Protective effect of ISO-1 with inhibition of RIPK3 up-regulation and neutrophilic accumulation on acetaminophen-induced liver injury in mice

Running title: ISO-1 ameliorates APAP liver injury

Tatsuya Ohkawara[a]*, Naoto Okubo[a], Osamu Maehara[a], Jun Nishihira[b], Hiroshi Takeda[a]

[a]Pathophysiology and Therapeutics, Hokkaido University Faculty of Pharmaceutical Sciences, kita-12, nishi-6, kita-ku Sapporo, 060-8638, Japan

[b]Department of Medical Management and Informatics, Faculty of Business Administration and Information Science, Hokkaido Information University, Nishinopporo, 59-2, Ebetsu, 069-8585, Japan

*Corresponding author at: Pathophysiology and Therapeutics, Hokkaido University Faculty of Pharmaceutical Sciences, kita-12, nishi-6, kita-ku Sapporo, 060-8638, Japan.

E-mail address: tokawara@pharm.hokudai.ac.jp (T.Ohkawara).

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Abbreviations: ALT, alanine aminotransferase; CCl_4, carbon tetrachloride; HSP, heat shock protein; IL-8, interleukin-8; ISO-1, (S,R)-3-(4-hydroxyphenil)-4,5-dihydro-5-isoxazole acetic acid methyl ester; MIF, macrophage migration inhibitory factor; MIP-2, macrophage inflammatory protein-2; RIPK, receptor-interacting protein kinase; TUNEL,

terminal deoxynucleotidyl transferase dUTP nick end labeling.

Abstract

Overdose use of acetaminophen (APAP) often occurs a severe liver injury, and its liver injury is lethal in some cases. Macrophage migration inhibitory factor (MIF) is expressed in a variety of cells and has multifunctional roles. However, the role of MIF in APAP-induced liver injury has not been fully investigated. In this study, we investigated whether treatment with (S,R)-3-(4-hydroxyphenil)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1), a MIF inhibitor, protected mice from acute APAP-induced liver injury. Acute liver injury was induced by injection of APAP (300mg/kg body weight). Mice were treated with a single injection of ISO-1(15mg/kg body weight) 1 hour (h) before APAP administration. Histological, biochemical and molecular analyses were performed in liver of mice 12 h after APAP administration. ISO-1 remarkably improved the histological findings of APAP-induced liver injury in mice. The increases in serum levels of alanine aminotransferase (ALT), and macrophage inflammatory protein-2 (MIP-2) by APAP were inhibited by ISO-1. In addition, ISO-1 reduced the increased number of the myeloperoxidase-staining cells and that of TUNEL-positive staining cells in the liver of mice with APAP-induced liver injury. Up-regulation of hepatic receptor interacting protein kinase (RIPK)3 and heat shock protein70 by APAP was suppressed in the liver of mice given ISO-1. These results provide the additional evidence that inhibition of MIF activity may be clinically effective for treatment of acute APAP-induced liver injury.

1. Introduction

Acetaminophen (APAP) is commonly used as an analgesic and anti-pyretic drug for treatment of several kinds of diseases. However, overdose use of APAP often causes severe drug-induced hepatic damage with inflammation (Lancaster et al., 2015). Although APAP-induced liver injury is usually improved by stopping APAP administration, severe APAP-induced liver injury is lethal in some cases (Lancaster et al., 2015). In the treatment of APAP-induced liver injury, several studies have been investigated, and N-acetylcysteine has been developed and used (Lancaster et al., 2015). However, some patients are resistant to current therapy. Thus, an additional treatment is expected.

Previous studies have been elucidated the mechanism by which APAP develops liver injury (Fantana et al., 2014; Lancaster et al., 2015; Stephens et al., 2014). In APAP-induced hepatotoxicity, oxidative stress induced by N-acetyl-*p*-benzoquinone imine (NAPQI) converted from APAP plays a causative role in the development of hepatocyte damage (Hinson et al., 2010). On the other hand, inflammatory mediators such as cytokines and chemokines are focused on their role for the development of APAP-induced liver injury (Blazka et al., 1996; Bourdi et al., 2002; Dambach et al., 2006; Pires et al., 2014). For instance, IL-8, as a neutrophilic chemoattractant, is up-regulated in the patients with APAP-induced liver injury (James et al., 2001 and 2005). In animal studies, macrophage inflammatory protein-2 (MIP-2, mouse IL-8 homologue) gene therapy attenuated acute APAP-induced liver injury, and over dose of APAP administration induce MIP-2 production in the liver of mice (Hogaboam et al., 1999; Lawson et al., 2000).

Macrophage migration inhibitory factor (MIF) was at first identified as a cytokine-derived activated T lymphocyte in vitro, and its various biological functions have been

elucidated since the cloning of MIF cDNA (Weiser et al., 1989). Interestingly, MIF plays an essential role in the processes of inflammation and cell proliferation in various diseases such as rheumatoid arthritis and inflammatory bowel diseases (Harris et al., 2019; Morand et al., 2005; Nishihira and Mitsuyama, 2009). In liver diseases, circulating and local MIF levels are increased in the patients with alcoholic and viral-infectious hepatitis (Kumagi et al., 2001; Zhang et al., 2002; Zhao et al., 2014). Previous studies using an anti-MIF antibody therapy or using MIF-deficient mice have clearly indicated that inhibition of MIF activity is beneficial for hepatic inflammation and its tissue damage (Bourdi et al., 2002; Kimura et al., 2006; Kobayashi et al., 1999; Nakajima et al., 2006). Recently, (S,R)-3-(4-hydroxyphenil)-4,5-dihydro-5-isoxazole acetic acid methyl ester (ISO-1) has been developed to inhibit MIF activity and used in various experiments including inflammatory disease models (Al-Abed and VanPatten, 2011). For instance, ISO-1 ameliorates the severities of experimental colitis, neuritis and sepsis (Al-Abed et al., 2005; Dagia et al., 2009; Yu et al., 2019). In carbon tetrachloride (CCl_4)-induced liver injury, treatment with ISO-1 improves tissue damage in the liver of mice (Xie et al., 2016), suggesting that ISO-1 is effective for protection of the other chemical-induced liver injury such as APAP. Thus, in this study, to develop the therapeutic option using ISO-1 in APAP-induced liver injury, we investigated the effect of ISO-1 on APAP-induced liver injury. Furthermore, we tried to clarify the additional role of MIF in APAP-induced liver injury.

2. Materials and Methods

2.1. Animals

Eight week-old male C57B6 mice were obtained from CLEA Japan Inc. (Tokyo, Japan).

All mice were bred and housed in standard cages in a well climinate-controlled room with comfortable temperature and adequate light-dark cycle. All procedures in animal experiments were strictly conducted in accordance with the guidelines of the Hokkaido University Institutional Animal Care and Use Committee under an approved protocol (13-0181).

2.2. ISO-1 treatment

At first, ISO-1 (Focus Biomolecules, PA) was dissolved in DMSO, and the solution was diluted in phosphate buffered saline (PBS) solution. Mice were intraperitoneally injected with ISO-1 (15mg/kg) in 0.2mL solution 1 hour before initial administration of APAP. In non-ISO-1-treated group, mice were injected with 0.5%DMSO solution diluted in PBS as a vehicle (0.2mL). To assess the effect of 0.5%DMSO solution on liver injury, the mice in another group were treated with PBS(0.2mL) solution only.

2.3. Induction of liver injury

To induce acute liver injury by APAP (Nakarai Tesque Inc., Kyoto, Japan), mice were intrapenitoreally injected with APAP (300mg/kg body) dissolved in PBS solution. Twelve hours after injection of APAP, liver tissues were removed, and blood samples were collected. The blood samples were coagulated at 4 ^{o}C overnight, and serum samples were separated from the blood samples after centrifugation at 3000g for 15 min. Collected serum samples and tissue samples for molecular analysis were stored at -20 ^{o}C until use.

2.4. Histological evaluation

Removed liver tissues were cut to adequate size, and the samples were fixed in 10% neutral buffered formalin solution for a few days. Next, fixed samples were embedded in paraffin blocks. Thin slice samples of liver tissues were stained with hematoxylin and eosin (H&E). In histological examination, the severity of liver injury was scored using modified scoring system on a scale of 0-5 (0: none lesion, 1: necrotic cells seen at a single cell layer adjacent to the central veins, and hyaline degeneration, 2: necrotic cells seen within 3 cells layers from the central veins, 3: necrotic cells seen at more than 3 layers from the central veins and limited within peripheral distribution, 4: the same as score 3, but with necrotic cells extending to another central veins, 5: more severe than score 4, with extensive centrilobular cell death throughout the section) as described previously (Pires et al., 2014).

2.5. Measurement of serum alanine aminotransferase and MIP-2

Serum levels of alanine aminotransferase (ALT) were determined with the Transaminase C2-test kits (Wako, Osaka, Japan) according to protocol. Serum levels of MIP-2 were determined with Quantikine ELISA kits (R&D systems Inc., Minneapolis, MN) according to protocols.

2.6. Total glutathione in liver tissue

The frozen liver tissue was homogenazied in cold PBS (10%wt/volume) and the supernatant was collected by centrifuge. The collected supernatant was deproteinated by 5% metaphosphoric acid solution and measured for the level of glutathione (GSH) concentration with GSH assay kit (Northwest Life Science Specialities, Vancouver,WA) according to the protocol.

2.7. Myeloperoxidase staining in liver tissues

A thin slice of liver tissue on a slide glass was deparaffined and incubated with an antigen activator (Nichirei Science, Tokyo) at 95 ºC for 20 min. After washing with Tris buffered saline (TBS) solution, the sample on the glass was incubated with 0.3% H_2O_2 at room temperature (RT) for 10min. Subsequently, the sample was incubated with an anti-myeloperoxidase (MPO) antibody (dilution ratio, 1:300) at 4 ºC over night following twice TBS washing. And then, the sample was further incubated with Histfine simplestain MAX-PO® (Nichirei Bioscience, Tokyo) at RT for 30min. The incubated sample was reacted with 3,3'-Diaminobenzidine Tetrahydrochloride at RT for 5min. Finally, the sample was stained with hematoxylin. The number of MPO-positive staining cells was counted in high power field (100x) at a light microscope. Three areas of liver in each mouse were evaluated in 5 mice.

