Naunyn-Schmiedeberg's Archives of Pharmacology (2020) 393:2129–2137
https://doi.org/10.1007/s00210-020-01930-1

ORIGINAL ARTICLE

ORY supplementation mitigates acetaminophen-induced acute liver failure in male mice: role of oxidative stress and apoptotic markers

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Received: 29 March 2020 / Accepted: 23 June 2020 / Published online: 29 June 2020
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Abstract

The aim of the present study was to assess the possible protective effect of γ-oryzanol (ORY) supplementation in a model of acute liver failure (ALF) induced by acetaminophen (APAP) in mice. Male Swiss strain mice were supplemented with ORY (10 and 50 mg/kg, per oral route) daily for 7 days. One hour after the last supplementation, animals received APAP (300 mg/kg, intraperitoneal). Twenty-four hours after APAP administration, mice were euthanized, and biochemical and histopathological determinations were performed. Histopathological analysis revealed that APAP caused vascular congestion, loss of cellular structure, and cellular infiltration in hepatocytes. Moreover, it caused oxidative damage (enzymatic and non-enzymatic analysis of oxidative stress), with loss of hepatic function leading to cell apoptosis (apoptotic parameters). ORY supplementation (ORY-10 and ORY-50) protected against all changes in ALF model. Thus, the protective effect of ORY supplementation was due to modulation of antioxidant defenses avoiding the apoptotic process.

Keywords Anti-apoptotic · Antioxidant · Hepatoprotective · Caspase · Nutraceutical

Introduction

The liver is essential for the human organism, because it is involved in several metabolic processes such as detoxification of drugs and xenobiotic compounds. Liver injury originated by occasional exposure (alcohol ingestion, viral infections), chemical exposure (drugs), or accidental exposure (hepatotoxins) (Chen et al. 2020) has become a public health problem, because it can lead to chronic hepatitis. Acetaminophen (APAP; N-acetyl-p-aminophenol) is a drug with analgesic and antipyretic action widely used worldwide. APAP is an easily accessible drug for population, being the cases of overdose this medication, the main cause of acute liver failure (ALF) in several countries (Larsen and Wendon 2014).

The metabolism of APAP occurs through cytochrome P450 enzymes that result in excessive formation of N-acetyl-q-benzoquinone imine (NAPQI). The NAPQI metabolite causes glutathione depletion (GSH), triggers oxidative stress (OS), and increased of inflammatory process with the activation of apoptotic pathways leading to hepatocellular necrosis (Gupta and Rawat 2017). In view of above, it is necessary to search for therapeutic or preventive alternatives for ALF APAP-induced. Thus, bioactive compounds can aid cell integrity against oxidative and inflammatory damage, improving aerobic metabolism or limiting the production of reactive oxygen species (ROS) (Suliman and Piantadosi 2016). Furthermore, they have little or no side effects when compared with the available drugs.

In this sense, γ-oryzanol (ORY) is a bioactive compound present in rice bran oil, being a mixture of trans-ferulic acid esters and phytosterols such as cycloartenol, β-sitosterol, 24-methylenecycloartenol, and campesterol (Minatel et al. 2016). In the last few years, several studies have demonstrated the antioxidant, anti-inflammatory, hypolipidemic, antitumoral, and hepatoprotective activities of ORY (Bhaskaragoud et al. 2016; Yu et al. 2019; De Gomes et al. 2019).

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00210-020-01930-1) contains supplementary material, which is available to authorized users.

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In view of aforementioned, the aim of the present study was to assess the possible protective effect of ORY supplementation (10 mg/kg and 50 mg/kg) in a model of APAP-induced ALF in mice. Moreover, it was investigated whether ORY supplementation would be able to mediate changes AFL-induced in histopathological analysis, OS, hepatic damage, and apoptotic markers.

Materials and methods

Reagents and compounds

APAP, glutathione reductase from baker's yeast, β-nicotinamide adenine dinucleotide phosphate reduced tetrasodium salt (NADPH), 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), and oxidized glutathione (GSSG) were purchased from Sigma (St. Louis, MO, USA). 1-Chloro-2,4-dinitrobenzene (CDNB) was purchased from Aldrich Chemical Co. (USA). ORY was purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). All other chemicals used in this study were of analytical grade.

