium bromide; DMSO, dimethyl sulfoxide; AV, annexin V; 7-AAD, 7-Amino-Actinomycin D; JC10, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrathylbenzimidazolyl carbocyanine iodide; TfR1, transferring receptor-1; HO-1, heme oxygenase-1; HGTD-P, Human growth and transformation-dependent protein; BNIP3, Bcl-2/adenovirus E1B 19 kDa-interacting protein 3; SDM, standard deviation of the mean; ANOVA, analysis of variance; RCI, respiratory control index; H_2O_2, hydroperoxide; FRL, free radical leak

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http://dx.doi.org/10.1016/j.freeradbiomed.2016.05.002
0891-5849/

impaired oxidative phosphorylation and ATP generation, increased ROS production and opening of the membrane permeability transition (MPT) pores, which in turn trigger apoptosis [5,6]. Hence, current approaches to reduce hepatic injury – such as antioxidants (*e.g.* glutathione or alpha-tocopherol), ischemic preconditioning, MPT inhibitors – target mitochondrial dysfunction and ROS production [3].

Anesthetic agents may impact mitochondrial function, ROS production and apoptosis during surgery. Inhaled anesthetics, such as sevoflurane and isoflurane, showed protective effects against I/R injury in several organs by improving mitochondrial function and energy metabolism, as well as by preventing ROS release [7,8]. On the other side propofol, a widely used intravenous anesthetic agent chemically similar to free radical scavengers such as alpha tocopherol, was associated with reduced damage and apoptosis in liver I/R injury [9]. Intravenous propofol was compared to inhaled desflurane during partial hepatectomy [10], but no data are available in I/R injury. Thus, we designed the present study to compare the protective effect of inhaled sevoflurane or intravenous propofol anesthesia on mitochondrial bioenergetics, redox balance and hepatocyte apoptosis in liver I/R injury. First, we reported that the mitochondrial dysfunction in hepatic I/R injury was limited by propofol but not by sevoflurane. Further, since the heterodimer hypoxia-inducible factor 1 alpha (HIF-1α) is the main molecular transducer of the hypoxic signal regulating the transcription of a variety of downstream genes involved in energy metabolism and apoptosis [11,12], we tested whether this transcription factor would be involved in the protective action exerted by propofol.

2. Methods

2.1. Animal experimentation

All animals received care in compliance with the *Principles of Laboratory Animal Care* formulated by the *National Society for Medical Research and the guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resources, published by the National Research Council (National Academy Press, revised 1996), as well as with Italian laws on animal experimentation. Adult male Wistar rats (Harlan, San Pietro al Natisone, Italy) weighing 150–200 g were caged individually in a temperature and light controlled environment with free access to food and water. Animals were randomly divided into three groups: (1) CTRL group (n=10), anaesthetized by intraperitoneal 40 mg/kg tiletamine/zolazepam; (2) PROP group (n=10), anesthetized by 1 mg/kg/min propofol administered in the femoral vein by the Marsh model; (3) SEVO group (n=10), anaesthetized with inhaled 2% sevoflurane [13]. 5 rats of each groups were subjected to 45 min of 70% hepatic ischemia followed by 60 min of reperfusion (I/R), using a Schwartz's clip as previously reported [14], while the remaining 5 rats underwent a sham operation (SHAM). This is the most preferred model of hepatic I/R since it is associated with almost 100% survival [15]. After surgery, the animals were sacrificed, their blood was drawn for the evaluation of alanine and aspartate aminotransferases (ALT and AST) activity, and their liver was processed.

2.2. Glutathione assay

Liver homogenate levels of reduced (GSH) and oxidized (GSSG) glutathione were measured by High Performance Liquid Chromatography, as previously reported [16].

2.3. Oxygraphic measurements

Freshly prepared liver mitochondria were assayed for oxygen consumption as previously reported [17]. Mitochondrial membrane potential ($\Delta\psi$) and proton leak analysis were measured as previously reported [17].

2.4. Evaluation of F_OF_1ATPase activity and tissue ATP content

F_OF_1ATPase activity was measured following ATP hydrolysis with an ATP-regenerating system coupled to NADPH oxidation [18]. The hepatic ATP concentration was assessed by bioluminescence (Enliten ATP assay kit - Promega Corporation, Madison, WI, USA) according to the method of Yang [19].

2.5. Measurement of mitochondrial H_2O_2 production and free radical leak

The rate of peroxide production and free radical leak were determined in isolated liver mitochondria following the oxidation of Amplex Red by horseradish peroxidase as previously reported [17].

2.6. Measurement of mitochondrial HNE-protein adducts

Liver mitochondria fluorescent adducts formed between 4-Hydroxy-2-Nonenal (HNE) and proteins were monitored by spectrofluorimetry, at Ex 360/Em 430 nm relative to 0.1 μg/ml quinine sulphate, as previously reported [14].

2.7. Mitochondrial membrane permeability transition pore (PTP) analysis

Opening of PTP was monitored by analyzing mitochondrial swelling, as previously reported [20]. In brief, mitochondrial swelling was assayed spectrophotometrically at 25 °C as a decrease in light absorbance at 540 nm under following additions of 20 μM Ca^{2+} every 90 s, and was expressed as a percentage of swelling with respect to the maximum swelling achieved by exposure to an external Ca^{2+} concentration of 1 mM. PTP opening was identified as a cyclosporin A (CsA)-sensitive decrease in light absorbance.

2.8. Western blot analysis

50 μg proteins from liver homogenates were loaded in a 3–8% SDS-PAGE and transferred to a nitrocellulose membrane, blocked for 1.5 h using 5% non-fat dry milk in TBS-t and incubated with a mouse anti-HIF-1α primary antibody (1:500; NB 100–105, Novus Biologicals Ltd, Cambridge, UK) overnight at 4 °C. Then, the membrane was incubated for 1.5 h with a rabbit HRP-conjugated anti-mouse secondary antibody (1:2000; Bio-Rad Laboratories Inc, Segrate (MI), Italy). Bands were detected by the Clarity™ Western ECL Blotting Substrate using a ChemiDoc MP system (Bio-Rad Laboratories Inc, Segrate (MI), Italy) and quantified by the Image Lab™ Software.

2.9. Cell culture and treatment

The liver cell line Huh-7 (Sigma Aldrich, St Louis, MO, USA) was cultured in high glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco, Carlsbad, CA, USA) at 37 °C in a humidified condition of 5% CO_2. The medium was supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were treated with 0.5–1 mM deferoxamine (DFX) or/and 10–50 μM propofol (PROP).

