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Running title: EPO and iron improves liver regeneration

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Co-administration of erythropoietin and iron complex improves late-phase liver regeneration

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ABSTRCAT

Erythropoietin and iron has individually shown some beneficial effects on early-phase liver regeneration following partial hepatectomy (PHx); however, there are limited data on their combined effect on late-phase liver regeneration after PHx. Here we examined the combined effects of recombinant human erythropoietin (rhEPO, 3,000 IU/kg) and iron isomaltoside (IIM, 40 mg/kg) on late-phase liver regeneration following PHx and investigated the possible underlying mechanism. Rats administrated with rhEPO showed significantly higher liver mass restoration, interleukin-6 (IL-6, a hepatocyte mitogen) levels, and Ki-67-positive hepatocytes on day 7 after PHx than saline-treated controls. These beneficial effects were further enhanced on days 7 and 14 by co-treatment with IIM. This combination also significantly improved the liver function indices, such as increased the albumin production and decreased the bilirubin levels, but did not alter the serum levels of toxic parameters, such as aspartate transaminase and alanine transaminase. This study demonstrated that the combination of rhEPO and IIM synergistically improves the late-phase liver regeneration and function after PHx, probably by promoting IL-6-mediated hepatocyte proliferation without adverse effects. Therefore, this combination treatment can be considered as a potential therapeutic strategy for patients undergoing resection for hepatic malignancies.

INTRODUCTION

Liver regeneration is a coordinated and homeostatic process that rapidly recoups lost functional liver mass, allowing the performance of extensive resection as a curative option for patients with primary or secondary hepatic cancers. However, as excessive resection can increase the risk of postoperative liver failure (1, 2) reducing or minimizing further damage to the remnant liver is essential. In addition, it is also important to successfully stimulate the regenerative function and capability of the remaining liver tissue after hepatectomy by exogenously supplying or endogenously inducing mitogenic and growth factors (3-7).

There are 2 types of cells responsible for liver regeneration: liver stem cells and hepatocytes. Liver stem cells participate in compensatory regeneration of the liver damaged by viral and chemical insults, and hepatocytes are important for liver growth or restoration after resection. Because hepatocytes, as fully differentiated and quiescent cells, exist in the G_0 phase of the cell cycle (8), liver regeneration after resection requires a priming event that would allow hepatocytes to re-enter the cell cycle from the G_0 to the G_1 phase, subsequently leading to hepatocyte proliferation. Interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and hepatocyte growth factor (HGF) are potential priming cytokines (9); however, the liver regeneration process can be triggered by multiple signaling cascades that are activated by coordinated actions with other cytokines, growth factors, and matrix remodeling, and are regulated by several positive and negative cross-talks among growth-related signals (8, 9).

The hematopoietic growth factor erythropoietin (EPO) not only stimulates erythropoiesis in the bone marrow (10) but also has other multiple functions, including anti-apoptotic and hepatoprotective actions (11). In addition, administration of recombinant human EPO (rhEPO) stimulates hepatic proliferation and liver regeneration after partial hepatectomy (PHx) (3-5). Similarly, iron supplementation has also been shown to increase hepatocyte

proliferation after PHx (6), probably by enhancing the catalytic activity of ribonucleotide reductase (12). Although rhEPO and iron have individually shown to improve early-phase liver regeneration in hepatectomized rats, their combination effect and underlying mechanism have not been clearly investigated in late-phase liver regeneration after PHx.

The objective of this study was to assess the combined effects of rhEPO and iron isomaltoside (IIM, an iron-carbohydrate complex) on late-phase liver regeneration, function, and toxicity in a rat model of 70% PHx, because, to our knowledge, this has never been done. Our results will assist in the clinical development of a therapeutic strategy for hepatic failure resulting from a small remnant liver volume.