Animals and exposure

Male adult Swiss strain mice (6 weeks old, 25–35 g) were used. Animals were kept at a temperature of 22 ± 2 °C under a 12-h light/dark cycle, with food and water ad libitum. Mice were used according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of UNIPAMPA, Uruguaiana, Brazil. (Protocol 021/2014). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Mice were randomly divided into six groups (6 animals per group):

Group 1 (*Control*): Canola oil (vehicle) + saline (vehicle);
Group 2 (*ORY-10*): ORY (10 mg/kg) + saline (vehicle);
Group 3 (*ORY-50*): ORY (50 mg/kg) + saline (vehicle);
Group 4 (*APAP*): APAP (300 mg/kg) + Canola oil (vehicle);
Group 5 (*ORY-10 + APAP*): APAP (300 mg/kg) + ORY (10 mg/kg);
Group 6 (*ORY-50 + APAP*): APAP (300 mg/kg) + ORY (50 mg/kg);

Mice received ORY supplementation (10 mg/kg or 50 mg/kg) per via oral (p.o., gavage) once a day for 7 days. The dose of ORY supplemented (Canola oil was used as vehicle) to animals is in agreement with previous studies of Sakamoto et al. 1987, Zolali et al. 2015, and De Gomes et al. 2018. Besides that, the treatment groups that received saline solution and canola oil (vehicles) were administered with a volume equivalent to the interaction groups. One hour after the last supplementation, mice received APAP (300 mg/kg, diluted in saline solution) via intraperitoneal injection (i.p.), for induction of ALF (Seo et al. 2020).

Plasma and liver tissue preparation

Twenty-four hours after APAP administration, mice were anesthetized using sodium pentobarbital (100 mg/kg, i.p.) and blood was collected by cardiac puncture into tubes containing heparin (1 UI/mL). Plasma was obtained by centrifuging of blood at 2400*g* for 12 min and used for evaluation of hepatic damage markers. After this procedure, livers were removed, dissected, and homogenized in 50 mM Tris-HCl, pH 7.4 (1/10 w/v), and centrifuged at 2400*g* for 15 min. Supernatant fraction (S_1) was used for biochemical analysis.

Measurement of oxidative stress index

Non-enzymatic markers: thiobarbituric acid reactive species, non-protein thiol, ascorbic acid, and 4-hydroxynonenal levels

Thiobarbituric acid reactive species (TBARS) levels were estimated in S_1 by measuring TBARS assay, a method previously proposed by Ohkawa et al. 1979. S_1 of the liver was incubated with 0.8% thiobarbituric acid (TBA), acetic acid buffer pH 3.4, and 8.1% sodium dodecyl sulfate at 95 °C for 2 h. The amount of TBARS produced was measured at 532 nm, using MDA as a biomarker of lipid peroxidation. Lipid peroxidation was expressed as nmol of MDA/mg protein.

Non-protein thiol (NPSH) levels were determined by a method previously proposed by Ellman 1959. A sample (S_1) aliquot was mixed (1:1) with 10% trichloroacetic acid. After the centrifugation, the protein pellet was discarded and free-SH groups were determined in the clear supernatant. An aliquot of supernatant was added in 1 M potassium phosphate buffer pH 7.4 and 10 mM DTNB. The color reaction was measured at 412 nm. NPSH levels were expressed as nmol NPSH/g tissue.

Ascorbic acid (AA) levels were measured in S_1 by a method previously proposed by Jacques-Silva et al. 2001, with some modifications. Briefly, S_1 was precipitated in 10% trichloroacetic acid solution. An aliquot of S_1 (300 mL) at a final volume of 575 mL of solution was incubated for 3 h at 38 °C, then 500 mL H_2SO_4 65% (v/v) and was added to the medium. The reaction product was determined using a color reagent containing 4.5 mg/mL dinitrophenyl hydrazine and $CuSO_4$ (0.075 mg/mL) at 520 nm. The AA content is related to the amount of tissue (mmol AA/g tissue).

Levels of 4-hydroxynonenal (4-HNE) were determined using commercially available ELISA assays (DuoSet Kits, R&D Systems; Minneapolis). Results are shown as pg/mg tissue.

Enzymatic markers: catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase, glutathione S-transferase, and δ-aminolevulinic acid dehydratase activities

Catalase (CAT) activity was assayed spectrophotometrically by the method of Aebi 1984, which involves monitoring the disappearance of H_2O_2 in presence of S_1 at 240 nm. Enzymatic reaction was initiated by adding an aliquot of S_1 20 μL and the substrate (H_2O_2) at a concentration of 0.3 mM in a medium containing 50 mM phosphate buffer, pH 7.0. The enzymatic activity was expressed in units of (1 U decomposes 1 μmol of H_2O_2 per minute at pH 7 at 25 °C).

Superoxide dismutase (SOD) activity was determined spectrophotometrically as described by Misra and Fridovich 1972. The color reaction was measured at 480 nm. One unit of enzyme was defined as the amount of enzyme required to inhibit the rate of epinephrine autoxidation by 50% at 37 °C.