2.10. MTT assay

Cell viability was determined by 3-(4,5-Dimethylthiazoly-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Cells were seeded in 96-well plate and treated with indicated concentrations of DFX and/or PROP for 3 h. After incubation with MTT solution for 12 h at 37 °C, the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). The absorbance at 546 nm and 650 nm were measured by a microplate reader (Molecular Devices LLC, Sunnyvale, CA, USA).

2.11. Flow cytometric analysis

Apoptosis was quantified by using annexin V (AV)/7-Amino-Actinomycin D (7-AAD) detection kit (Beckman Coulter Inc, Indianapolis, IN, USA) according to the manufacturer's instructions. Cells were treated with DFX and/or PROP for 3 h and harvested. The cells were resuspended in 1 × binding buffer and stained with AV and 7-AAD solution at +4 °C for 30 min. Mitochondrial injury and changes in the $\Delta\psi$ were assessed by staining with 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetrathylbenzimidazolyl carbocyanine iodide (JC-10; Abcam Inc, Cambridge, UK), according to the instructions of the manufacturer. Samples were analyzed by a FlowSight Imaging flow cytometer (Merck Millipore, Darmstadt, Germany).

2.12. Gene expression analysis by real-time RT-PCR

Real-time RT-PCR was performed on RNA extracted from liver tissue, using SYBR Green I assay in Bio-Rad iCycler detection system as previously reported [17]. A PCR master mix was used containing the following specific primers: rat transferrin receptor-1 (TfR1) forward 5′-ATA CGT TCC CCG TTG TTG AGG-3′, reverse 5′-GGC GGA AAC TGA GTA TGG TTG A-3′; rat heme oxygenase-1 (HO-1) reverse 5′-CAG AAG GGT CAG GTG TC-3′, reverse 5′-AGT AAC TCC CAC CTC GT-3′; human growth and transformation-dependent protein (*HGTD-P*) forward 5′-ATG TTT CCT CGC CAC TAA-3′, reverse 5′-CAG CGT CAA GCA TCT CAA-3′; human Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (*BNIP3*) forward 5′-GCC CGG GAT GCA GGA GGA GA-3′, reverse 5′-GAG CAG CAG AGA TGG AAG GAA AAC-3′; human *GAPDH* forward 5′-AGG GCT GCT TTT AAC TCT GGT-3′, reverse 5′-CCC CAC TTG ATT TTG GAG GGA-3′. The threshold cycle (CT) was determined, and the relative gene expression subsequently was calculated as follows: fold change $=2^{-\Delta(\Delta CT)}$, where $\Delta CT = CT - CT$ target housekeeping and $\Delta(\Delta CT) = \Delta CT - \Delta CT$ treated control.

2.13. Statistical analysis

Data were expressed as mean ± standard deviations of the mean (SDM). Since the data from animal experiments were not paired, differences among means between all groups were analyzed using one-way analysis of variance (ANOVA) after Gaussian distribution evaluation by the Kolmogorov-Smirnov test. The Tukey-Kramer multiple comparison test for all pairs of columns was applied as a post hoc test. Furthermore, the 2-way ANOVA was performed referring to "I/R" as the between variable and "treatment" as the within variable. Bonferroni's post hoc test was used where appropriate to perform multiple comparisons. In all instances, $P < 0.05$ was taken as the lowest level of significance. The package GraphPad Prism 6 for Windows (GraphPad Software Inc., San Diego, CA, USA) was used to perform all the statistical analysis.

3. Results

Hepatic ischemia-reperfusion injury induced a dramatic increase in the serum level of both AST and ALT in all the animal groups studied. However, the increase was significantly limited in rats anesthetized with propofol with respect to controls and sevoflurane group (Fig. 1).

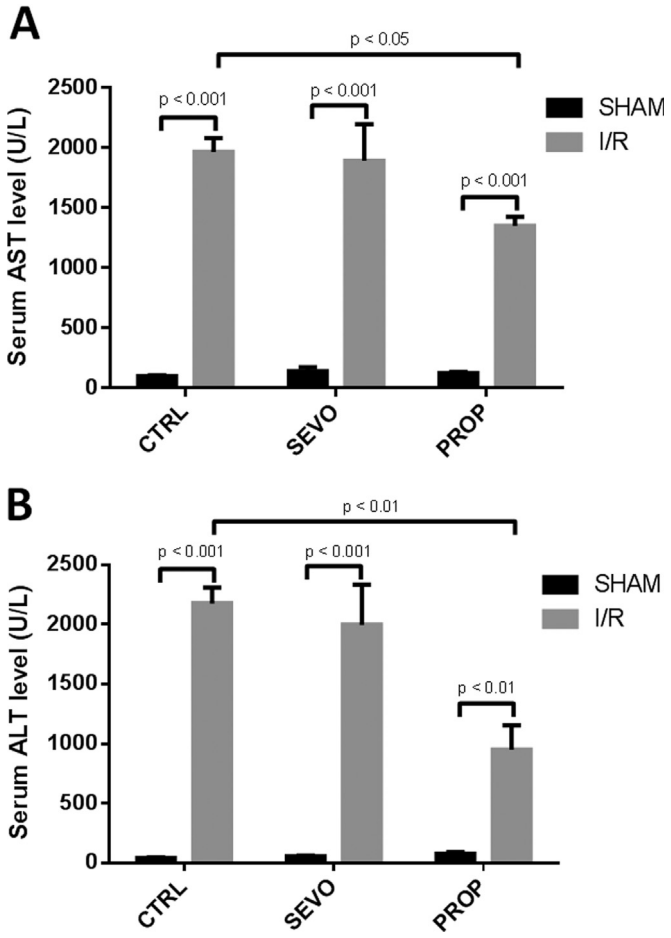

Fig. 1. Serum level of Alanine (ALT) and Aspartate (AST) aminotransferase in sham or ischemia/reperfusion (I/R) rats anesthetized with tiletamine/xylazine (CTRL), sevoflurane (SEVO) or propofol (PROP). Data are espressed as mean ± SDM of five separate experiments. Statistical differences were assessed using two-way analysis of variance (ANOVA) and Tukey-Kramer as a post-hoc test.

3.1. Effects of propofol and sevoflurane on liver oxidative stress and mitochondria function

Reduced (GSH) and oxidized (GSSG) glutathione were measured in the liver from sham and I/R rats undergoing different anesthetic protocols. GSH and GSSG were similar in all sham groups; however, when measured in I/R groups, both SEVO and PROP associated with a limitation in the decrease of GSH hepatic levels. Nevertheless GSSG/GSH ratio, calculated as the expression of glutathione balance, was significantly reduced only in the I/R PROP group as compared to both I/R CTRL and I/R SEVO (Table 2).