RESULTS

Co-administration of rhEPO and IIM promotes late-phase liver restoration

The control remnant livers reached approximately 72% of the relative liver weight on day 7 after PHx, and this recovery effect was further increased by treatment with rhEPO alone and strongly augmented by the co-administration of rhEPO and IIM (Fig. 1). On day 14 after PHx, the regeneration rate of control livers reached approximately 81%, and this rate was slightly increased, but not significantly, by treatment with rhEPO alone and significantly enhanced by co-administration of rhEPO and IIM (Fig. 1). Their combination also slightly increased both hemoglobin and hematocrit levels by about 110% (11.94 ± 0.07 vs. 12.98 ± 0.35 g/dl and 38.83 ± 0.48 vs. 42.38 ± 1.07%) only on day 14 compared with untreated control, but did not alter the numbers of white blood cells and platelets. Collectively, these results show that rhEPO promotes late-phase liver restoration after PHx and that this effect is further augmented by co-treatment with IIM.

Combination of rhEPO and IIM prolongs late-phase hepatocyte proliferation

To compare the relationship between liver regeneration and hepatocyte proliferation in rats co-administrated with rhEPO and IIM following PHx, we performed immunohistochemical examination of the expression of the proliferation marker Ki-67 in liver tissues. On day 7 after PHx, only a few Ki-67-positive hepatocytes appeared in liver tissues from control hepatectomized rats, whereas the Ki-67-positive index was significantly increased by rhEPO compared with control, and this effect was further augmented by co-administration of rhEPO and IIM (Fig. 2A and 2B). However, Ki-67-positive cells were barely or insignificantly detected in all experimental groups on day 14 after PHx (Fig. 2A and 2B). These results suggest that co-administration of rhEPO and IIM potentially stimulates and prolongs hepatocyte proliferation, thus contributing to improvement of late-phase liver regeneration after PHx.

Co-treatment with rhEPO and IIM maintains high levels of IL-6 but not HGF

Among many cytokines and growth factors, IL-6 and HGF are major priming factors of hepatocyte proliferation (13, 14). We determined the serum levels of IL-6 and HGF in hepatectomized rats. The serum HGF levels were not significantly altered by the administration of rhEPO alone or by the combination of rhEPO and IIM on days 7 and 14 after PHx compared with those in both sham and saline controls (Fig. 3A). However, the serum IL-6 levels were increased, but not statistically significant, by rhEPO in hepatectomized rats on day 7 after surgery, and further significantly enhanced by co-treatment with rhEPO and IIM; however, these effects disappeared on day 14 (Fig. 3B). The present data suggest that co-administration of rhEPO and IIM maintains high serum IL-6 levels during the late phase of liver regeneration.

Co-treatment with rhEPO and IIM improves late-phase liver function

The activities of the hepatic injury marker enzymes aspartate transaminase (AST) and alanine

aminotransferase (ALT) were slightly increased, but not statistically significantly, in the sera of hepatectomized rats on days 7 and 14, compared with those in sham animals, and these enzyme levels were not altered by the administration with rhEPO or by the combination of rhEPO and IIM (Fig. 4A and 4B). We next examined the serum levels of albumin and total bilirubin, as markers of liver function. On day 7 after PHx, the serum levels of albumin were decreased in hepatectomized control rats compared with those in sham animals, and this decrease was partially recovered, but not significantly, by treatment with rhEPO alone and restored to sham control levels by the co-administration of rhEPO and IIM (Fig. 4C). Conversely, the serum levels of total bilirubin were significantly elevated in hepatectomized control animals on day 7, and this increase was slightly reduced by the administration of rhEPO and further decreased to sham control levels by the co-treatment with rhEPO and IIM (Fig. 4D). However, no significant differences in the serum levels of albumin and total bilirubin among all groups were observed on day 14 after PHx (Fig. 4C and 4D). These results suggest that co-administration of rhEPO and IIM effectively restores late-phase liver function in hepatectomized rats.