2.8. Immunostaining for F4/80 in the liver

Immunohistochemistry for F4/80 was analyzed using a Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA) as previously described (Ohkawara et al., 2008). In brief, the shin slice samples of paraffin-embedded liver tissues were incubated with 3% H_2O_2 for 10 min at 4 ºC and given 10% normal goat serum for 30 min at RT followed by overnight incubation with the anti-F4/80 antibody (diluted 100:1, Biosource, Camarillo, Calif., USA) at 4 ºC. F4/80-positive staining was visualized with diaminobenzidine as a chromogen. After F4/80 staining, the number of positive stained cells was counted in high power field (100x) using a light microscope. Three areas of liver in each mouse were evaluated in 5 mice.

2.9. TUNEL staining in liver tissue

The paraffin-embedded liver tissue sections were dewaxed, rehydrated and treated with protease. Then, In Situ Cell Death Detection Kit (Roche, Mannheim, Germany) was used to identify apoptotic cells in the liver tissues by the TUNEL analysis according to the manufacture's protocol. For evaluation, the number of TUNEL positive staining cells was counted in a high power field (100x) at a light microscope.

2.10. Evaluation of Receptor Interacting Serine Threonine Kinase 3 and heat shock protein 70 in liver

The liver tissue in Lysis buffer 3 (Cloud-Clone Corp. Houston, TX, USA) was homogenated with Shakeman homogenizer (BMS, Tokyo, Japan), and the supernatant was isolated and collected from the homogenated sample by centrifugation for 5 min at 10,000 g. The level of RIPK3 in the collected supernatant was measured with Enzyme-linked immunosorbent assay (ELISA) kits for RIPK3 (Cloud-Clone Corp. Houston, TX, USA) according to the manufacture's protocol. On the other hand, the level of HSP70 in the supernatant was measured with ELISA kits for HSP70 (Enzo Biochem. Inc., New York, NY, USA) according to the manufacture's protocol.

2.11. Statistical analysis

All results were shown as average ± standard error. One analysis of variance (ANOVA) was performed followed by Turkey's test. Values were considered as significant at $p<0.05$.

3. Results

3.1. Effect of ISO-1 on APAP induced liver injury

Histological findings were evaluated in the liver of mice. Vehicle- or ISO-1-treated mice had no histological findings of liver injury without APAP administration (Fig. 1A, B). Vehicle-treated mice showed the severe liver injury in mice 12h after APAP administration. Histological findings showed the severe hepatocyte destruction and infiltration of inflammatory cells into the liver tissues (Fig.1A). In the histological scores of liver damage, the scores in the PBS- treated mice and in the vehicle- and APAP-treated mice were significantly increased compared to the mice without APAP administration (4.4 ±0.24 and 4.25±0.48, respectively). (Fig. 1B). On the other hand, 15mg/kg and 30mg/kg of ISO-1 markedly reduced the severity of liver injury of mice given APAP (0.75±0.48 and 1.20 ±0.20 respectively, $p<0.01$ vs. APAP- and vehicle-treated mice) (Fig.1A, B).

The serum levels of ALT were remarkably increased in mice treated with PBS or vehicle 12h after APAP administration (2073.3±410.8 and 2220.9±113.4 IU/L, respectively) (Fig.1C). On the other hand, 15mg/kg and 30mg/kg of ISO-1 significantly inhibited the increases of serum ALT level in mice 12 after APAP administration (106.5 ±10.6 U/L and 115.8±19.9 IU/L, respectively, $p<0.01$ vs. APAP-and vehicle-treated mice) (Fig.1C).

3.2. ISO-1 restores the depletion of hepatic GSH by APAP

Hepatic GSH levels were decreased in the liver of mice 12 h after APAP administration compared to the mice given only vehicle (1.28±0.23 and 8.71±1.59 μmol/g tissue, respectively, $P<0.01$) (Fig.2). ISO-1 (15mg/kg) recovered the hepatic GSH levels in mice

given APAP with a significance (6.54±1.25 μmol/g tissue, $p<0.05$ vs APAP- and vehicle-treated mice)(Fig.2). Only ISO-1 treatment did not alter the hepatic GSH levels compared to the mice given vehicle (9.30±3.51 μmol/g tissue)(Fig.2).

3.3. ISO-1 inhibits the infiltration of neutrophils by APAP but not F4/80 macrophage in the liver

To evaluate the content of inflammatory infiltration in liver, MPO-staining and immunohistochemistry for F4/80 were performed in the liver tissues. The number of MPO-positive staining cells remarkably was increased in the liver of mice 12 h after APAP administration (69.2 ±1.7/HPF, $p<0.01$ vs. vehicle-treated mice) (Fig. 3A, B). On the other hand, ISO-1 (15mg/kg) treatment reduced the number of infiltrated MPO-positive staining cells in the liver of mice 12h after APAP administration compared with the mice given vehicle (5.2 ±1.7/HPF, $p<0.01$ vs. APAP- and vehicle-treated mice) (Fig. 3A, B). In F4/80 staining, APAP administration tented to decrease the number of F4/80 positive staining cells infiltrated in the liver (25.0±1.3/HPF), but there was statistically no significant difference compared to the mice without APAP administration (Fig. 3C, D). ISO-1 also essentially unchanged the number of F4/80 positive staining cells in the liver (34.2 ±3.8/HPF) (Fig. 3C, D).

3.4. ISO-1 suppresses the increase of circulating level of MIP-2 by APAP

To assess the effect of chemoattractant migrating neutrophils, the serum level of MIP-2 was measured. The MIP-2 levels were markedly increased in the serum of mice 12h after APAP administration (238.8 ±3.1 pg/mL, $p<0.01$ vs. vehicle-treated mice) (Fig.4). On the other hand, ISO-1 significantly suppressed the increased serum levels of

MIP-2 by APAP (114.0 ± 1.3 pg/mL, $p<0.01$ vs. APAP- and vehicle-treated mice) (Fig. 4).

3.5. ISO-1 inhibits the increase of TUNEL-positive staining cells by APAP in liver

In order to further evaluate the liver damage, TUNEL staining was performed in the liver tissue of mice. Considerable increase of TUNEL-positive staining cells was mainly observed in the hepatic lobule of mice 12h after APAP administration (76.2±10.9/HPF, $P<0.01$ vs. only vehicle-treated mice) (Fig. 5A, B). On the other hand, ISO-1 markedly reduced the increased number of TUNEL-positive staining cells by APAP in the liver of the mice (5.0±0.8/HPF, $p<0.01$ vs. APAP- and vehicle-treated mice) (Fig. 5A, B).

3.6. ISO-1 inhibits the up-regulation of RIPK3 by APAP in liver

Based on our results in TUNEL study, to further analyze the hepatocyte death process by APAP, hepatic RIPK3 level was measured by ELISA. The levels of hepatic RIPK3 were increased in mice 12 hours after APAP administration (393 ± 79 μg/mg protein, $p<0.01$ vs. vehicle-treated mice) (Fig. 6). On the other hand, ISO-1 significantly inhibited the increased hepatic RIPK3 levels by APAP administration in mice (185 ± 33 μg/mg protein, $p<0.01$ vs. APAP- and vehicle-treated mice) (Fig. 6).

3.7. ISO-1 increases the hepatic level of HSP70 in liver of mice given APAP

The levels of HSP70 which is known to have a cytoprotective effect were measured in the liver tissues by ELISA. APAP markedly increased the hepatic HSP70 levels in vehicle-treated mice (272.1±57.4 and 33.1±3.1 ng/mg protein, respectively, $p<0.05$)(Fig. 7). On the other hand, pre-treatement with ISO-1 inhibited the up-regulation of hepatic

HSP70 levels in mice with APAP-induced liver injury (76.9±13.8 ng/mg protein, $p<0.05$vs. APAP- and vehicle-treated mice) (Fig. 7).

4. Discussion

In this study, we found that ISO-1 suppressed the severity of acute liver injury induced by APAP in mice. ISO-1 clearly improved the severity of histological hepatic damage and biochemical parameter induced by APAP in mice. Furthermore, ISO-1 inhibited increase of neutrophilic infiltration in the liver of mice given APAP. ISO-1 suppressed up-regulation of circulating MIP-2 known as a neutrophilic chemoattractant. In addition, ISO-1 reduced the increased number of TUNEL-positive hepatocyte and suppressed the up-regulation of RIPK3 expression in the liver from mice treated with APAP. Our results suggest that the regulation of MIF activity by ISO-1 is beneficial for control of APAP-induced liver injury.

MIF has been first reported to be an inhibitor of random migration of macrophages and plays an essential role for the development and enhancement of inflammatory process (Nishihira, 2000). Up-regulation of MIF expression is observed in the patients and animals with various liver diseases (Kimura et al., 2006; Kobayashi et al., 1999; Kumagi et al., 2001; Zhang et al., 2002; Zhao et al., 2014). Blockade of MIF bioactivity with an anti-MIF antibody ameliorates the severe hepatic damage in acute hepatic failure induced by BCG and lipopolysaccharide in mice (Kobayashi et al., 1999). Additionally, MIF-deficient mice were resistant to hepatic damage induced by APAP or concanavalin A and exhibit the less severity of APAP-induced liver injury (Bourdi et al., 2002; Nakajima et al., 2006). On the other hand, *in vivo* and *in vitro* studies have demonstrated that ISO-1 known as a MIF inhibitor has an anti-inflammatory effect (Al-Abed et al., 2005; Al-Abed

and VanPatten, 2011; Dagia et al., 2009; Xie et al., 2016; Yu et al., 2019). In a mouse model of liver injury induced by CCl_4, ISO-1 reduced the severity of liver damage (Xie et al., 2016). Consistent with the previous *in vivo* study, in this study, we found that ISO-1 improved the histological findings of severe liver injury and inflammatory infiltration induced by APAP in mice. Moreover, the increase of serum ALT level by APAP was suppressed in mice treated with ISO-1. Our histological and biochemical results clearly showed the effect of ISO-1 on APAP-liver injury.