The respiratory activity was studied in isolated liver mitochondria using glutamate + malate as complex I-linked substrates, or succinate (in the presence of rotenone as complex I inhibitor) as complex II-linked substrate. As shown in Table 1, I/R injury caused a reduction in the Respiratory Control Index (RCI), which represents the ratio between state 3 (coupled, in the presence of ADP) and state 4 (uncoupled, without ADP) mitochondrial respiration, both with complex I and with complex II-linked substrates. Very interestingly, the RCI after liver I/R was significantly reduced in in SEVO group but was preserved in the PROP. We observed also an increase in both state 3 and state 4 respiration in the SEVO group after I/R as compared to CTRL and PROP groups,

Table 1
Respiratory activity, Respiratory Control Index (RCI) and Mitochondrial Membrane Potential ($\Delta\psi$) in isolated liver mitochondria from sham or ischemia/reperfusion (I/R) rats anesthetized with tiletamine/xylazine (CTRL), sevoflurane (SEVO) or propofol (PROP), using Glutamate-Malate or Succinate as Complex I- or Complex II-linked substrates respectively. Data are expressed as mean ± SDM of five separate experiments. Statistical differences were assessed using one-way analysis of variance (ANOVA) and Tukey-Kramer as a post-hoc test.

	SHAM			I/R		
	CTRL	SEVO	PROP	CTRL	SEVO	PROP
Complex I						
State 4 (nmolO_2/min/mg prot)	6.13 ± 0.93	7.96 ± 2.17	3.81 ± 1.17	6.98 ± 4.45	11.82 ± 2.03*	4.10 ± 0.37^
State 3 (nmolO_2/min/mg prot)	31.90 ± 7.09	41.43 ± 13.4	16.73 ± 2.59	12.59 ± 5.81	26.03 ± 15.0*	16.03 ± 5.40^
RCI	5.17 ± 0.61	5.22 ± 0.68	4.52 ± 0.61	1.84 ± 0.12	2.12 ± 0.91	4.01 ± 1.76^
Complex II						
State 4 (nmolO_2/min/mg prot)	14.34 ± 7.34	19.93 ± 5.30	13.24 ± 6.27	18.59 ± 4.59	21.69 ± 6.28	12.66 ± 2.45*,^
State 3 (nmolO_2/min/mg prot)	50.79 ± 21.0	57.66 ± 19.5	46.85 ± 6.57	38.87 ± 18.4	56.28 ± 36.5	45.12 ± 14.7
RCI	3.72 ± 0.58	2.86 ± 0.39	4.03 ± 1.64	2.24 ± 0.27	2.91 ± 0.91	3.52 ± 0.68*
$\Delta\psi$ (mV)	188.4 ± 25.0	192.9 ± 13.5	200.9 ± 21.2	158.8 ± 12.1	164.1 ± 14.4	174.5 ± 18.3*

* $p < 0.05$ *vs* CTRL.
^ $p < 0.05$ *vs* SEVO.

Table 2
Reduced (GSH) and oxidized (GSSG) glutathione levels in liver homogenates from sham or ischemia/reperfusion (I/R) rats anesthetized with tiletamine/xylazine (CTRL), sevoflurane (SEVO) or propofol (PROP). Data are expressed as mean ± SDM of five separate experiments. Statistical differences were assessed using one-way analysis of variance (ANOVA) and Tukey-Kramer as a post-hoc test.

	SHAM			I/R		
	CTRL	SEVO	PROP	CTRL	SEVO	PROP
GSSG (nmol/g prot)	106.1 ± 24.9	97.6 ± 22.7	103.1 ± 31.8	298.3 ± 44.4	211.8 ± 52.3*	164.0 ± 40.7**
GSH (μmol/g prot)	3.99 ± 1.09	4.23 ± 1.34	4.03 ± 0.59	2.29 ± 0.55	2.26 ± 0.50	3.13 ± 0.54
GSSG/GSH (%)	2.67 ± 0.61	2.32 ± 0.86	2.52 ± 0.16	13.0 ± 3.12	9.32 ± 2.91	5.21 ± 0.76**

* $p < 0.05$ *vs* CTRL.
** $p < 0.01$ *vs* CTRL.

which was significant using complex I-linked substrates (Table 1).

To verify whether changes in membrane potential and permeability occurred after I/R injury, isolated liver mitochondria were incubated with succinate as complex II-linked substrate and rotenone as complex I inhibitor in the presence of oligomycin, which selectively inhibits mitochondrial ATP synthase. As shown in Table 1, hepatic mitochondria from CTRL and SEVO groups were not able to maintain the same membrane potential after I/R injury, while it was preserved in PROP rats. The profile of the flow-force relationship in mitochondria, illustrated in Fig. 2, showed that treatment did not influence the normal bi-phasic relationship between the rate of respiration and the extent of the membrane potential in SHAM animals, where at a relatively low respiratory rate a linear increase of the membrane potential was measured up to 150 mV, while further increases of the respiratory activity resulted in a lower enhancement of the membrane potential (Fig. 2A, B and C). When liver mitochondrial were studied after I/R injury, the profile of the flow-force relationship was different in CTRL and SEVO groups, where a significant change in the correlation slope was found resulting in a much higher oxidation rate of succinate required to settle a given membrane potential when compared to SHAM (Fig. 2D and E). This change did not occur in the PROP group, where the normal bi-phasic relationship was maintained (Fig. 2F).

3.2. Propofol, but not sevoflurane, limits liver mitochondria ROS production and free radical leak enhancement after I/R injury

Release of mitochondrial hydroperoxide (H_2O_2) was measured using pyruvate+malate or succinate as complex I or complex II-linked substrates. The rate of H_2O_2 synthesis was significantly higher in I/R liver mitochondria from all the groups studied when both substrates were used. However, in the PROP group the rate of H_2O_2 production was significantly lower than in CTRL and SEVO groups (Fig. 3A and B).

The percentage of electrons in the respiratory chain directed to ROS generation (free radical leak, FRL) was significantly enhanced in I/R liver mitochondria both with the complex I and the complex II-linked substrates, but the increase was limited in PROP as compared to CTRL or SEVO groups (Fig. 3C and D).

Free radicals may oxidize polyunsaturated fatty acyl groups of membrane phospholipids producing various aldehydes such as HNE, reactive mediator of free radical damage. Then, we observed an increase in the level of mitochondrial HNE-protein adducts after hepatic I/R injury in CTRL and SEVO rats, but not in PROP animals (Fig. 3E).