Glutathione peroxidase (GPx) activity was measured in a system containing reduced GSH, reduced form of NADPH and glutathione reductase (GR), as previously described by Wendel 1981. The decline in the concentration of NADPH can be monitored at 340 nm. Activity of GPx is given by the consumption of NADPH in nmol/min/mg of protein.

GR activity was determined spectrophotometrically as described by the method of Carlberg and Mannervik, 1985. In this assay, GSSG is reduced by GR at the expense of NADPH consumption, which is followed at 340 nm. An aliquot of 50 μL of S_1 was added in the system containing 0.15 M potassium phosphate buffer, pH 7.0, 1.5 mM EDTA, and 0.15 mM NADPH. After the basal reading, 50 μL of 20 mM GSSG (substrate) was added. Enzymatic activity was expressed as nmol NADPH/min/mg protein.

Glutathione S-transferase (GST) activity was assayed through the conjugation of glutathione with CDNB at 340 nm as described by Habig et al. 1974. An aliquot (100 μL of 0.1 M potassium phosphate buffer (pH 7.4), with CDNB as the substrate) was added to 50 mM GSH. The enzymatic activity was expressed in μmol/min mg protein.

δ-Aminolevulinic acid dehydratase (δ-ALA-D) activity was determined by measuring the formation of porphobilinogen (PBG), according to the method of Sassa 1982, except that 45 mM sodium phosphate buffer and 2.2 mM ALA were used. Samples were homogenized in 0.9% NaCl in the proportion (w/v) 1/5 and centrifuged at 2400×g for 15 min. An aliquot of 50 μL of homogenized tissue was incubated for 2 h at 37 °C. PBG formation was detected with the addition of modified Erlich's reagent at 555 nm.

Hepatic function analysis

Aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, gamma-glutamyl transferase, and lactate dehydrogenase activities

The enzyme activities in plasma were measured using a commercial kit (LABTEST, Diagnostica S.A., Minas Gerais, Brazil). The enzymatic activities were expressed as U/L.

Apoptotic markers

Caspase 3 and 9 activities

Caspase 3 and 9 activities in S_1 were measured using a Caspase-Glo assay kit (Promega, Madison, USA) according to the manufacturer's instructions. The activities were calculated as pmol/min/mg protein.

Protein analysis

Protein concentration was measured by the method of Bradford (1976), using bovine serum albumin (1 mg/mL) as pattern.

Histopathological analysis

Small pieces of liver tissues from individual mice were fixed in 10% formalin. For optical microscopy examination, tissues were embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin. Liver sections were graded to assess the histological features for degree of acute hepatic injury. Zonal necrosis (damage of several liver cells around the central vein), vascular congestion, megalocytosis, cell infiltration, eosinophilic cells, and tumefaction of hepatocytes were characterized (Bancroft et al. 1996).

Statistical determinations

The data distribution was verified by applying Shapiro-Wilk test. Results are expressed as means ± S.E.M. Comparisons between experimental and control groups were performed by two-way analysis of variance (ANOVA) followed by Newman–Keuls test when appropriate. A value of $p < 0.05$ was significant. Statistical analysis was performed using the software GraphPad Prism version 6 (San Diego, CA, USA).

Table 1 Assessment of ORY supplementation on TBARS, NPSH, AA, and 4-HNE levels in ALF model induced by APAP

Groups	TBARS (nmol MDA equivalents/g tissue)	NPSH (μmol/g tissue)	Ascorbic acid (μg AA/g tissue)	4-HNE (pg/mg tissue)
Control	14.62 ± 1.22	33.80 ± 2.13	298.0 ± 13.82	1.26 ± 0.11
ORY-10	15.48 ± 1.42	42.00 ± 2.98	295.2 ± 22.65	1.04 ± 0.06
ORY-50	14.92 ± 1.67	42.80 ± 4.81	321.8 ± 18.79	1.10 ± 0.07
APAP	41.02 ± 3.37[a]	9.01 ± 2.12[a]	199.2 ± 12.74[a]	3.56 ± 0.21[a]
ORY-10 + APAP	29.38 ± 3.99[ab]	31.01 ± 2.91[b]	279.0 ± 10.27[b]	2.16 ± 0.08[ab]
ORY-50 + APAP	26.88 ± 3.98[ab]	33.60 ± 3.32[b]	299.8 ± 13.90[b]	1.72 ± 0.09[ab]

Data are expressed as means ± S.E.M (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). [a] Denoted $p < 0.05$ as compared with the control group. [b] Denoted $p < 0.05$ as interaction groups when compared with APAP