DISCUSSION

On the basis of the understanding of the mechanisms of liver regeneration, a number of successful strategies have been developed to counteract postoperative liver insufficiency or post-hepatectomy liver failure (15-17). Despite a successful hepatectomy, insufficient remnant liver volume and function are central factors in the etiology of fulminant liver failure after major liver resection (18). Therefore, some studies have proposed potential therapeutic treatments, such as high-dose insulin therapy and portal vein embolization, which effectively increase the regenerative capacity of the remaining liver after resection, subsequently improving the outcome of patients undergoing liver surgery (16, 17).

Among many possible strategies, a number of growth factors, cytokines, and drugs have

been considered as potential candidates for enhancing the proliferative capability of remnant parenchymal hepatocytes (3-7). There is some evidence showing that supplementation with either rhEPO or iron improves early-phase liver regeneration and function in hepatectomized rats (3-6, 19); however, the combined effect of rhEPO and iron on liver regeneration has not been studied, particularly in the late phase of liver regeneration after PHx. Similarly to previous studies in the early phase of liver regeneration (3-5), our study shows the beneficial effect of rhEPO on hepatocyte proliferation and liver mass restoration in the late phase of liver regeneration following PHx. More notably, the effect of rhEPO was further improved by co-administration with IIM, suggesting a synergistic action between rhEPO and IIM in late-phase liver regeneration.

Because hepatocytes are quiescent, highly differentiated cells, they are not normally proliferative (8). Special stimulants are required for priming hepatocytes to re-enter the cell cycle into the G_1 phase from the G_0 phase, and to initiate their proliferation (9). Priming and auxiliary factors activate hepatocytes in the remnant liver to promote the proper progression of the cell cycle by triggering the signaling pathways of cell proliferation. These pathways are known to be stimulated by several cytokines and growth factors, including IL-6, TNF-α, and HGF, which are endogenously produced after hepatectomy (13, 14). Therefore, inadequate production and maintenance of those factors delay liver regeneration and can cause hepatic failure. This unfavorable phenomenon can be overcome by means of exogenous supplementation of hepatocyte-activating factors such as rhEPO and IL-6 (3, 13). The activity of rhEPO in liver regeneration was reported to be synergistically or additively elevated by co-treatment with the antioxidant curcumin (7), suggesting that there are several modes of action, e.g., positive or synergistic cooperation, among the growth-related signaling pathways. Our data showed that the combination of rhEPO and IIM maintains high levels of circulating IL-6 in the late phase of liver regeneration, which plays a pivotal role in priming hepatocytes for proliferation both in the early and late phases of liver regeneration (8, 20). This suggests that the combination of rhEPO and IIM potentiates late-phase liver

regeneration and function through a mechanism of hepatocyte proliferation associated with the long-term maintenance of high IL-6 levels after PHx.

Hepatocytes specifically synthesize albumin, urea, and α-fetoprotein, as well as take up bilirubin from blood circulation, which subsequently conjugates with glucuronic acid and is finally secreted into bile (21). Indeed, serum albumin levels were decreased by an impaired liver function in hepatectomized mice (22), whereas serum bilirubin levels were increased by insufficient liver mass and function (23). Thus, changes in the serum levels of albumin and total bilirubin are considered potential indices of the progression of liver regeneration after PHx. Our data showed that rhEPO resulted in an increase in serum albumin levels and a decrease in serum total bilirubin levels in the late stage of liver regeneration, and their circulating levels were further restored to the levels of the sham control group by the co-administration of IIM. These results suggest that the combination of rhEPO and IIM promotes hepatic maturation and function during the late phase of liver regeneration (Fig. 4D).

As the hepatocytic amino acid-metabolizing enzymes AST and ALT are secreted under conditions of liver injury or damage, the circulating activities of both enzymes have been used as indicators of liver injury and toxicity. In the present study, we found that rhEPO alone or in combination with IIM did not significantly increase the serum levels of AST and ALT in the late phase of liver regeneration after PHx, suggesting that the combination treatment does not induce any hepatotoxicity. Therefore, we conclude that co-administration of rhEPO and IIM stimulates hepatocyte proliferation and maturation during the late phase of liver regeneration without causing hepatotoxicity.