3.3. Propofol, but not sevoflurane, prevents liver ATP depletion occurring after I/R injury

Since the mitochondrial RCI and proton leak express the coupling between substrate oxidation and ATP synthesis by measuring, respectively, generation and dissipation of the proton motive force to be used by the complex V in the ATP synthesis, we investigated whether sevoflurane and propofol might affect the level of ATP stores. As expected, I/R dramatically depleted liver ATP store and significantly affected mitochondrial ATPase function that was recovered, almost in part, by the use of propofol but not by sevoflurane (Fig. 4A). The specific ATP synthase activity (complex V) was also severely affected by I/R injury, even though the PROP

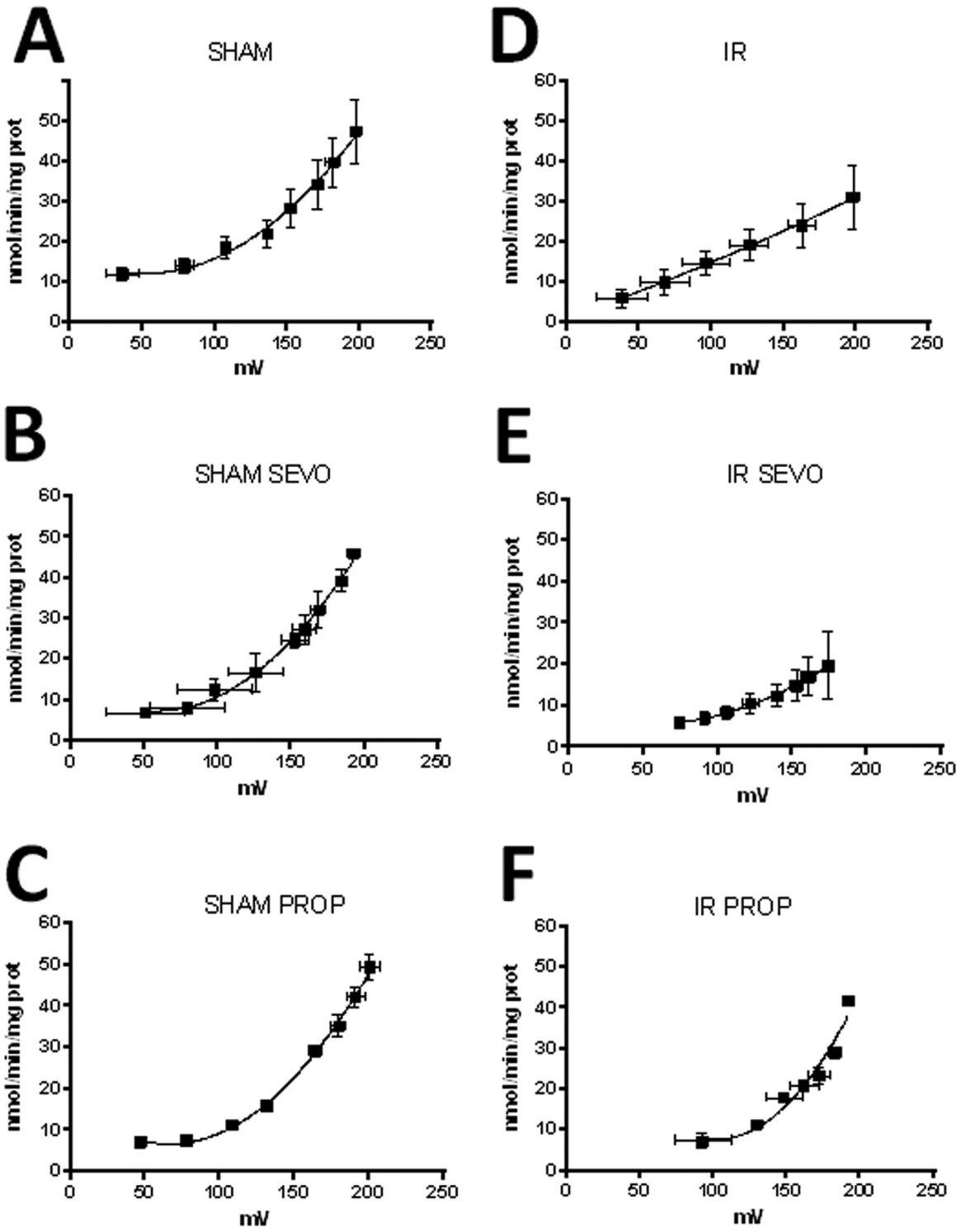

Fig. 2. Proton permeability in isolated liver mitochondria from sham or ischemia/reperfusion (I/R) rats anesthetized with tiletamine/xylazine (CTRL), sevoflurane (SEVO) or propofol (PROP). The membrane potential dependence of the proton leak rate was determined in mitochondria oxidising succinate (10 mM) in the presence of rotenone. Oligomycin was added to inhibit ATP synthase activity. Data are espressed as mean ± SDM of five separate experiments.

group showed a less dramatic decrease as compared to CTRL and SEVO (Fig. 4B).

3.4. The mitochondrial PTP opening after liver I/R injury was prevented by propofol but not by sevoflurane

An additional cause of mitochondrial membrane potential reduction is the opening of the Permeability Transition Pore (PTP), a protein pore that is located in the inner mitochondrial membrane, which determines an increase in mitochondrial permeability to molecules which can increase the osmotic load of the organelle, leading to swelling and consequent rupture of the outer membrane, with further release of cytochrome c and activation of the pro-apoptotic cascade [21]. Because the induction of mitochondrial permeability transition is a crucial factor in the I/R injury, we also measured MPTP in mitochondria isolated from animals