Results

Measurement of oxidative stress index

Non-enzymatic markers: TBARS, NPSH, AA, and 4-HNE levels

Two-way ANOVA of TBARS, NPSH, AA, and 4-HNE-levels showed a significant ORY-10 × APAP interaction ($F_{1,36} = 5.07$; $p < 0.03$), ($F_{1,36} = 7.20$; $p < 0.01$), ($F_{1,36} = 7.02$; $p < 0.01$), and ($F_{1,36} = 19.78$; $p < 0.001$) respectively. Similarly, a two-way ANOVA of TBARS, NPSH, AA, and 4-HNE-levels revealed a significant ORY-50 × APAP interaction ($F_{1,36} = 6.61$; $p < 0.02$), ($F_{1,36} = 5.63$; $p < 0.03$), ($F_{1,36} = 6.56$; $p < 0.02$), and ($F_{1,36} = 38.52$; $p < 0.001$). Newman–Keuls post hoc test demonstrated that ORY supplementation (ORY-10 and ORY-50) protected against alterations in non-enzymatic markers of OS in ALF induced by APAP (Table 1).

Enzymatic markers: CAT, SOD, GPx, GR, GST, and δ-ALA-D activities

Statistical analysis revealed no significant difference of CAT, SOD, GPx, GR, GST, and δ-ALA-D activities (ORY-10 × APAP); ($F_{1,36} = 1.81$; $p < 0.19$), ($F_{1,36} = 0.10$; $p < 0.75$), ($F_{1,36} = 2.12$; $p < 0.16$), ($F_{1,36} = 3.80$; $p < 0.06$), ($F_{1,36} = 2.77$; $p < 0.11$), and ($F_{1,36} = 0.04$; $p < 0.83$) (Table 2 and Fig. 1a–c, respectively). For CAT, SOD, GPx, GR, GST, and δ-ALA-D activities, a two-way ANOVA showed a significant ORY-50 × APAP interaction ($F_{1,36} = 6.15$; $p < 0.02$), ($F_{1,36} = 5.12$; $p < 0.03$), ($F_{1,36} = 13.83$; $p < 0.001$), ($F_{1,36} = 13.35$; $p < 0.001$), ($F_{1,36} = 21.30$; $p < 0.001$), and ($F_{1,36} = 4.52$; $p < 0.04$). Post hoc analysis revealed that ORY-10 supplementation was not able to modulate alterations caused by APAP in enzymatic markers of OS. On the contrary, post hoc analysis revealed that ORY-50 supplementation modulated enzymatic activities in ALF induced by APAP (Table 2 and Fig. 1a–c).

Hepatic function markers: AST, ALT, ALP, GGT, and LDH activities

Statistical analysis of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and lactate dehydrogenase (LDH) activities showed a significant ORY-10 × APAP interaction ($F_{1,36} = 11.47$; $p < 0.001$), ($F_{1,36} =$

Table 2 Effect of γ-ORY supplementation on CAT, SOD, and δ-ALA-D activities in the model of ALF induced by APAP

Groups	CAT (U/mg protein)	SOD (U/mg protein)	δ-ALA-D (nmol PBG/mg protein/h)
Control	33.70 ± 2.73	11.06 ± 0.56	11.04 ± 0.65
ORY-10	34.18 ± 1.72	11.14 ± 0.60	12.02 ± 0.59
ORY-50	36.15 ± 3.20	12.58 ± 1.08	12.09 ± 0.97
APAP	20.15 ± 1.27[a]	5.70 ± 0.67[a]	8.05 ± 0.57[a]
ORY-10 + APAP	25.91 ± 1.80[a]	6.20 ± 0.79[a]	8.74 ± 0.90[a]
ORY-50 + APAP	34.56 ± 1.96[b]	10.66 ± 0.59[b]	12.11 ± 0.54[b]

Data are expressed as means ± S.E.M (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). [a] Denoted $p < 0.05$ as compared with the control group. [b] Denoted $p < 0.05$ as interaction groups when compared with APAP group