In the previous study using MIF deficient mice in APAP-induced liver injury, MIF deficient mice were resistant to APAP-induced lethal and showed the inhibition of serum ALT up-regulation (Bourdi et al. 2002). Additionally, the expressions of several cytokines were not suppressed in the liver of MIF deficient mice given APAP. Bourdi et al. suggested that one of the mechanisms by which the deletion of MIF reduced APAP-induced liver injury was due to induction of heat shock proteins (HSPs). ISO-1 induced HSP70 in the liver of mice given APAP. HSP is an early stress responsive protein protecting the cells and tissues from hepatocyte damage (Tolson et al. 2006, Nishida et al., 2006). In addition, HSP70 deficient mice exhibited the more severe liver injury by APAP compared to WT mice (Martin-Murphy et al.,2010). Our previous study also demonstrated that the induction of HSP was enhanced in MIF deficient mice in experimental colitis (Ohkawara et al. 2006). In this study, we found that APAP markedly up-regulated the induction of HSP70 in liver of mice. Conversely, ISO-1 remarkably inhibited the up-regulation of HSP70 expression in liver of mice treated with APAP. Although we have not fully clarified the reason why the results using a MIF antagonist was different from the previous results in MIF deficient mice in APAP-induced liver injury, amelioration of stressors against hepatocytes such as oxidative stress by ISO-1

may suppress the induction of HSP70 because HSPs are induced by stress response. Our results of HSP70 suggest that ISO-1 may protect from liver damage by APAP independent of cytoprotective effect of HSP. We herein could not elucidate the discrepancy on HSP induction between MIF inactivation by ISO-1 and complete endogenous MIF deletion. Further study is needed.

In APAP-induced hepatotoxicity, the reduction of hepatic GSH level by NAPQI metabolized from APAP results in liver injury via oxidative stress (Fontana et al., 2014; Lancaster et al. 2015; Stephens et al., 2014). In previous studies, MIF acts as a glutathione-s-transferase (GST) (Blocki et al., 1993; David, 1993). In a cardiac ischemia/reperfusion model, deletion of MIF increased oxidized GSH in heart (Koga et al., 2011). However, our study showed that ISO-1 moderately recovered the reduction of hepatic GSH level in mice with APAP-induced liver injury. Although our results were discontent with the previous study, our data suggest that various experimental designs may be different such as time to take a sample and the way to induce oxidative stress, Moreover, ISO-1 may indirectly affect the GSH levels in liver, or may affect with independence of MIF activity. However, in this study we could not fully clarify the reason why ISO-1 recovered the hepatic GSH depletion. Further study is needed.

To further evaluate the contents of inflammatory infiltration, in this study, we assessed the infiltration of neutrophils and macrophages in liver. Lee *et al.* showed that infiltration of MPO-staining cells into the liver were elevated in APAP-induced mice (Lee et al., 2018). In this study, we confirmed that APAP administration markedly increased the number of MPO-positive staining cells in the liver. Moreover, ISO-1 treatment significantly inhibited the infiltration of MPO-staining cells into the liver of mice with APAP-induced liver injury. However, several studies have indicated that neutrophils do

not primarily contribute to the development of hepatic damage and that influence the regeneration of liver tissue (Jaeschke et al., 2020). Neutrophils cleans up debris such as damage-associate molecular patterns (DAMPs). Thus, neutrophilic function is controversial in APAP-induced liver injury. Although our study did not elucidate the effect of ISO-1 on the function of neutrophils in APAP-induced liver injury, ISO-1 may suppress the further expansion of liver damage by modulating excessive infiltration of neutrophils into liver in APAP-induced liver injury.

In F4/80 positive staining cells, Kobayashi *et al.* found that suppression of MIF by anti-MIF antibody could not change the infiltration of F4/80-positive staining cells in the liver in mouse models of BCG and LPS-induced fulminant hepatitis (Kobayashi et al., 1999). In this study, the infiltration of F4/80 positive staining cells tended to reduce in the liver tissue of mice in APAP- and vehicle-treated mice and restored in mice given ISO-1. In liver tissue, F4/80 presenting cells are known to be mainly Kupffer's cells. The study by Zigmond et al. showed that APAP decreased the number of resident Kupffer's cells in liver at early acute phase, and that monocyte-derived macrophages affected the damage of liver tissue at the resolution phase (Zigmond et al., 2014). Compared to the report, our observation in F4/80 staining may be only finding of mild reduction of Kupffer's cells in liver at early acute phase of APAP challenging. Thus, our study could not conclude that ISO-1 has any effect on the macrophage infiltration in APAP-induced liver injury because we did not evaluate the expression and function of another macrophage. Further study is needed to clarify the contribution to the monocytes and macrophages in APAP-induced liver injury.

In several disease models, ISO-1 suppresses the up-regulation of inflammatory cytokines and chemokines (Al-Abed and VanPatten, 2011). Especially, treatment with

ISO-1 remarkably inhibits the increasing level of IL-8 in endometriosis in mice (Knoufache et al., 2012). IL-8 mainly enhances neutrophilic infiltration into the lesions in inflammatory diseases. In animal models, inhibition of MIF activity reduces the expression of MIP-2. Deletion of MIF in mice dose not up-regulate the expression of MIP-2 in colitis and arthritis (Ichiyama et al., 2004; Ohkawara et al., 2008). Takahashi *et al.* have demonstrated that ISO-1 treatment decreased MIP-2 levels in alveolar in pulmonary inflammation (Takahashi et al., 2009). In this study, ISO-1 suppressed the increased circulating MIP-2 level in mice given APAP, suggesting that the inhibition of MIF activity by ISO-1 reduced the severity of APAP-induced liver injury partly via suppression of serum MIP-2 level. Thus, the results from MPO staining and MIP-2 suggest that modulation of MIF activity by ISO-1 down-regulates neutrophilic infiltration with inhibition of MIP-2 secretion. However, our study has not fully clarified the role of MIP-2 down-regulation by ISO-1 in APAP-induced liver injury. Although further study is needed to clarify these phenomenon, down-regulation of MIP-2 may partly affect the further expansion of hepatic damage by APAP.

In addition to analysis of inflammatory process in this study, we evaluated the effect of ISO-1 on protection from cell damage in the liver of mice given APAP. TUNEL analysis reflects the degree of DNA fragmentation to assess cell death such as apoptosis. ISO-1 suppresses the increase of TUNEL-positive staining cells induced by recombinant MIF treatment in cardiomyocyte (Dhanantwari et al., 2008). Consistent with the previous study, in this study, ISO-1 decreased the increase in the number of TUNEL-positive staining cells in mice given APAP. Our results suggest that ISO-1, at least, directly or indirectly attenuates the cell death in the liver in mice with APAP-induced liver injury.

However, in our observation, the morphological findings in the liver of mice given

APAP overdose seemed to be necrotic findings with cell swelling and rupture. Several studies have suggested that not only necrosis but also necroptosis occurred in APAP-induced liver injury (Liao et al., 2020; Ramachandran et al., 2013). Necroptosis is known to be programed cell death with morphological similarity to necrosis. In molecular analysis, RIPK3 plays an important role in the development of necroptosis (Lee et al., 2018; Ramachandram et al., 2013). Escecially, Ramachandran *et al.* have shown that RIPK3 is overexpressed in the liver of mice with APAP-induced liver injury, and that down-regulation of RIPK3 by morpholinos or RIPK3-deficient mice show the less severity of liver injury by APAP (Ramachandram et al., 2013). Recently, Soppert *et al.* have demonstrated that co-treatment with soluble CD74 and recombinant MIF decreased living myofibroblast cells via activation of RIPK3 (Soppert et al., 2018). In this study, consistent with the previous study, we found that the hepatic RIPK3 levels were increased in the mice with APAP-induced liver injury. Interestingly, ISO-1 treatment suppressed the up-regulation of RIPK3 in the liver of mice given APAP. Compared with the study by Soppert *et al.*, our observation may have some differences in the effect of MIF on the regulation of RIPK3. Compared to the previous study, there are some differences in our experimental designs including *in vivo* study, target organs and inducers. Although our current data did not fully elucidate the mechanism by which ISO-1 inhibited the increase of TUNEL-positive cell death and the up-regulation of RIPK3 in mice with APAP-induced liver injury, our results are suggestive in the roles of ISO-1 and MIF in necroptosis in APAP-induced liver injury.

In this study, our findings of the effect of ISO-1 on APAP-liver injury have the limitation to develop the clinical application of MIF inhibitors such as ISO-1 in APAP-induced liver injury. Our current experiments in this model are pre-treatment study. Thus,

for the development of clinical implication, the study concerning the effect of the treatment of ISO-1 after APAP administration on liver injury is needed.

In summary, we demonstrated that ISO-1 protected mice from APAP-induced liver injury, suggesting that inhibition of MIF activity is effective for treatment of APAP-induced liver injury. ISO-1 inhibited the up-regulation of RIPK3, HSP70 and the neutrophilic immune response in APAP-induced liver injury. Although the treatment with ISO-1 after APAP challenge is needed to develop the clinical implication with MIF inactivators, treatment with a MIF inhibitor such as ISO-1 is expected to be an option for APAP-induced liver injury.

Conflict of interest

The authors declare no conflict of interest regarding the study.

Author contributions

T.O., J.N. and H.T. conceived and conducted the experiments. T.O., N.O. and O.M. performed animal experiments. T.O., N.O., O.M. and H.T. performed the molecular experiments and analyzed the results. All authors reviewed the manuscript.