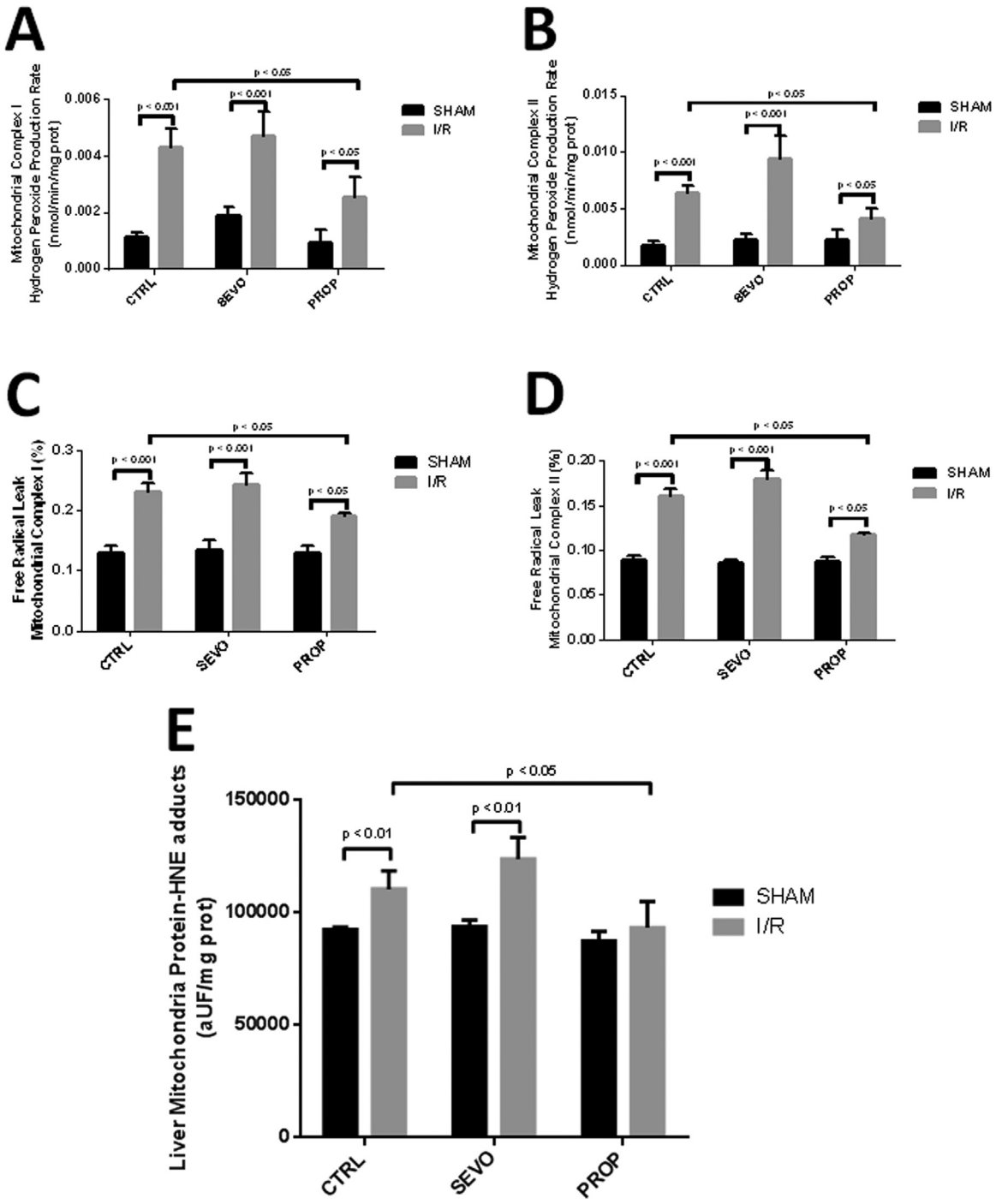

Fig. 3. (A and B) Rates of peroxide production in isolated liver mitochondria from sham or ischemia/reperfusion (I/R) rats anesthetized with tiletamine/xylazine (CTRL), sevoflurane (SEVO) or propofol (PROP), using Glutamate-Malate (A) or Succinate (B) as Complex I- or Complex II-linked substrates respectively. (C and D) Free radical leak of the same samples in A and B. The free radical leak is the percentage of the total electron flow in the respiratory chain directed to oxygen radical generation. (E) mitochondrial levels of 4-hydroxy-2-nonenal (HNE)-protein adducts from the same samples. Data are espressed as mean ± SDM of five separate experiments. Statistical differences were assessed using two-way analysis of variance (ANOVA) and Tukey-Kramer as a post-hoc test.

treated with sevoflurane or propofol. As shown in Fig. 5, the PTP opening in isolated liver mitochondria from I/R animals was induced by increasing concentration of Ca^{++}. In I/R animals concentrations higher than 60 μM induced mitochondrial PTP opening. SEVO did not induce any change as compared to I/R; however, animals anaesthetized by propofol seemed to have significant resistance to mitochondrial PTP. *In vitro* pre-treatment with the PTP inhibitor CsA prevented mitochondrial swelling induced by calcium, demonstrating that the swelling was caused by PTP induction (data not shown). Very interestingly, the swelling was reduced in I/R liver mitochondria isolated from PROP groups (Fig. 5).

3.5. *The hepatoprotective effect of propofol involves the Hypoxia-Inducible Factor 1α (HIF-1α)*

The cellular response of the liver to I/R may be modulated by HIF-1α, a transcription factor induced by hypoxia/ischemia that stimulates the transcription of multiple genes [22]. We observed that I/R induced the expression of HIF-1α in rat liver homogenates,

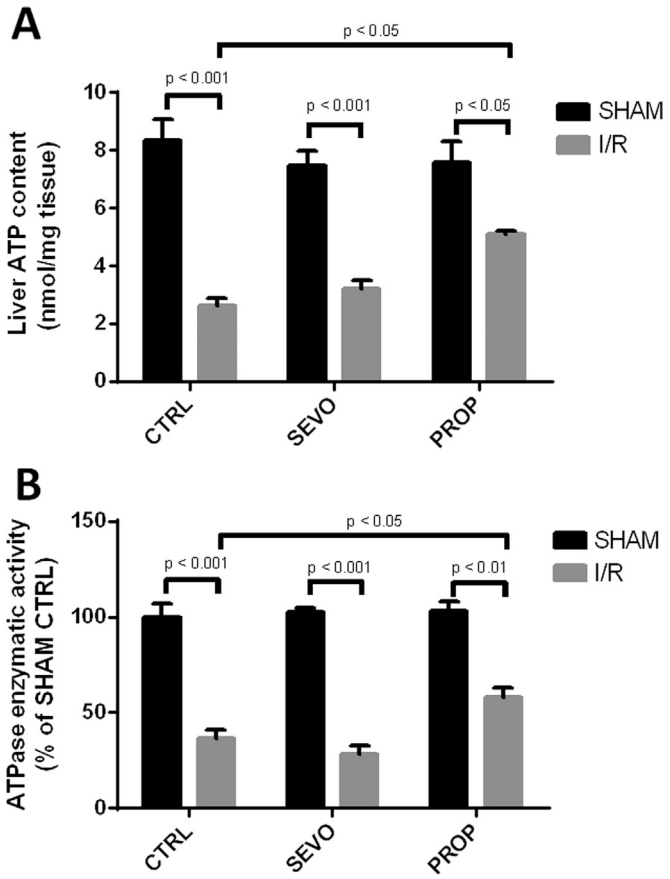

Fig. 4. Hepatic ATP content (A) and specific ATPase activity (B) in isolated liver mitochondria from sham or ischemia/reperfusion (I/R) rats anesthetized with tiletamine/xylazine (CTRL), sevoflurane (SEVO) or propofol (PROP) Data are espressed as mean ± SDM of five separate experiments. Statistical differences were assessed using two-way analysis of variance (ANOVA) and Tukey-Kramer as a post-hoc test.