A number of studies have shown that rhEPO treatment is safe and well tolerated in patients with anemia and stroke, improving the clinical remission of anemia and neurologic symptoms (24, 25). Although iron is an essential component or a cofactor of many critical proteins and enzymes, such as hemoglobin, cytochrome P450s, ribonucleotide reductase, and many other enzymes, biological free iron causes toxicity by generating reactive oxygen

species or free radicals through redox-based reactions. Despite the presence of a biological detoxification mechanism, intravenous infusion or administration of free iron is prohibited. Thus, only a few types of iron complex can be intravenously administrated in the form of iron-carbohydrate complex containing a polynuclear Fe^{3+} hydroxide, which is stabilized by carbohydrate ligands (26), because it is chemically inert and does not generate reactive oxygen species. In this study, rats were intravenously administrated with IIM (Monofer®), which had been recently developed for treating anemia across different therapeutic strategies with a pharmacological safety profile (27, 28). We also found that IIM combined with rhEPO did not cause hepatotoxicity in the late phase of liver regeneration.

In summary, our results showed that rhEPO administration enhances liver regeneration and function to some extent through the maintenance of high levels of serum IL-6 and subsequent stimulation of hepatocyte proliferation for a prolonged period until the late phase of liver regeneration. Notably, these effects were further significantly augmented by co-treatment with IIM, without inducing hepatotoxicity. This strategy may offer potential benefits for the enhancement of liver regeneration and recovery in patients with hepatic malignancy after PHx and in donor patients undergoing living-donor liver transplantation.

MATERIALS AND METHODS

Animals

Inbred male Sprague-Dawley rats (Orient Bio Inc., Sungnam, South Korea), 7–8 weeks of age and weighing 300–350 g, were used in the study. The animals were housed in a specific pathogen-free animal facility with a constant temperature of 21 ± 2°C, relative humidity of 45 ± 15%, and a 12 h-light/dark cycle and given standard food and water *ad libitum*. The experimental protocol was approved by the Animal Ethics Committee of Hanyang University (approval number 2017-0260A). All the experimental procedures were carried out in

accordance with international guidelines for care and use of laboratory animals.

Experimental design

The rats were randomly divided into a sham group and 3 experimental groups by using the sealed envelope method. The sham group consisted of 8 rats, whereas the experimental groups consisted of 16 animals per group. Each group was divided into 2 subgroups (8 animals in each subgroup). The experimental groups underwent a 70% PHx and were treated with saline, subcutaneous (s.c.) rhEPO (3,000 IU/kg, Eporon®; Dong-A ST Co., Ltd., Seoul, Korea), or s.c. rhEPO in combination with intravenous (i.v.) IIM (40 mg/kg, MonoFer®; Pharmacosmos A/S, Holbaek, Denmark). The rats were sacrificed on days 7 and 14 after surgery.

Surgical procedure

The rats were anesthetized through intraperitoneal administration of 80 mg/kg ketamine and 10 mg/kg xylazine, and their weights were recorded. After placing the rats in the supine position, 24-G angiocatheters (BD Angiocath Plus; Becton Dickinson Medical, Singapore) were inserted into the left femoral artery for blood collection, and the blood samples were used for serological and biochemical analyses. A 70% PHx resecting the left and median lobes of the liver was performed according to Higgins and Anderson's method (29). The hepatectomized specimens were weighed and kept at -80°C, and some specimens were preserved in 3.7% formaldehyde (10% formalin) solution. The abdomen was closed with continuous stitches using 4-0 silk sutures and cleaned with povidone iodine. The rats were given s.c. and i.v. injections of saline for the saline control group, and s.c. rhEPO (3,000 IU/kg) alone or in combination with i.v. IIM (40 mg/kg) for the treatment groups. After finishing the surgical procedures, all animals were subcutaneously injected with 5 mg/kg caprofen (Ramadyl®; Pfizer Inc., New York, NY, USA) to relive pain. The rats were sacrificed on postoperative days 7 and 14, intracardiac blood was obtained for biochemical

analysis. In addition, the livers were completely excised, weighed, and divided into 2 pieces: one piece was kept at -80°C and another in 3.7% formaldehyde solution until use.