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Figure Legends

Fig. 1. Inhibition of histological hepatic damage by ISO-1 treatment in mice with APAP-induced liver injury. (A) The representative picture of liver tissue. The slices of liver tissues were stained with H&E. magnification x 100. (B) Histological scores in the liver tissues. Data were shown as the mean ± SE. n=5. (C) The serum levels of alanine aminotransferase (ALT) were shown. The serum ALT levels were measured by the method described in the Materials and Methods. The values were shown as the mean ± SE. APAP; acetaminophen-treatment, vehicle; solution without ISO-1, ISO-1; solution with ISO-1. *$p<0.01$ vs. only vehicle-treated mice

Fig. 2. The effect of ISO-1 on the depletion of GSH in liver of mice with APAP-induced liver injury. The hepatic GSH levels were measured by the method described in the Materials and Methods section. The values were shown as the mean ± SE. n=5. APAP; acetaminophen-treatment, vehicle; solution without ISO-1, ISO-1; solution with 15mg/kg of ISO-1. *$p<0.05$ vs. only vehicle-treated mice. **$p<0.05$ vs. vehicle- and APAP-treated mice

Fig. 3. The effect of ISO-1 on infiltration of myeloperoxidase (MPO) and F4/80 staining cells in liver of mice with APAP-induced liver injury. The cell count was performed in 3 fields in 5 mice. (A) The representative picture in the MPO-positive staining cells into the liver tissues (magnification x 100). (B) The number of MPO-positive staining cells infiltrating into the liver tissues in the high power field. (C) The representative pictures in the F4/80-positive staining cells into the liver tissues (magnification x 100). (D) The number of F4/80-positive staining cells infiltrating into

the liver tissues in the high power field. The values were shown as the mean ± SE. APAP; acetaminophen-treatment, vehicle; solution without ISO-1, ISO-1; solution with 15mg/kg of ISO-1. *p<0.01 vs. only vehicle-treated mice

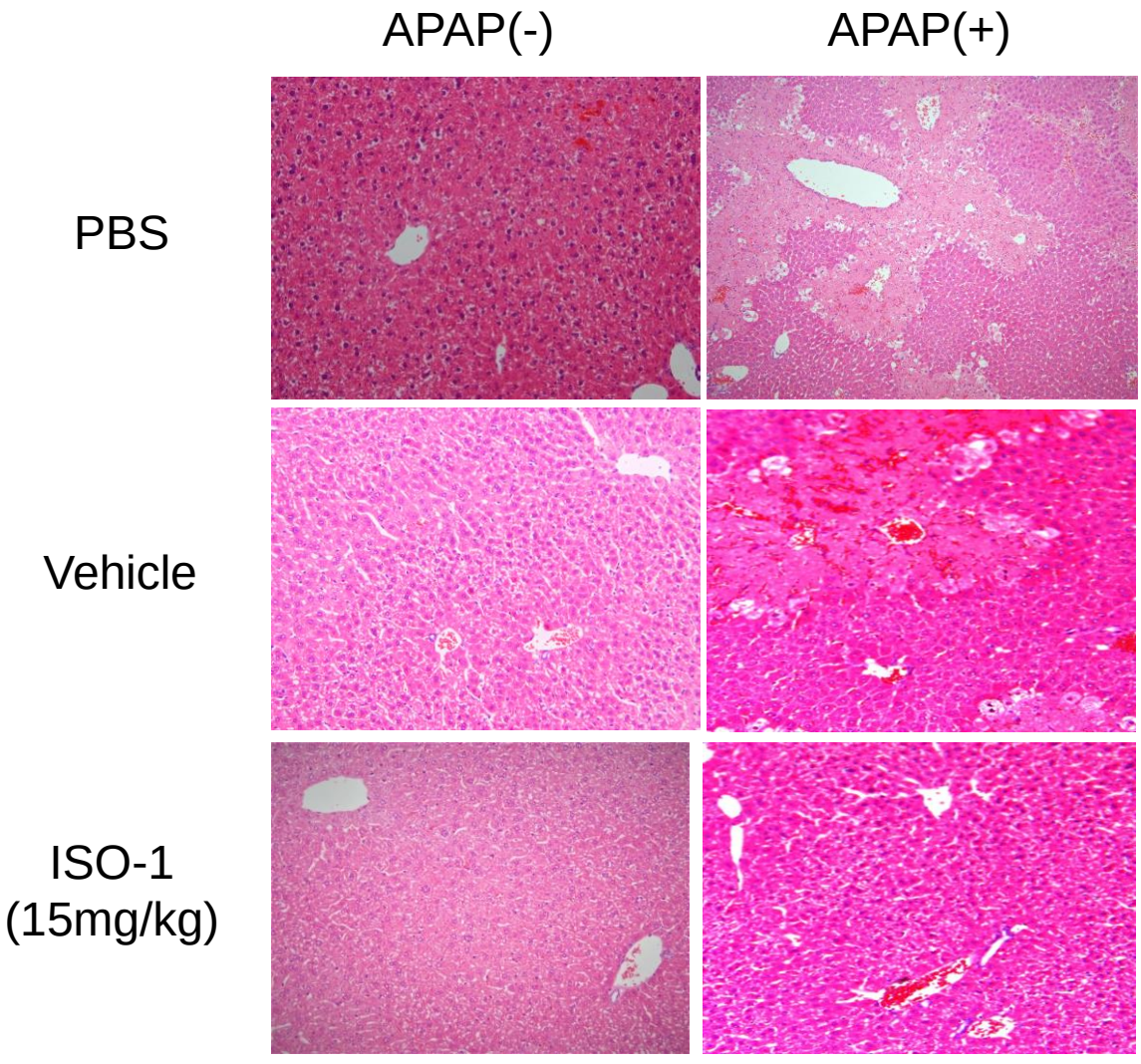
Fig. 4. The effect of ISO-1 on serum levels of MIP-2 in mice with APAP-induced liver injury. The serum levels of MIP-2 were measured in 5 mice in each group by the methods described in the Materials and Methods section. The values were shown as the mean ± SE. APAP; acetaminophen-treatment, vehicle; solution without ISO-1, ISO-1; solution with 15mg/kg of ISO-1. *p<0.01 vs. only vehicle-treated mice

Fig. 5. The effect of ISO-1 on the up-regulation of TUNEL-positive staining in liver of mice with APAP-induced liver injury. TUNEL staining was performed in 5 mice in each group by the methods described in the Materials and Methods section. (A) Representative pictures of TUNEL staining were shown in the liver of mice. (B) The numbers of TUNEL positive staining cells in the liver in the high power field. The data were shown as the mean ± SE. APAP; acetaminophen-treatment, vehicle; solution without ISO-1, ISO-1; solution with 15mg/kg of ISO-1. *p<0.01 vs. only vehicle-treated mice

Fig. 6. The effect of ISO-1 on the hepatic RIPK3 up-regulation by APAP in liver of mice. Hepatic RIPK3 levels were measured by ELISA in 5 mice in each group by the methods described in the Materials and Methods section. The data were shown as the mean ± SE. APAP; acetaminophen-treatment, vehicle; solution without ISO-1, ISO-1; solution with 15mg/kg of ISO-1. *p<0.01 vs. only vehicle-treated mice

Fig. 7. The effect of ISO-1 on the hepatic HSP70 levels in liver of mice. Hepatic HSP70 levels were measured by ELISA in 4-5 mice in each group by the methods described in the Materials and Methods section. The data were shown as the mean ± SE. APAP; acetaminophen-treatment, vehicle; solution without ISO-1, ISO-1; solution with 15mg/kg of ISO-1. *$p<0.05$ vs. only vehicle-treated mice