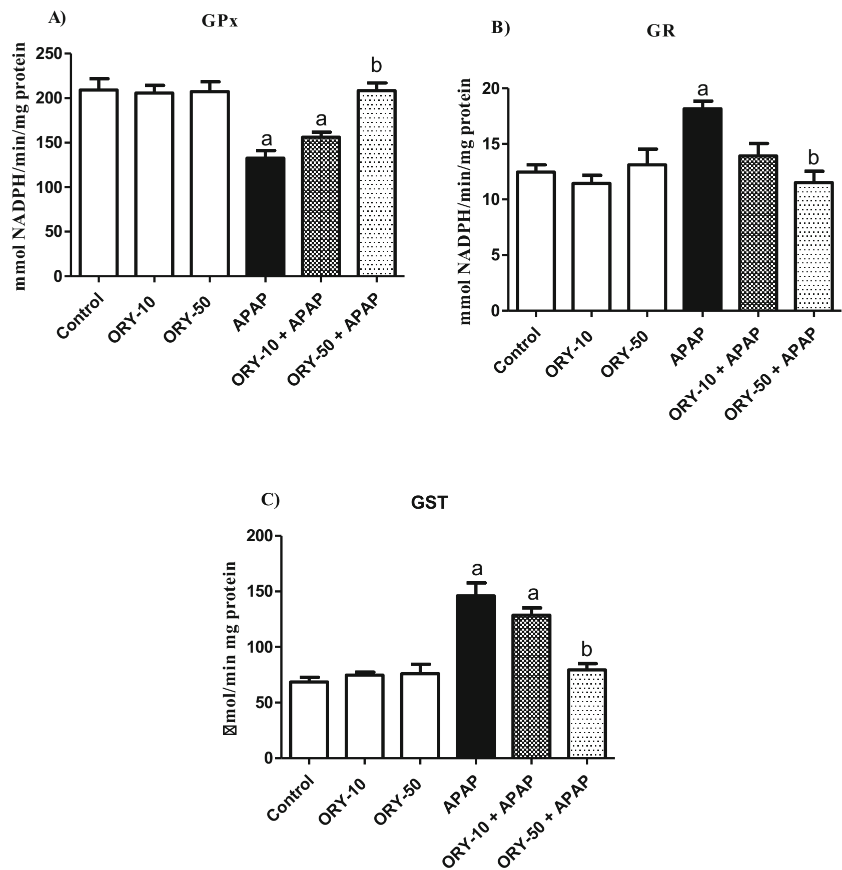

Fig. 1 Effect of ORY supplementation (ORY-10 and ORY-50) in GPx (**a**), GR (**b**), and GST (**c**) activities in ALF model induced by APAP. Data are expressed as means ± S.E.M (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). [a]Denoted $p < 0.05$ as compared with the control group. [b]Denoted $p < 0.05$ as interaction groups when compared with APAP

13.12; $p < 0.001$), ($F_{1,36} = 27.81$; $p < 0.001$), ($F_{1,36} = 20.20$; $p < 0.001$), and ($F_{1,36} = 15.40$; $p < 0.001$). In similar manner, a two-way ANOVA of TBARS, NPSH, AA, and 4-HNE-levels revealed a significant ORY-50 × APAP interaction ($F_{1,36} = 25.22$; $p < 0.001$), ($F_{1,36} = 39.48$; $p < 0.001$), ($F_{1,36} = 26.08$; $p < 0.001$), ($F_{1,36} = 33.48$; $p < 0.001$), and ($F_{1,36} = 31.47$; $p < 0.001$). Post hoc test demonstrated that ORY supplementation (ORY-10 and ORY-50) protected against alterations in enzymatic of hepatic function in ALF induced by APAP (Table 3).

Apoptotic markers: caspase 3 and 9 activities

Statistical analysis and two-way ANOVA of caspase 3 and 9 activities revealed a significant ORY-10 × APAP interaction ($F_{1,36} = 21.12$; $p < 0.001$) and ($F_{1,36} = 18.85$; $p < 0.001$) respectively. Equally, a two-way ANOVA of caspase 3 and 9 activities demonstrated a significant ORY-50 × APAP interaction ($F_{1,36} = 30.42$; $p < 0.001$) and ($F_{1,36} = 19.49$; $p < 0.001$). Newman–Keuls post hoc test demonstrated that ORY supplementation (ORY-10 and ORY-50) protected

Table 3 Evaluation of γ-ORY supplementation on AST, ALT, ALP, GGT, and LDH activities in a model of APAP-induced ALF

Groups	AST (U/L)	ALT (U/L)	ALP (U/L)	GGT (U/L)	LDH (U/L)
Control	44.40 ± 5.14	30.80 ± 4.11	237.4 ± 18.21	25.20 ± 2.88	272.4 ± 25.51
ORY-10	35.80 ± 5.64	31.00 ± 5.16	228.0 ± 24.44	27.60 ± 3.62	277.0 ± 44.92
ORY-50	47.60 ± 4.53	38.40 ± 3.64	229.2 ± 21.22	32.00 ± 6.04	249.2 ± 49.10
APAP	166.6 ± 16.52[a]	164.4 ± 14.56[a]	873.6 ± 45.72[a]	129.4 ± 8.98[a]	879.6 ± 51.97[a]
ORY-10 + APAP	92.60 ± 6.45[ab]	99.40 ± 8.26[ab]	498.6 ± 42.28[ab]	79.40 ± 5.80[ab]	579.2 ± 25.88[ab]
ORY-50 + APAP	74.60 ± 6.26[ab]	71.60 ± 3.61[ab]	413.6 ± 70.38[ab]	59.80 ± 6.98[ab]	396.4 ± 31.01[b]