Regeneration rate

The relative liver regeneration rates were calculated as previously described (30). Basically, the body weights of the rats were measured before PHx ($BW_{pre\text{-}Hpx}$) and at euthanasia ($BW_{euthanasia}$), and the preoperative estimated liver weight ($ELW_{pre\text{-}HPx}$) was calculated from the relative ratio between the resected liver weight and the relative extent of PHx [($LW_{resection}$/70) × 100]. Regenerated liver weights ($LW_{euthanasia}$) were measured at euthanasia. The liver regeneration rate was defined as follows: rate (%) = [($LW_{euthanasia}$/ $BW_{euthanasia}$)/($ELW_{pre\text{-}Hpx}$/$BW_{pre\text{-}Hpx}$)] × 100.

Immunohistochemistry

Hepatocyte proliferation was determined by means of immunohistochemical counting of Ki-67-positive cells. Liver tissues fixed in 3.7% formaldehyde solution were embedded in Optimal Cutting Tissue compound (Leica Biosystems, Richmond, IL, USA) and subsequently frozen in liquid nitrogen. The tissues were sectioned at a thickness of 10 μm and washed in phosphate-buffered saline (PBS) containing 0.1% Triton X-100, followed by incubation with blocking solution (Dako North America, Carpinteria, CA, USA) for 30 min. The sections were incubated with the primary mouse monoclonal antibody against Ki-67 (Abcam, Burlingame, CA, USA; 1:200) for 2 h. After washing with PBS, the sections were incubated with Alexa Fluor® 488-labeled goat anti-mouse immunoglobulin G (Invitrogen, Carlsbad, CA, USA) for 1 h. The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). After mounting with Mounting Medium (Dako North America), images were taken and analyzed using a confocal fluorescence microscope. The percentage of Ki-67-positive cells quantified using ImageJ software (NIH, Bethesda, MD, USA).

Biochemical analysis

Blood samples were collected into tubes containing EDTA and centrifuged at 3,000 rpm at 4°C for 15 min (ScanSpeed 1730R; Labogene, Lillerød, Denmark), and sera were stored at -80°C until use. The serum levels of AST and ALT were determined using a colorimetric assay kit (Asan Pharmaceutical, Seoul, Korea) according to the manufacturer's instructions. The serum levels of HGF and IL-6 were analyzed using mouse/rat HGF Quantikine enzyme-linked immunosorbent assay (ELISA) kit (cat. no. MHG00) and a rat IL-6 Quantikine ELISA kit (cat. no. R6000B), which were purchased from R&D Systems (Minneapolis, MN, USA). The serum levels of albumin and total bilirubin were determined using ELISA kits purchased from Shibayagi Corporation (Gunma, Japan; cat. no. AKRAL-120) and Sigma-Aldrich (St. Louis, MO, USA; cat. no. MAK126), respectively.

Statistical analysis

Quantitative data are expressed as the mean ± standard error of the mean of samples from 8 rats in each group. Data were analyzed using GraphPad Prism 6 software (GraphPad Inc., La Jolla, CA, USA). The data met the assumptions of the test, and the variance between the statistically compared groups was similar. Statistical differences were tested using 2-way analysis of variance with Holm-Sidak post-hoc test. $P < 0.05$ was considered significant.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

FIGURE LEGENDS

Fig. 1. Co-administration of rhEPO and IIM synergistically promotes late-phase restoration of liver mass in hepatectomized rats. Rats were subjected to PHx and were administrated with saline, s.c. rhEPO (3,000 IU/kg), or s.c. rhEPO in combination with i.v. IIM (40 mg/kg). The livers were removed on days 7 and 14 after liver resection. The liver regeneration rate was calculated using the protocol described in Materials and Methods. n = 8, $*P < 0.05$ and $**P < 0.01$.