A



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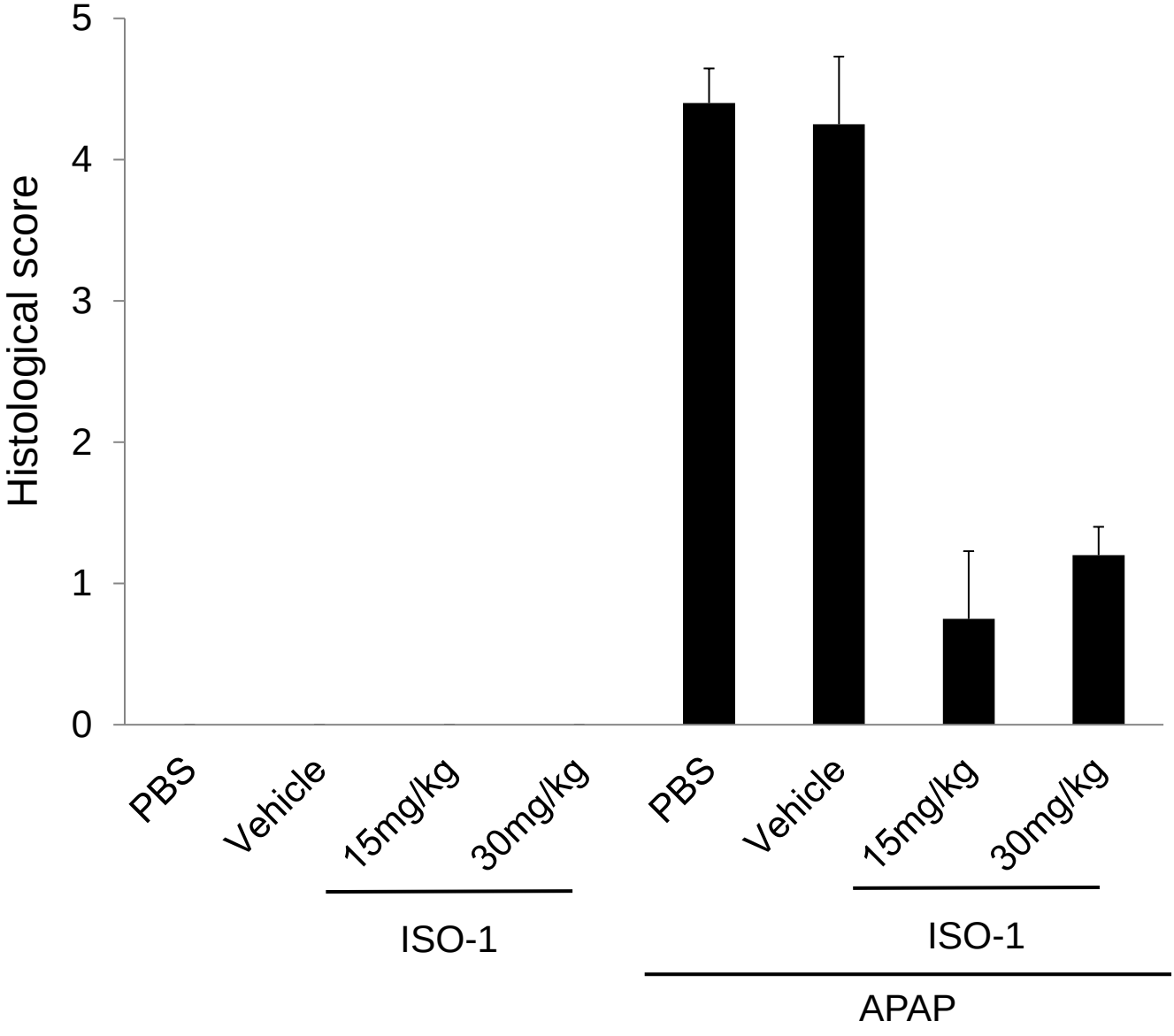
Histological score
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4
3
2
1
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15mg/kg
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PBS
Vehicle
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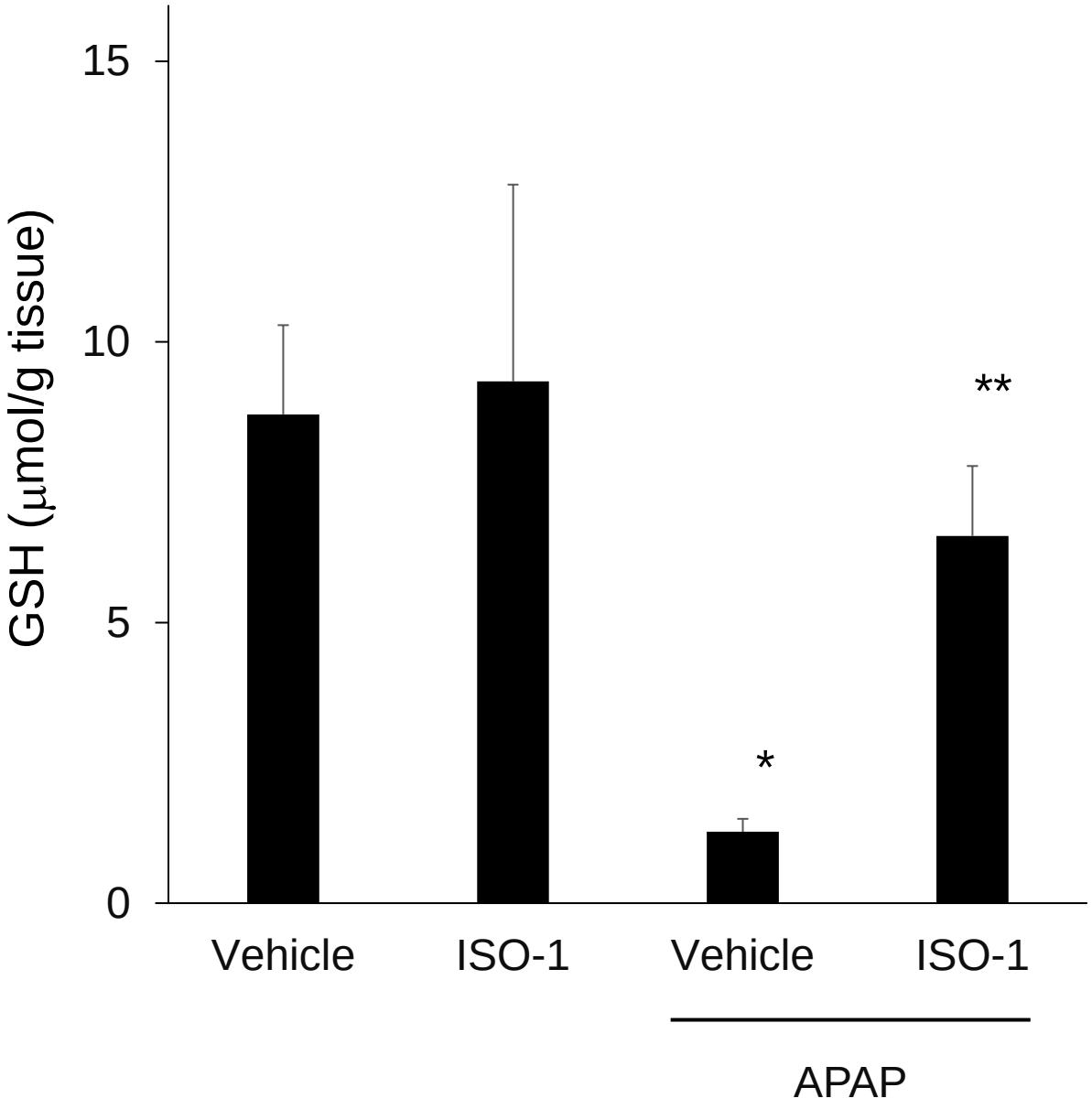
ISO-1
ISO-1
APAP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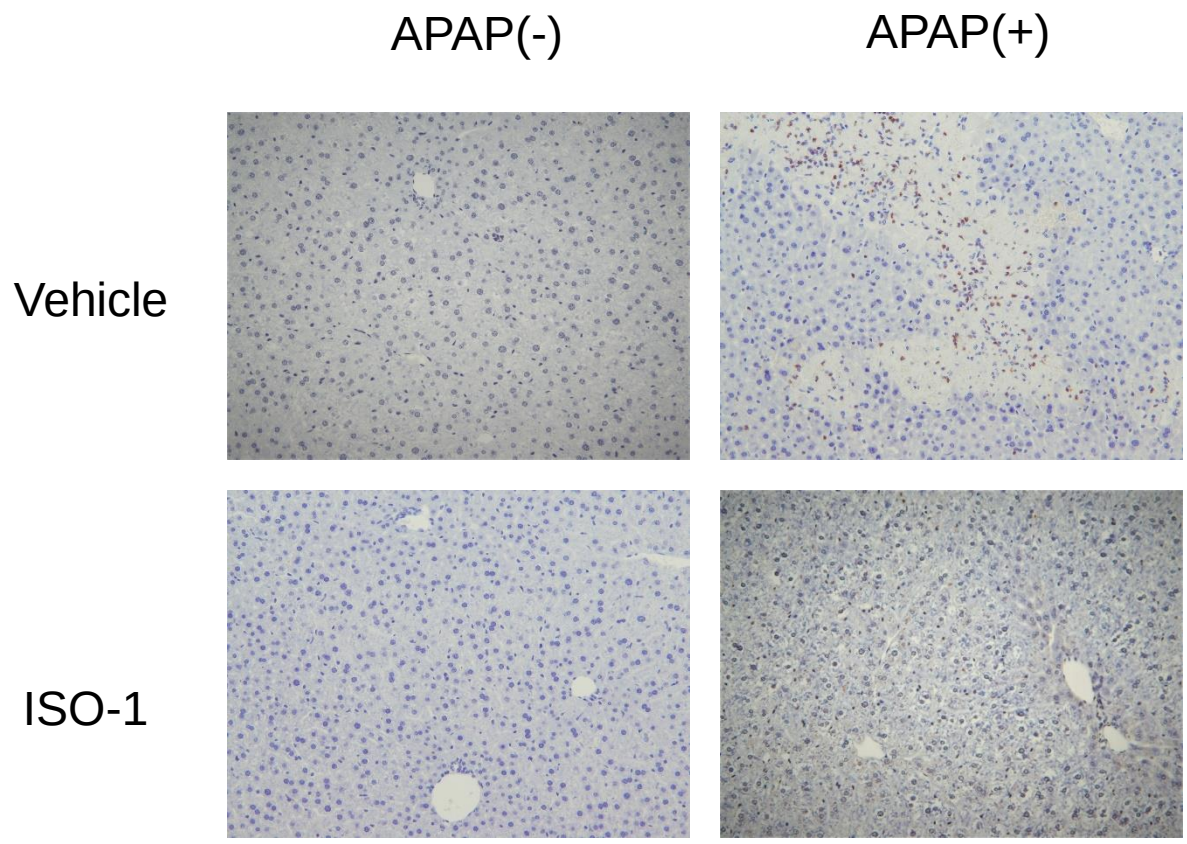
A