Data are expressed as means ± S.E.M (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). [a] Denoted $p < 0.05$ as compared with the control group. [b] Denoted $p < 0.05$ as interaction groups when compared with APAP

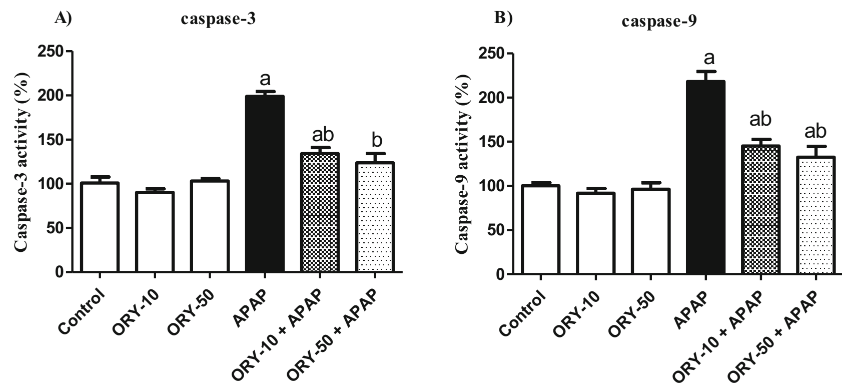

Fig. 2 Evaluation of ORY supplementation (ORY-10 and ORY-50) in caspase-3 (**a**) and caspase-9 (**b**) activities in a model of APAP-induced ALF. Data are expressed as means ± S.E.M (two-way ANOVA/"Newman-Keuls Multiple Comparison Test"). [a]Denoted $p < 0.05$ as compared with the control group. [b]Denoted $p < 0.05$ as interaction groups when compared with APAP group

against alterations of apoptotic markers in ALF induced by APAP (Fig. 2a, b).

Histopathological analysis

Histological evaluation demonstrated a normal aspect of liver morphology in control, ORY-10, and ORY-50 supplementation groups (Fig. 3a–c). APAP group presented extensive liver injuries, characterized principally by loss of cellular structure, infiltration of inflammatory cells (fibrosis), and vascular congestion (CV) (Fig. 3d). Evaluation of the liver from mice exposed to ORY-10 and ORY-50 supplementation + APAP showed cell hepatic morphology more preserved than those from the APAP group. ORY supplementation significantly protected against ALF and markedly ameliorated the degree injuries (Fig. 3e, f).

Discussion

In the current study, ORY supplementation (ORY-10 and ORY-50) protected against oxidative and histopathological changes in ALF model APAP-induced. The overdose of APAP caused ALF through alteration of non-enzymatic (TBARS, NPSH, AA, and 4-HNE) and enzymatic markers (CAT, SOD, GPx, GR, GST, and δ-ALA-D) of OS. Still, it caused an imbalance in liver function markers (AST, ALT, ALP, GGT, and LDH) and started apoptotic process via activation of caspases (caspases 3 and 9). All changes aforementioned are corroborated by histopathological analysis.

When an APAP overdose occurs, hepatoxicity is mediated by formation of NAPQI metabolite that causes depletion in antioxidant defenses, generating OS, increased inflammatory process, and activation of apoptotic pathways (Mandal et al. 2016). NAPQI metabolite easily depletes glutathione and then binds to cellular proteins inducing toxic reactions, such as lipid peroxidation and mitochondrial dysfunction, leading to severe damage in hepatocytes (Seo et al. 2020). It is known that the lipid peroxidation is involved in ALF physiopathology (McGill and Jaeschke 2014).

Thus, in the present study, ALF was APAP-induced, causing an increase in lipid peroxidation mediated by an increase of TBARS and depletion in NPSH and AA levels. Other metabolite of lipid peroxidation is 4-HNE that accumulates in liver tissue during OS process (Zheng et al. 2014). Hence, APAP administration caused an increase in 4-HNE levels, in liver tissue of animals. The results demonstrated that ORY supplementation (ORY-10 and ORY-50) attenuated lipid peroxidation (TBARS and 4-HNE levels) and also restored NPSH and AA levels. Moreover, it is known that AA is essential for maintaining glutathione levels and also 90% of NPSH are formed by glutathione, preventing OS (Meister, 1984). Therefore, it is inferred that ORY supplementation modulated hepatic glutathione depletion, being the main mechanism involved in ALF pathophysiology induced by APAP. Similar results for parameters exposed above were found in the study of Shu et al. 2019; however, with a different dose of ORY, the effect of ORY supplementation was attributed to the activation of NRF2 pathway (pathway involved with antioxidant defenses). Furthermore, these results highlighted earlier (TBARS and 4-HNE levels) for ORY supplementation corroborate with previous studies of De Gomes et al. 2018 and De Gomes et al. 2019. Still, in relation to dose, it has been demonstrated that chronic ORY supplementation has not caused toxicological effects in animals (2 g/kg), attesting to the safety of use per oral route (Moon et al., 2016).