Fig. 2. Co-administration of rhEPO and IIM augments hepatocyte proliferation during the late phase of liver regeneration after PHx. Rats were administrated with saline, rhEPO, or rhEPO in combination with IIM following PHx. The livers were removed on days 7 and 14 after liver resection. Liver tissues obtained on days 7 and 14 after PHx were immunohistochemically stained with an anti-Ki-67 antibody and a secondary antibody labeled with Alexa Fluor 488. The nuclei were also counterstained with DAPI. (A) Fluorescence images were captured using a confocal microscope. Arrows indicate cells stained by an antibody for Ki-67 (n = 8). (B) Ki-67-positive cells were quantified by counting the total number of nuclei per high-power field (n = 8). $**P < 0.01$. ns, not significant.

Fig. 3. Co-administration of rhEPO and IIM increases the serum levels of IL-6 during the late phase of liver regeneration. Rats were subjected to sham operation or PHx and were administrated with saline, rhEPO, or rhEPO in combination with IIM. Blood samples were collected on days 7 and 14 after surgery. The serum levels of HGF (A) and IL-6 (B) were determined using ELISA kits (n = 8). $*P < 0.05$. ns, not significant.

Fig. 4. Combined effect of rhEPO and IIM on the serum levels of AST, ALT, albumin, and total bilirubin during the late phase of liver regeneration. Rats were subjected to sham operation or PHx. Hepatectomized rats were administrated with saline, rhEPO, or

rhEPO in combination with IIM. Blood samples were obtained on days 7 and 14 after resection, and sera were prepared by centrifugation. Serum levels of AST (A), ALT (B), albumin (C), and total bilirubin (D) were determined using colorimetric assay kits and ELISA kits (n = 8). (E) Time kinetics of hepatocyte proliferation and liver regeneration in the early (hepatocyte priming), middle (hepatocyte proliferation), and late phases (liver function restoration) after co-administration of rhEPO and IIM. *$P < 0.05$ and **$P < 0.01$. ns, not significant.

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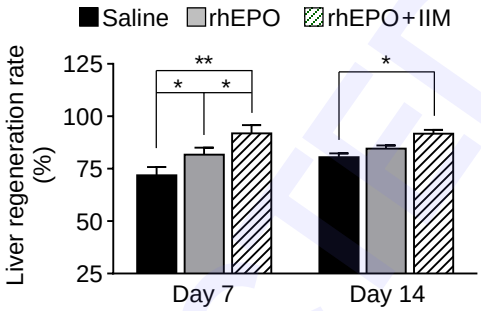