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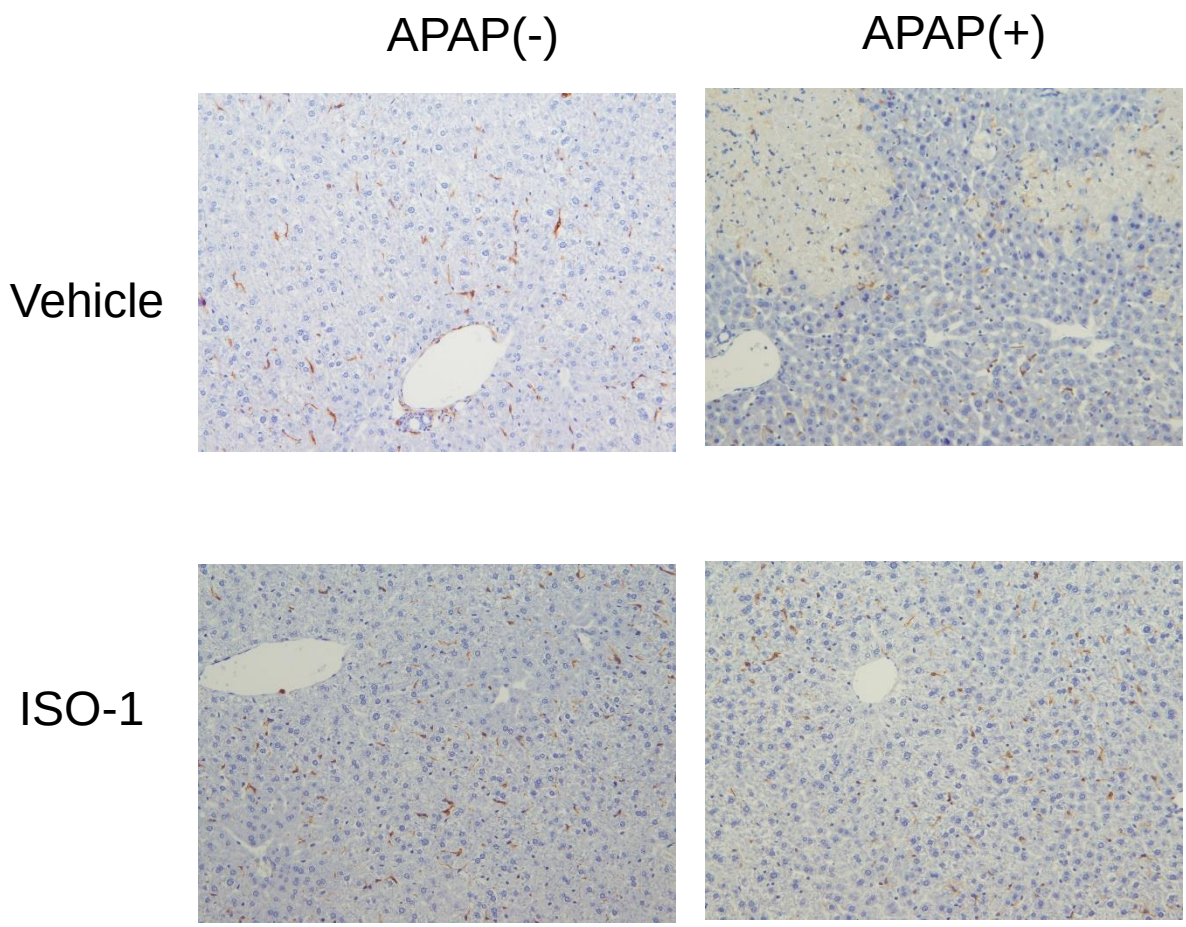

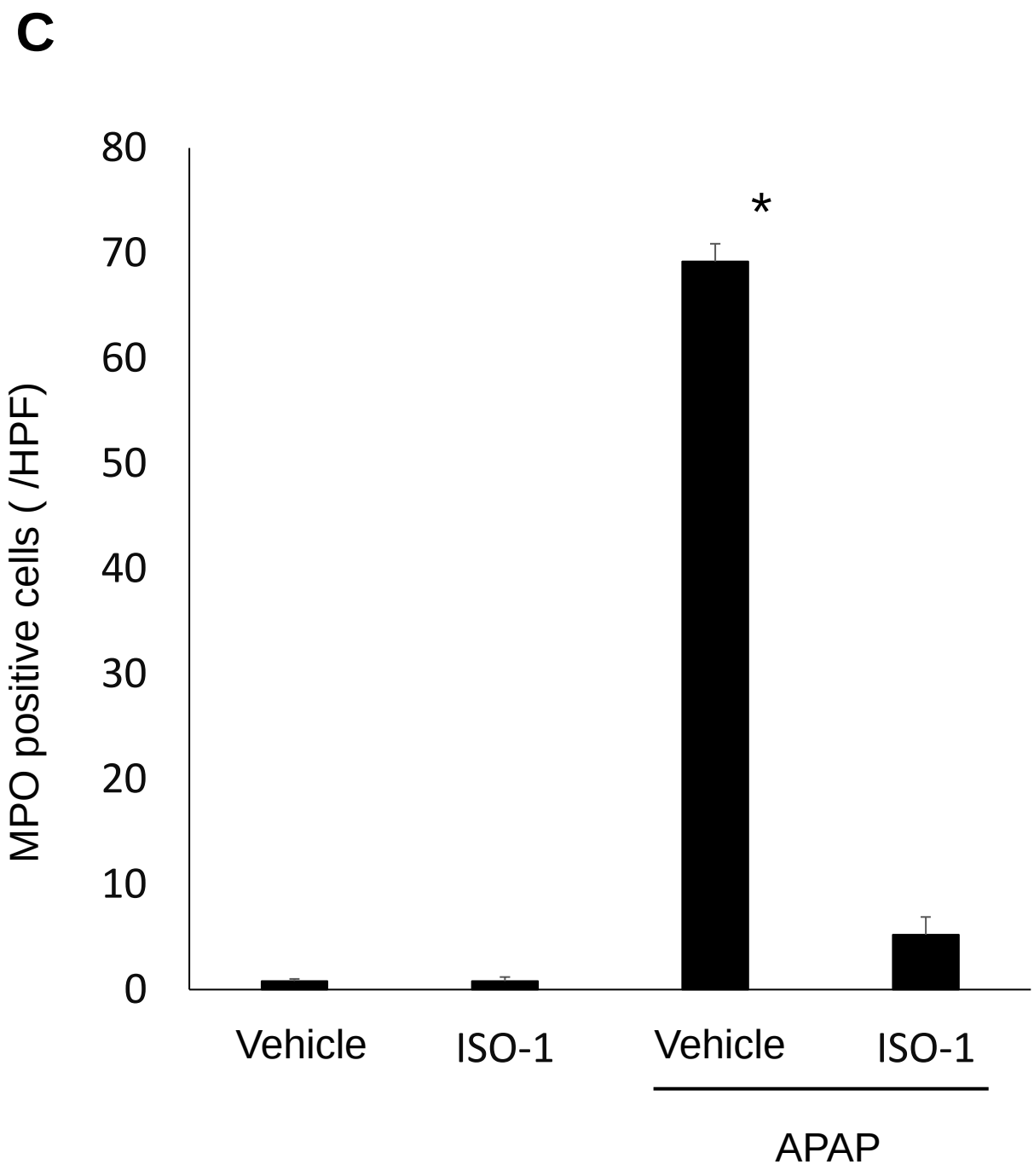
C
80
70
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MPO positive cells (/HPF)
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Vehicle
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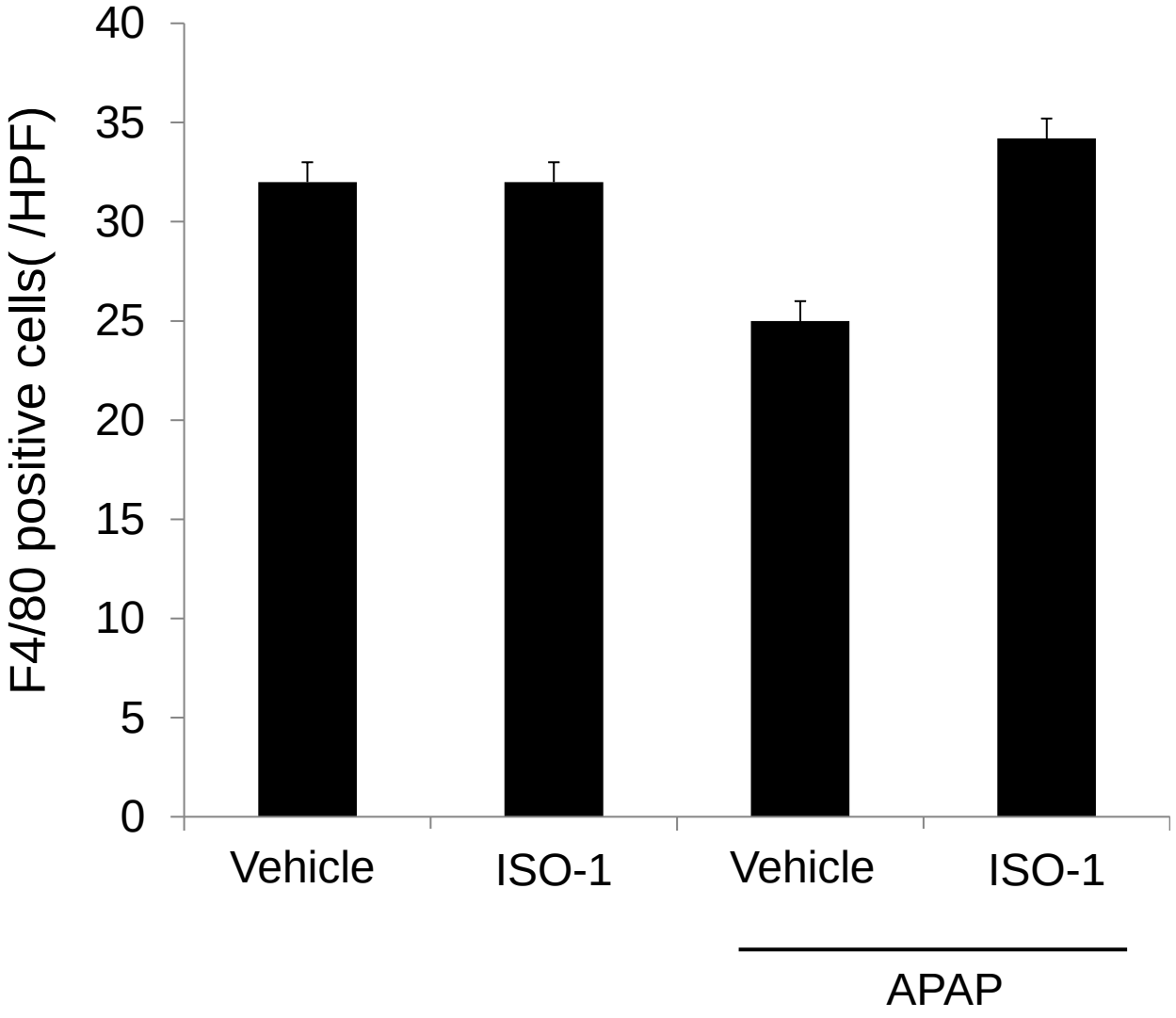
ISO-1
APAP