It is known that antioxidant enzymes function cooperatively on different tissue/cells to avoid the deleterious effects OS-caused (Ohara et al., 2001). For example, the liver is an organ which has several antioxidant enzymes (CAT, SOD, GPx, GR, GST, and δ-ALA-D) that function together to maintain redox equilibrium. However, for some enzymes to function as GPx, glutathione is an essential factor (Gupta and Rawat 2017). When glutathione is oxidized to form glutathione disulfide, is GR that reduces oxidized form by restoring glutathione with expense of NADPH. In this sense, SOD enzyme is responsible for catalyzing the dismutation of superoxide radical forming hydrogen peroxide and oxygen, while CAT or GPx catalyze hydrogen peroxide originated by SOD activity (Southorn and Powis, 1988).

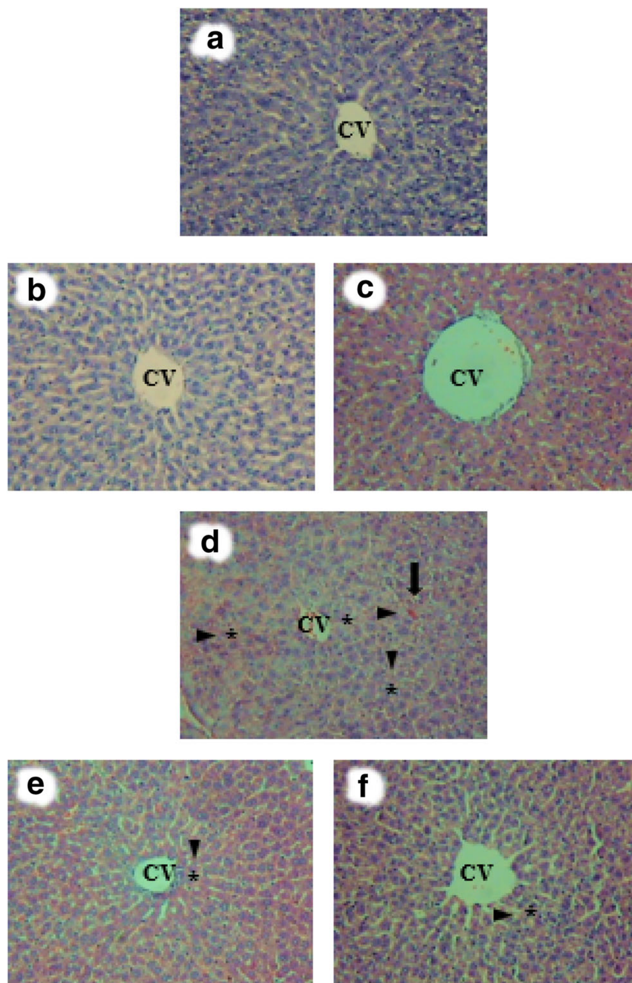

Fig. 3 Representative photomicrographs of the liver (H. E.; × 40). **a** Photomicrography of hepatic lobe segment in control group. Hepatic lobe with normal aspect and without histological damages. **b** Photomicrography of hepatic lobe segment in ORY-10 group. Hepatic lobe with normal aspect and without vascular congestion (CV). **c** Photomicrography of hepatic lobe segment in ORY-50 group. Hepatic lobe with normal aspect and without vascular congestion (CV). **d** Photomicrography of hepatic lobe segment in APAP group. One can observe around the centrilobular vein some hepatocytes with vacuolation, cell infiltration, and loss cellular structure (arrows). Characteristic damages present in the ALF. **e** Photomicrography of hepatic lobe segment in ORY-10 + APAP interaction. Supplementation prevented the characteristic histological damages present in the ALF. **f** Photomicrography of hepatic lobe segment in ORY-50 + APAP interaction. Supplementation prevented the characteristic histological damages present in the ALF

In view of above, APAP overdose caused inhibition of enzyme activities, markers of OS index studied, through glutathione depletion and free radical formation, and also generated a decrease in CAT, SOD, ALA-D, and GPx activities while increasing GR and GST activities. In the case of GR, the antioxidant defenses are required to maintain glutathione levels. This increase in GST activity is explained by conjugation with NAPQI metabolite, to make the molecule more hydrophilic, facilitating your excretion. ORY-10 supplementation was unable to protect against enzymatic activity changes in ALF model. It is inferred that ORY-10 was not enough to reestablish the glutathione levels for antioxidant enzymes, even modulating non-enzymatic markers of OS. Thus, a longer time of ORY-10 supplementation may be necessary. In the study of Panchal et al. 2017, a similar dose (ORY-10) was tested, and they used ORY in an acute treatment, discovering that the low dose was unable to restore enzymatic antioxidant defenses. On the contrary, ORY-50 supplementation was able to protect against enzymatic activity changes APAP-induced. Besides that, it prevented the decrease in activities of all enzymes and did not differ from the control group. In view of findings, ORY-50 supplementation had a strong antioxidant effect in the present study. Therefore, this effect on all enzyme markers of OS corroborates with studies of Zolali et al. 2015, De Gomes et al. 2018, Shu et al. 2019, and De Gomes et al. 2019.