Figure 1

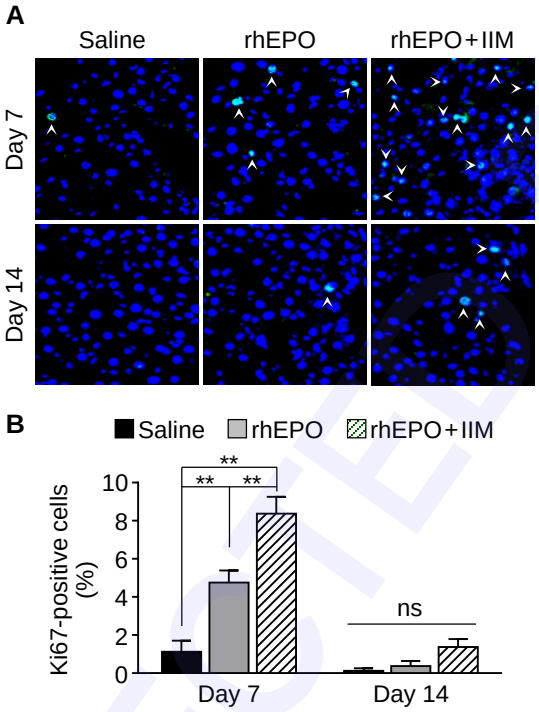

Figure 2

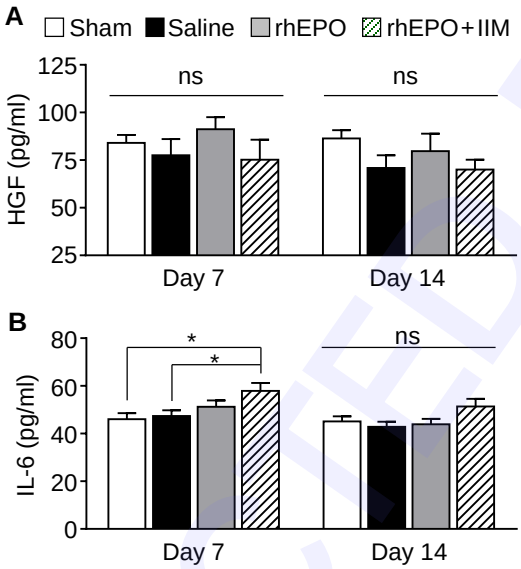

Figure 3

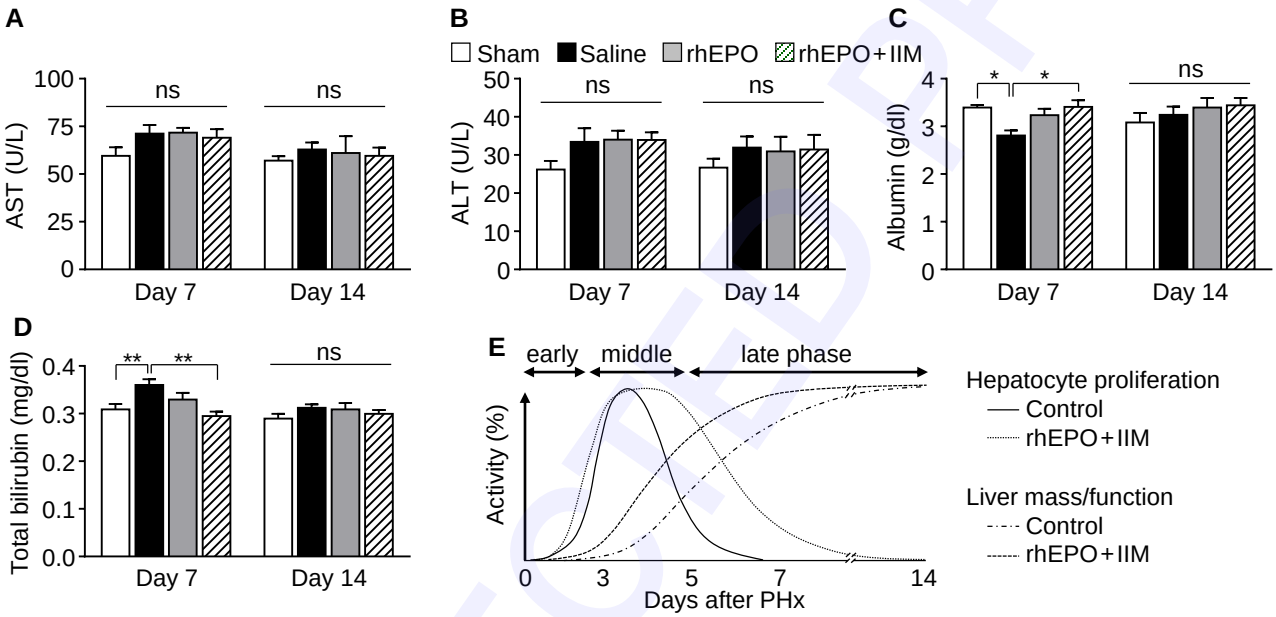

Figure 4