D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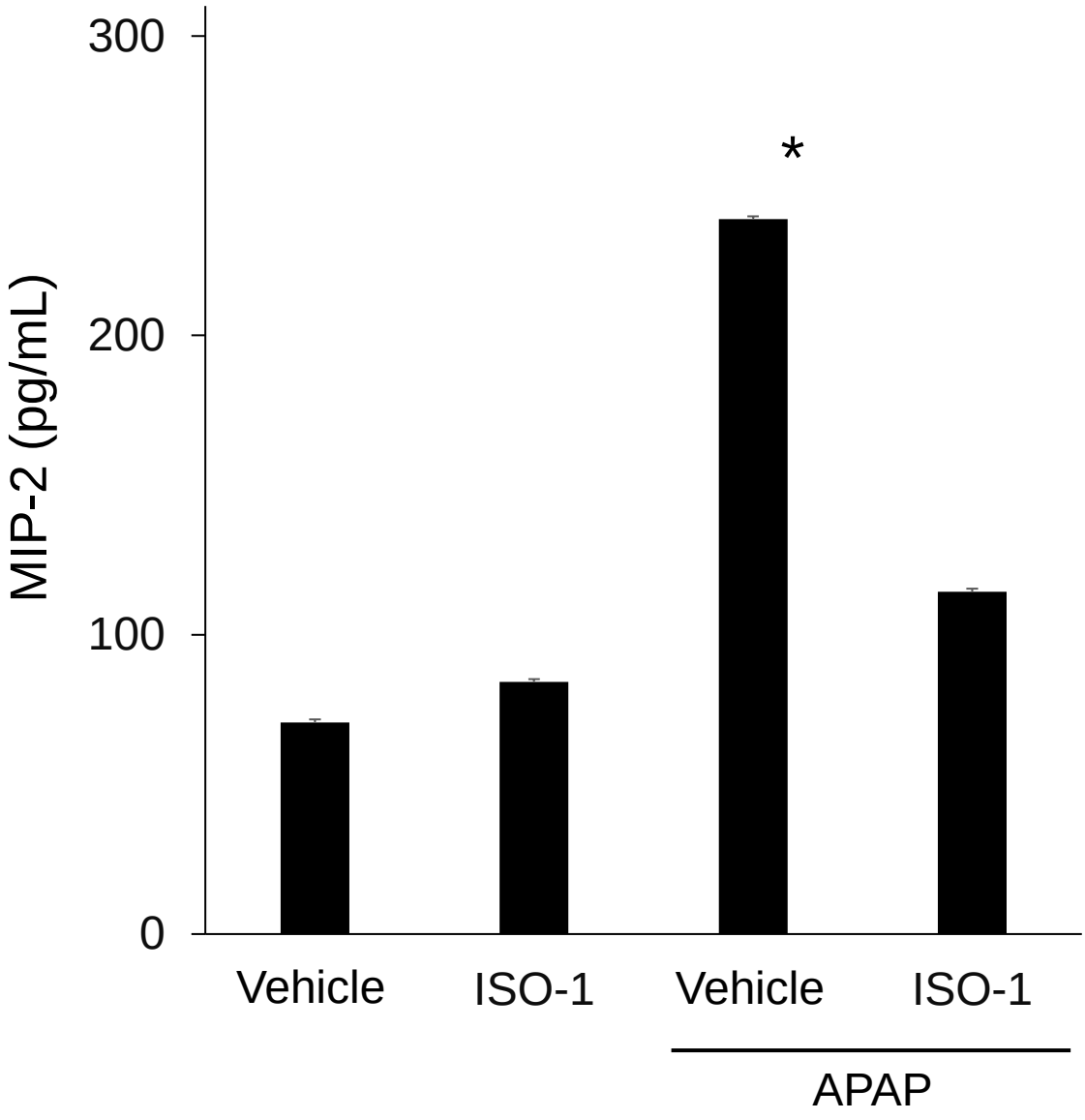
Vehicle
ISO-1
Vehicle
ISO-1
APAP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MIP-2 (pg/mL)
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Vehicle
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ISO-1
APAP

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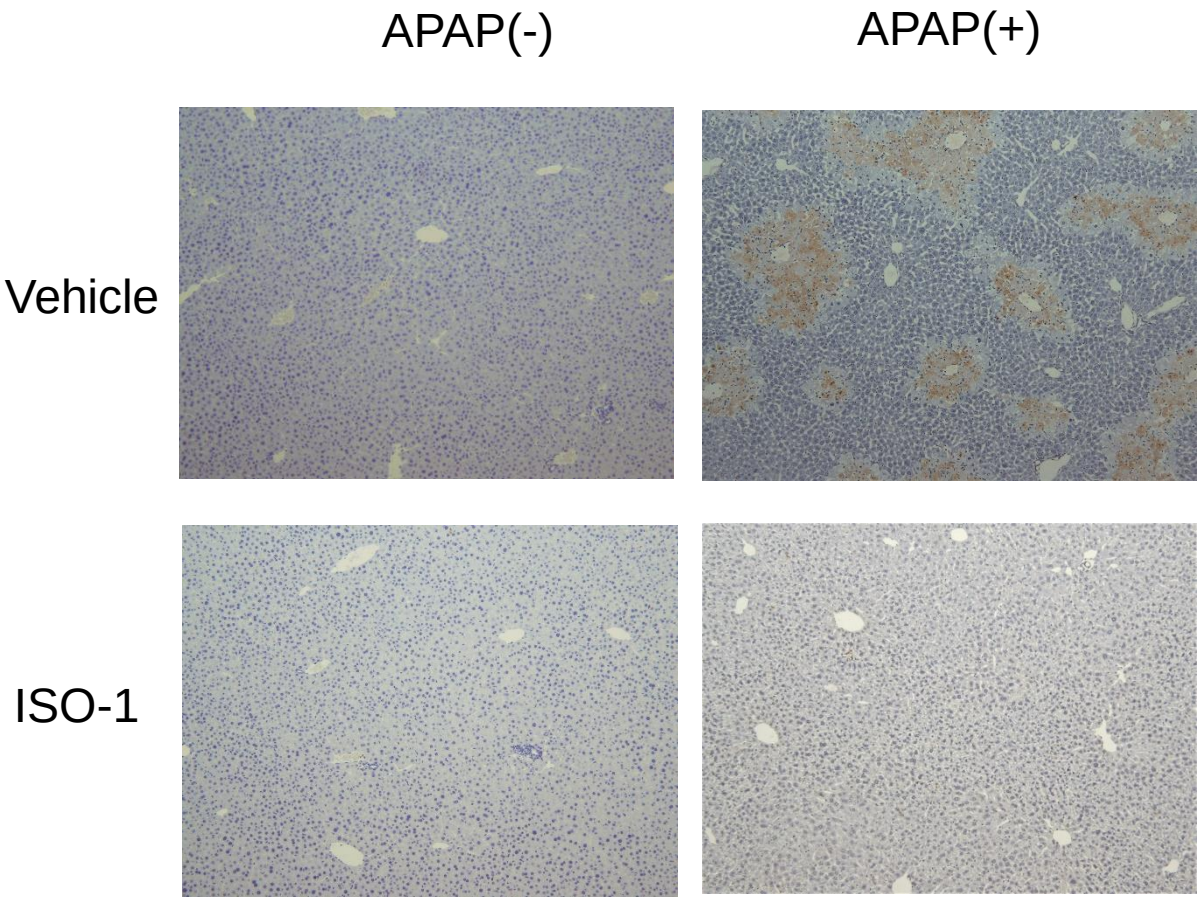