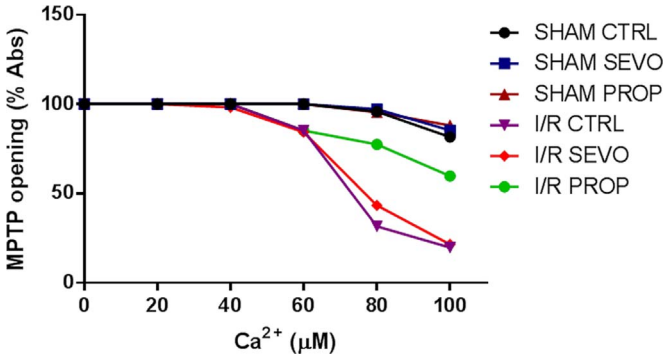

Fig. 5. Mitochondrial permeability transition pore (PTP) opening in isolated liver mitochondria from sham or ischemia/reperfusion (I/R) rats anesthetized with tiletamine/xylazine (CTRL), sevoflurane (SEVO) or propofol (PROP). Calcium-induced mitochondrial swelling was measured as a percentage in absorbance decrease at 540 nm. In-vitro pretreatment with cyclosporin A prevented the swelling, demonstrating that it was dependent on PTP opening (not shown).

but this induction was lower in the I/R group anesthetized with intravenous propofol (Fig. 6A and B). Very interestingly, the expression of two target genes of HIF-1α such as transferrin receptor (TfR) and heme oxygenase-1 (HO-1) was increased in the liver of I/R rats, but not in those anesthetized with propofol (Fig. 6C).

To elucidate the involvement of HIF-1α in the protective effect of propofol during liver I/R, we used an *in vitro* model of HIF-1α induction, treating Huh-7 cells with 0.5–1 mM deferoxamine (DFX) [23,24]. Even though 0.1 mM DFX has been reported to be the lower dose able to up-regulate HIF-1α, we used the lower toxic dose accordingly to our experiments in order to observe the protective effects of propofol. Very interestingly, we observed that propofol prevented the toxicity, apoptosis and mitochondrial depolarization induced by DFX in liver cells (Fig. 7A, C-D). Moreover, it limited the expression of *HGTD-P* and *BNIP3*, two main pro-apoptotic HIF-1α target genes (Fig. 7B).

4. Discussion

The morbidity associated with liver transplantation and major hepatic resections is partly a result of I/R injury, which may lead to acute or chronic liver failure. In conditions of liver ischemia, the cellular damage in hypoxic liver is highlighted by the restoration of blood flow and oxygen delivery [25]. Minimizing the adverse effect of I/R injury is of great importance in order to improve the outcome of warning surgical procedures associated with an increased risk of post-operative dysfunction [26].

Animal models of hepatic I/R are valuable tools for studying its pathophysiology and discovering novel therapeutic targets and drugs [27]. These models allow to understand that early free radicals release during reperfusion initiates a chain of deleterious cellular responses leading to inflammation and cell death [28]. Mitochondria are particularly susceptible to free radicals, which may lead to dysfunction and injury during hepatic damage [4]. Impaired mitochondria may in turn produce significant amounts of ROS, which play a central role in triggering the deleterious cascade of events associated with the hepatic I/R injury [29].

Different anesthetics could influence ROS production and antioxidant capacity during liver surgery, since both volatile and intravenous anesthetic agents have been demonstrated to improve redox balance through different mechanisms. Even if halothane, isoflurane and sevoflurane were protective in an isolated liver perfusion model [30], the same effect was not confirmed *in vivo* either in terms of liver damage or in terms of oxidative stress markers [31]. Sevoflurane is more protective than isoflurane in terms of hepatic redox balance and energy metabolism [8]. Propofol has previously been demonstrated to protect against I/R injury in several organs, such as the heart [32,33], brain [34], kidney [35], but it has also improved liver histology and serum markers in an *in vivo* hepatic I/R model in rabbits [36]. Moreover, propofol is effective in reducing lipid peroxidation, protecting mitochondrial function and preventing PTP opening in rat liver I/R injury [37,38].

Our data demonstrate that propofol, with respect to sevoflurane, reduces hepatocellular injury by preserving mitochondrial bioenergetics and reducing oxidative stress. This may be dependent on the different pharmacokinetics and pharmacodynamics of the two compounds. Compared to other volatile anesthetics, sevoflurane presents a lower solubility in the blood; its hepatic metabolism results in the formation of inorganic fluoride [39]. Propofol is chemically similar to phenol-based free radical scavengers such as the endogenous antioxidant vitamin E, while its lipophilic nature allows its rapid access to cellular and subcellular membranes compartment [40]. A line of studies reported the protective effect of propofol based on its potent antioxidant activity in heart, lung, skeletal muscle and kidney [41–44]. The limitation in the increase of GSSG as well as the GSSG/GSH ratio provides evidence that propofol may improve the hepatic glutathione balance after I/R *via* its antioxidant property. Given the similarity of propofol molecular structure to that of alpha tocopherol, subsequent studies using such as Trolox C as control will better estimate the effect of this compound as a free radical scavenger.

Propofol is able to restore the activity of mitochondrial respiratory chain complexes and ROS production in cardiac I/R injury