One of characteristics in ALF model is the increase in enzyme activity markers of liver function. In our study, APAP caused an increase in AST, ALT, ALP, GGT, and LDH activities. It is known that a slight increase in AST, ALT, and GGT activities indicates hepatotoxicity (Chotimarkorn and Ushio 2008). Thus, APAP through NAPQI metabolite caused a toxicity to hepatocytes. ORY-10 and ORY-50 supplementation was able to protect against increased in AST, ALT, ALP, GGT, and LDH activities. In fact, this indicates that ORY supplementation promoted the functional recovery of organ. In view of aforementioned, these results are corroborated of histopathological analysis in which APAP group demonstrated extensive loss of cellular structure, vascular congestion, and cellular infiltration (hepatic fibrosis), while ORY-10 and ORY-50 interaction groups presented opposite characteristics, indicating the preservation of hepatic function (shown in Fig. 3). In the study of Shu et al. 2019, similar results for ORY supplementation were obtained for the markers of hepatic function (AST, ALT, ALP, and GGT) and histological analysis. Our results for these parameters exposed above (markers of hepatic function and histology) are in accordance with those found in studies of De Gomes et al. 2018, Shu et al. 2019, and De Gomes et al. 2019.

Overall, it is known that apoptosis is a mechanism maintained during the evolution of mammals, related to programmed cell death, and is essential for cell maintenance and development. It has already been demonstrated that apoptosis is directly involved with ALF pathophysiology induced by APAP (El-Hassan et al. 2003; Zhang et al. 2018). Generally, cell apoptosis is initiated through proapoptotic pathways, being caspases the main activator of routes. The intrinsic pathway is initiated with oxidative damage that generated a mitochondrial alteration, leading to release of cytochrome c, activation of caspase-9 (initiator) and subsequent activation of caspase 3 (terminator) (Opdenbosch and Lamkanfi 2019). In our study, APAP induced an increase of

caspase 3 and 9 activities, activating the apoptosis of hepatocytes and generating deficit of organ functionality; this result is supported by histological analysis and biochemical determinations. Our findings are in agreement with the scientific literature, which demonstrated that apoptosis is one of mechanisms in liver injury APAP-induced (Woolbright and Jaeschke 2017; Jaeschke et al. 2018; Helal and Samra 2020). ORY-10 supplementation was effective in preventing the increase in caspase activities (3 and 9), protecting mice from apoptosis of hepatocytes. A similar result for apoptotic markers was also found in the study of Shu et al. 2019; however, the ORY dose was higher (14 mg/kg). In similar manner, ORY-50 supplementation prevented the increase in caspase activities. These results (ORY-50) corroborate with the studies of De Gomes et al. 2018 and De Gomes et al. 2019. Taken the data together, ORY-10 and ORY-50 supplementation was able to attenuate ALF trough modulation of apoptotic markers. Moreover, the hepatoprotective effect presented by ORY supplementation appears to be dose dependent. Thus, ORY supplementation mitigated the OS and apoptosis in ALF model APAP-induced.

Conclusion

In present contribution, ORY supplementation (ORY-10 and ORY-50) protected against hepatoxicity in ALF APAP-induced. ORY supplementation prevented oxidative (enzymatic and non-enzymatic), apoptotic, and histological changes in the liver. Therefore, in the present study, we demonstrated pharmacological and nutraceutical effects of ORY supplementation. Thus, these beneficial effects of ORY should be explored in other models that cause hepatoxicity.

Acknowledgments Special thanks to C.R.J for making the study possible.

Author contributions M.G.G: Conceptualization and writing. L.D.F., A.R.G., L.C.S., S.P.B, and C.R.J.: experimental methodology. All listed authors have agreed to the final submission of manuscript. In addition, the authors declare that all data were generated in-house and that no paper mill was used.

Funding information This study was financially supported by the Federal University of Pampa. This study was financed in part by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) – Finance Code 001.

Compliance with ethical standards

Mice were used according to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and guidelines of the Committee on Care and Use of Experimental Animal Resources of the Federal University of UNIPAMPA, Uruguaiana, Brazil. (Protocol 021/2014).

Conflict of interest The authors declare that they have no conflicts of interest.

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