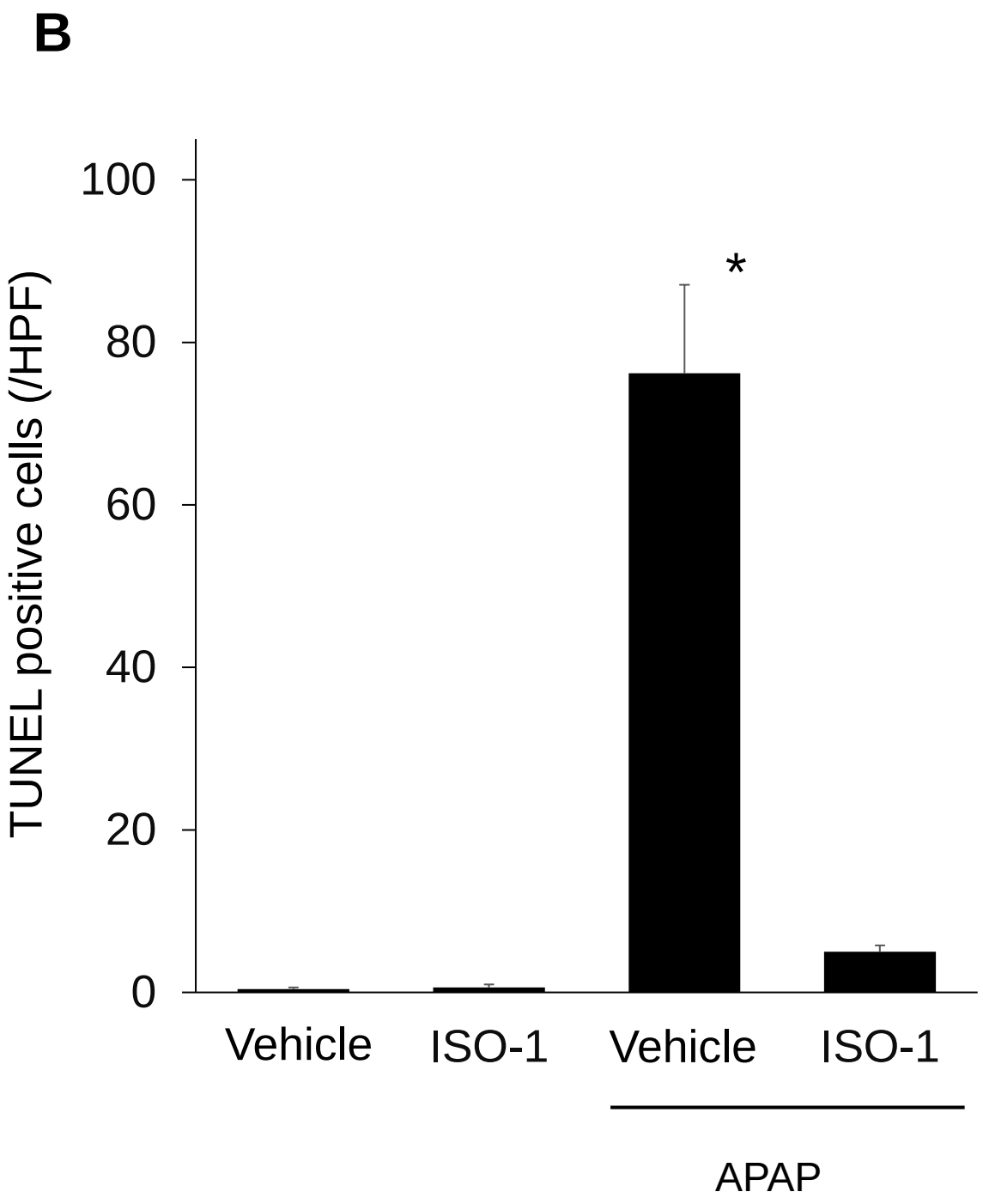
B
TUNEL positive cells (/HPF)
100
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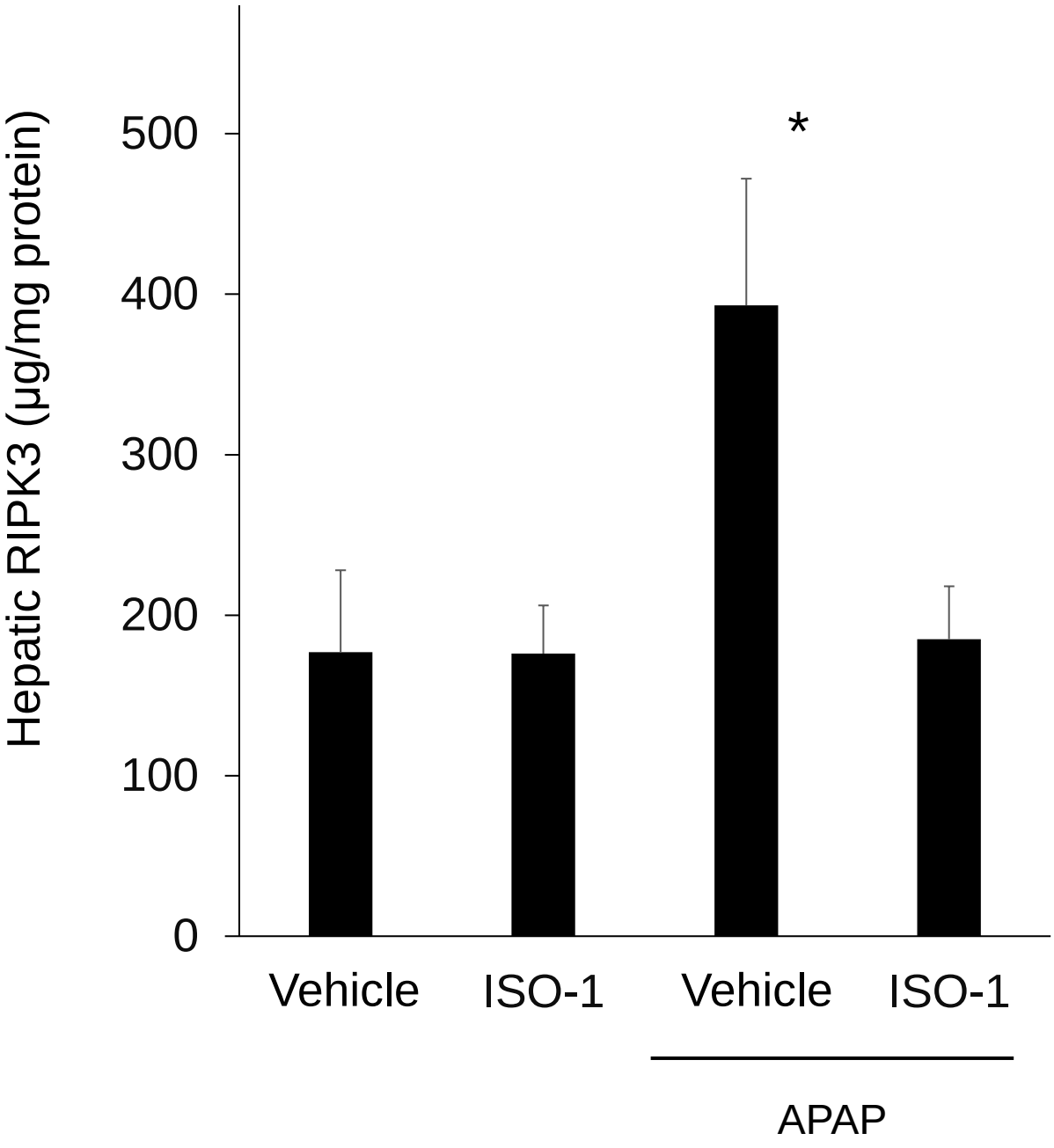
*
Vehicle
ISO-1
Vehicle
ISO-1
APAP


Hepatic RIPK3 (μg/mg protein)
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400
300
200
100
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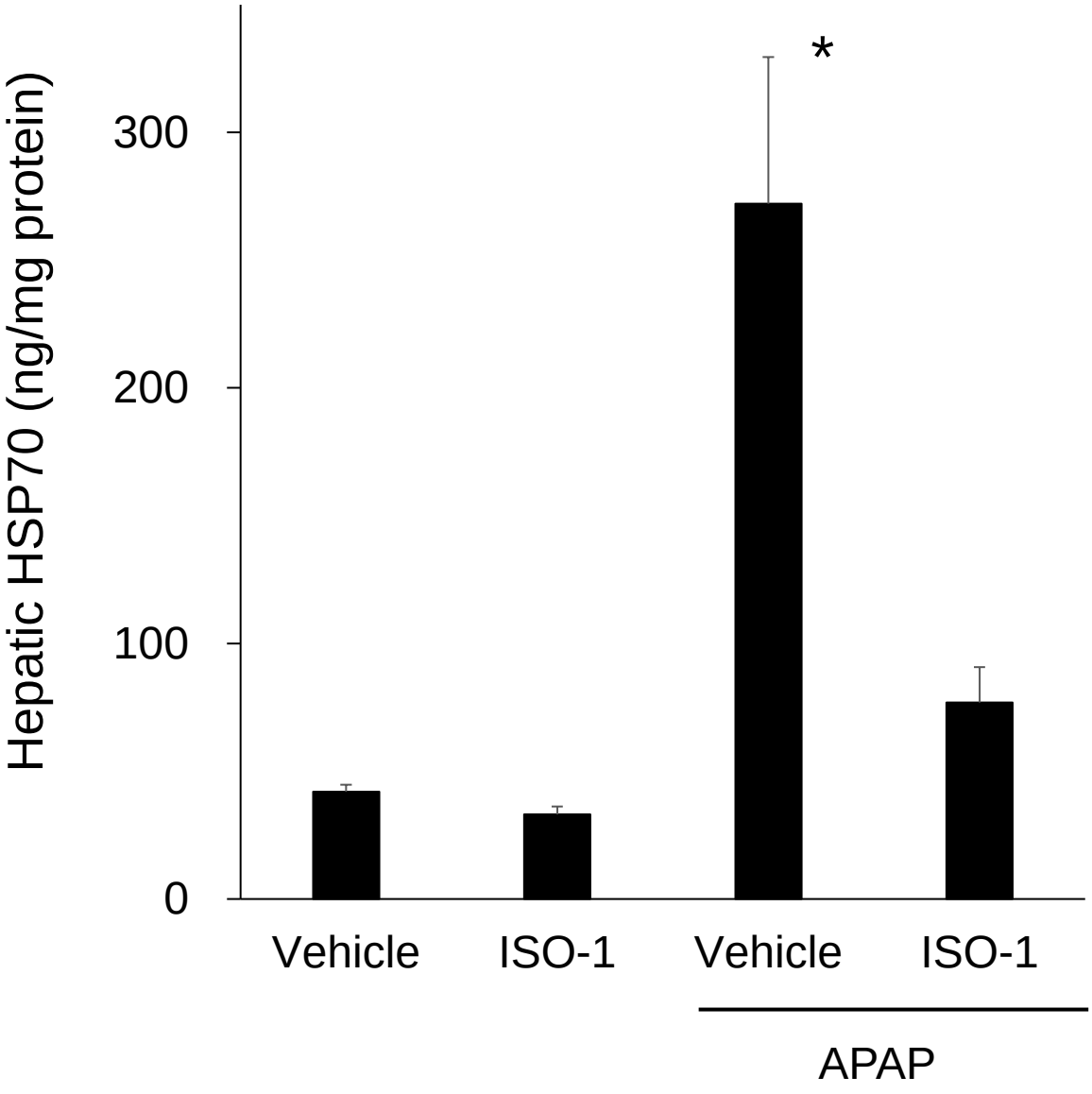
Vehicle
ISO-1
Vehicle
ISO-1
APAP


Hepatic HSP70 (ng/mg protein)
300
200
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Vehicle
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ISO-1
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