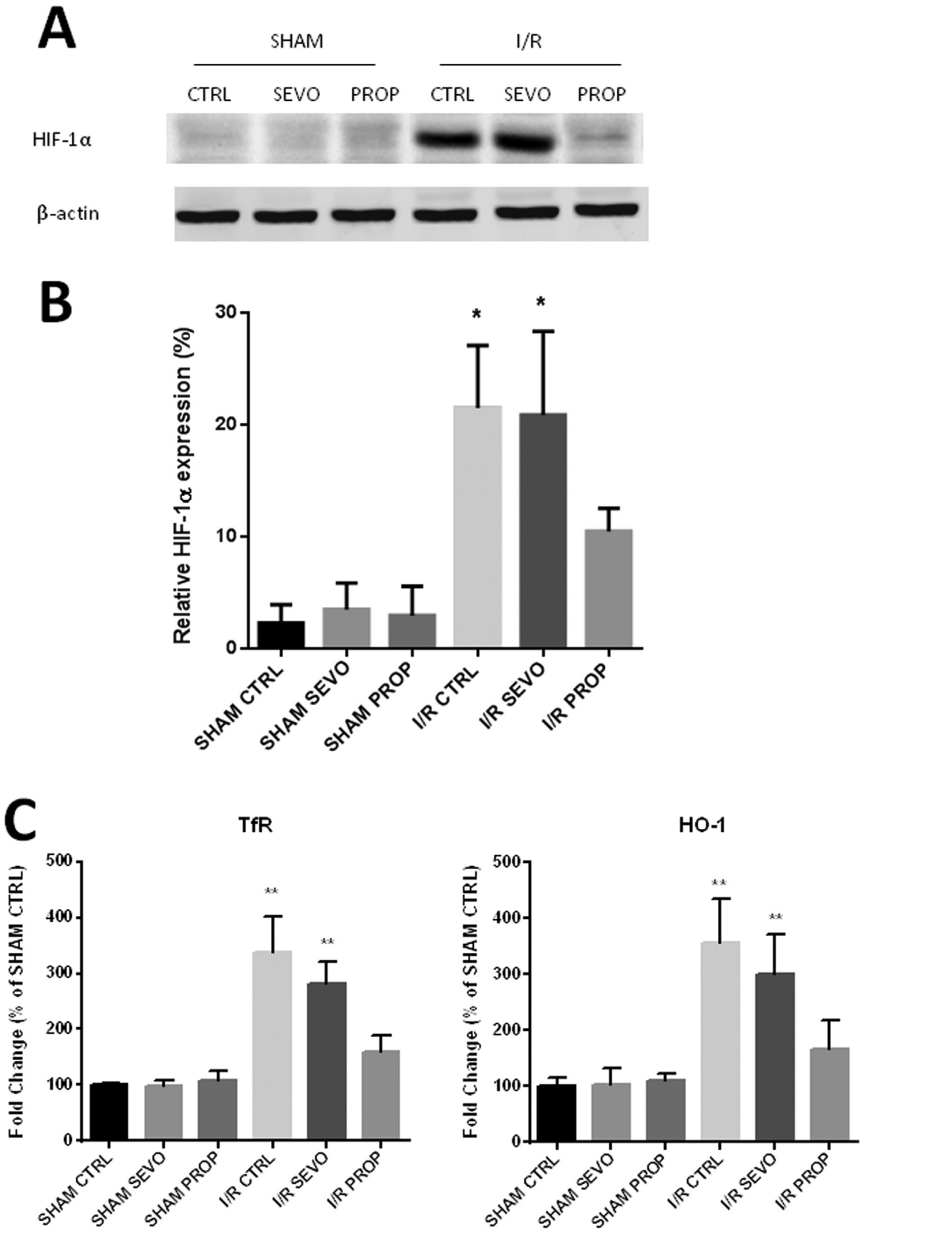

Fig. 6. Representative Western blot (panel A) and relative densitometric analysis (panel B) of HIF-1α compared to β-actin in liver homogenates from sham or ischemia/reperfusion (I/R) rats anesthetized with tiletamine/xylazine (CTRL), sevoflurane (SEVO) or propofol (PROP). Panel C: gene expression of transferrin receptor (TfR) and heme oxygenase-1 (HO-1) in the liver of the same groups. Data are expressed as mean ± SDM. Statistical differences were assessed by one-way ANOVA and Tukey-Kramer as post-hoc test.

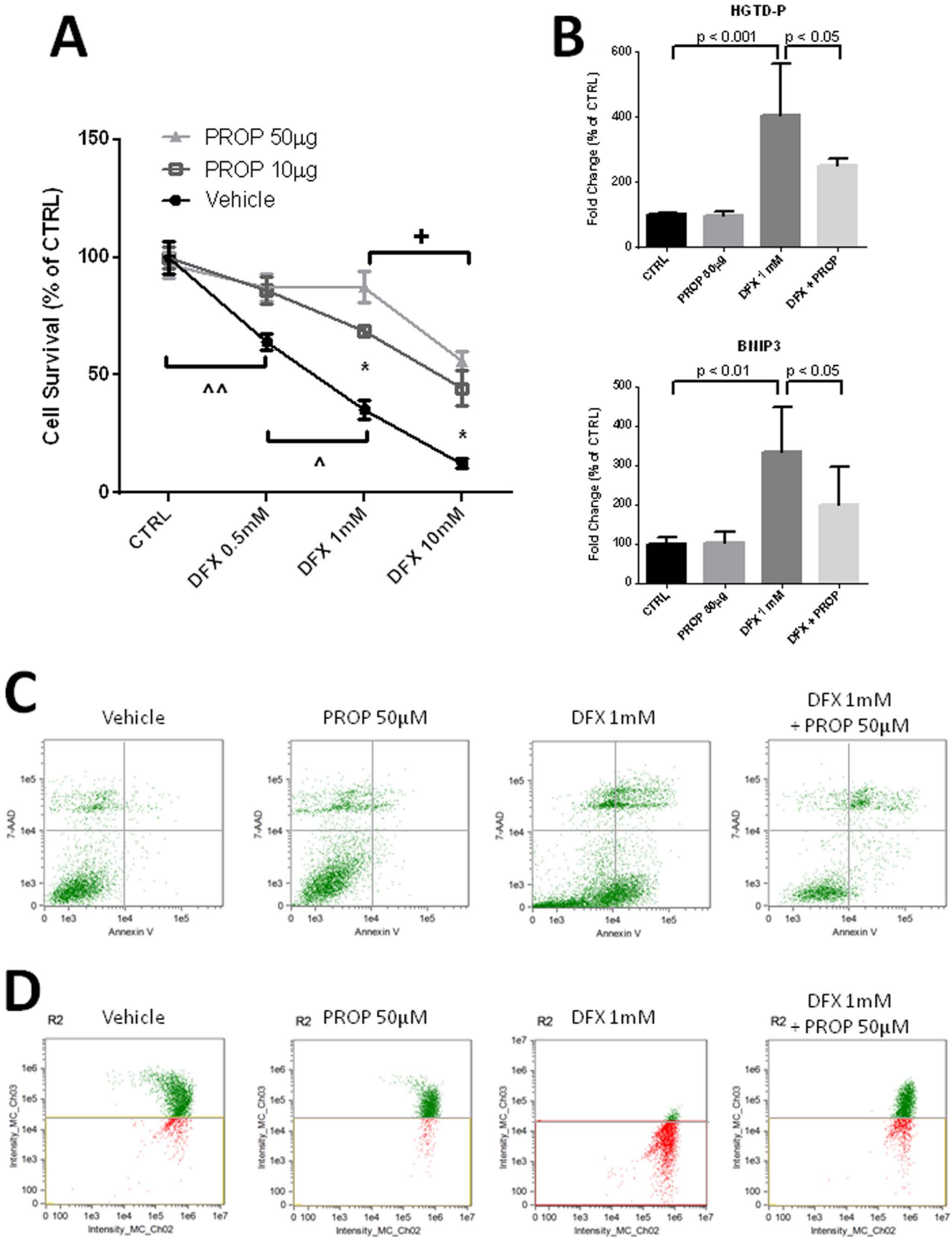

Fig. 7. Panel A: cell viability of Huh-7 cells after 3 h incubation with deferoxamine (DFX) ± vehicle or propofol (PROP), assessed by the MTT assay. Data are expressed as mean ± SDM. Statistical differences were assessed by one-way ANOVA and Tukey-Kramer as post-hoc test. Panel B: Effect of 3 h incubation with deferoxamine (DFX), propofol (PROP) or both on Huh-7 cells gene expression of Human growth and transformation-dependent protein (HGTD-P) and Bcl-2/adenovirus E1B 19 kDa-interacting protein 3 (BNIP3) evaluated by Real Time RT-PCR. Panel C: Effect of 3 h incubation with deferoxamine (DFX), propofol (PROP) or both on Huh-7 cells apoptosis and necrosis. Apoptotic or necrotic cells were detected by flow cytometry after staining with annexin V and 7-AAD. Panel D: Effect of 3 h incubation with deferoxamine (DFX), propofol (PROP) or both on Huh-7 cells mitochondrial potential, detected by flow cytometry after staining with Jc-10.

[40]. Here we report that this mechanism also occurs in liver, where propofol (but not sevoflurane) prevents mitochondrial dysfunction by protecting the respiratory chain function as well as the mitochondrial membrane potential. Studies related to the effects of propofol on mitochondrial function are controversial, but discrepancies could be related to the experimental conditions (different dosages, cell types, *in-vitro* or *ex-vivo* evaluations). Even though some studies show an impairing effect of propofol on macrophages and aortic endothelial cells when used *in vitro* at higher dosages [45,46], this compound is able to prevent the membrane potential collapse in mitochondria isolated from liver and brain subjected to partial ischemia [38,47]. Moreover, our data show that propofol administration limits hepatic ATP depletion, dependent on a less impact on the Complex V activity and on mitochondrial uncoupling induced by I/R injury.

The present study shows that intravenous propofol limits liver damage. However, several studies comparing the effect of different anesthesia regimens on surgery have provided contrasting results [10,48]. In fact, the use of sevoflurane for pharmacological preconditioning in liver surgery has been previously studied in a randomized clinical trial, showing a protective effect [49]. Nevertheless, a retrospective study did not provide any protection of continuous volatile anesthesia when compared to intravenous anesthesia during hepatic I/R [50].

Short-time exposure of sevoflurane during pre-conditioning improved mitochondrial bioenergetics and reduced mitochondrial ROS formation in isolated heart [7]; the same effect was also reported during post-conditioning [51]. However, to mimic a human administration regimen, in our protocol sevoflurane was administered during all the ischemia/reperfusion time. It is conceivable that the long-term administration of sevoflurane could alter the hepatic mitochondria respiratory chain activity [52]. It is also possible that the inorganic fluoride resulting from hepatic metabolism of sevoflurane could modify ROS production and lipid peroxidation, increase GSH/GSSG ratio and reduce the activity of antioxidant enzymes with resulting oxidative stress, as well as cause ATP depletion [53].

The protection of mitochondrial function exerted by propofol during liver I/R is also sustained by an inhibition of the permeability transition pore (PTP) opening. Closed in the ischemic period, the mitochondrial PTP opens during reperfusion leading to membrane potential dissipation, ROS overproduction, uncoupled oxidative phosphorylation with ATP depletion, and release of apoptotic factors such as cytochrome c [54]. The modulation of hepatic mitochondria PTP opening by propofol in I/R injury may be dependent on the suppression of glycogen synthase kinase 3β, as previously demonstrated [38]. Sevoflurane may inhibit the PTP opening in isolated rat heart or cardiomyocytes [55,56], however it may induce mitochondria-dependent apoptosis in human T-lymphocytes and lung alveolar epithelial cells [57,58]; moreover, it promotes apoptosis in the developing brain [59]. In our model, we did not observe any protective effect of sevoflurane probably because inorganic fluorides resulting from sevoflurane hepatic metabolism trigger the disruption of mitochondrial outer membrane and cause the swelling, as suggested elsewhere [53].

Hypoxia-inducible factor-1 alpha (HIF-1α) is a master regulator of cellular responses to hypoxia [12]. Even though several contributions show protective effects of HIF-1α against I/R injury in heart and kidney [60,61], the response to hypoxia varies by cell type [62] and the role of HIF-1α is still debated on neuronal ischemic injury [63]. HIF-dependent upregulation of the transferrin gene contributes to ROS formation and liver injury in reperfusion, likely through iron-dependent reactive species accumulation [64]. The present study shows that propofol anaesthesia is associated with reduced expression of TfR and HO-1 genes in I/R liver, suggesting that this compound could also protect against this redox-based pathway *via* HIF-1α.

Our data confirm the involvement of HIF-1α in the liver of I/R models, and intravenous propofol administration was associated with a lower induction of HIF-1α. This observation is in accordance with previous reports on the reversible inhibition of HIF-1α gene expression as well as activity by propofol [65,66]. Apoptosis plays an important role in I/R injury, and HIF-1α may trigger liver apoptosis following I/R through the induction of hypoxically regulated genes [67]. Our results suggest that the propofol reduces apoptosis and mitochondrial dysfunction induced by liver I/R by a mechanism involving the inhibition of HIF-1α, as evidenced by a limited up-regulation of *HGTD-P* and *BNIP3* genes. *HGTD-P* gene is a HIF-1α-responsive mediator in the mitochondrial apoptotic pathway [68], and the induction of *BNIP3* by HIF-1α determines its localization to mitochondria, triggering a loss of membrane potential and an increase in ROS, leading to cell death [69]. Evidently, the detailed elucidation of this protective pathway by specifically silencing or inhibiting HIF-1α and/or target genes goes beyond the scope of this study.

In conclusion, the present study provides novel evidences that propofol but not sevoflurane may limit liver injury during hepatic ischemia/reperfusion acting at mitochondrial level, where it is able to protect the respiratory chain function and limit both ATP depletion and proton leak, suggesting a partial induction of HIF-1α dependent pathway. The potential impact in patients undergoing liver surgery merits further investigation.

Financial support

The present research was funded by the University of Foggia.

Competing interests

The authors declare no competing interests.

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