Title:

Platelet administration via the portal vein promotes liver regeneration in rats after 70% hepatectomy

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Short running title:

Platelet therapy for liver regeneration.

Introduction

Many studies over the past two decades have investigated hepatotropic factors that could prevent hepatic failure after extended hepatectomy or other types of liver damage[1, 2]. However, such factors have not proven clinically applicable.   Platelets have thrombotic effects and also play important roles in wound healing and tissue regeneration[3-6].   We recently reported that platelets play a very important role in liver regeneration after extended hepatectomy[7-9].   Furthermore, a low platelet count in humans is associated with delayed recovery of liver function after partial liver resection[10].   Platelets contain many growth factors, such as PDGF, HGF, VEGF, EGF, and TGF-β[11-16], that are required for tissue regeneration[3, 4, 17], and we reported that growth factors in platelets, such as HGF, and IGF-1, are critical for hepatocyte proliferation[8].   We showed that endogenous platelets accumulate in the livers of mice immediately after 70% hepatectomy and that platelets migrate from sinusoidal spaces into Disse's spaces through the fenestration of sinusoidal endothelial cells[7].   We also reported that platelets accumulate in the rat liver after 70% hepatectomy and that residual liver regenerates sooner in such rats[7].   Endogenous platelets accumulate in the damaged liver and actively translocate into Disse's spaces in response to lipopolysaccharides[7, 18-23].   These findings indicated that platelet accumulation would provide a natural delivery system of growth factors and that platelets could prevent hepatic failure due to hepatocyte apoptosis.   We therefore speculated that platelets could promote liver regeneration after massive hepatectomy.

Here, we postulated that exogenous platelets infused via the portal vein could promote liver regeneration and investigated the clinical applicability of platelet therapy for liver regeneration by infusing platelet-rich plasma via the portal vein after 70% hepatectomy and evaluated residual liver regeneration in rats.   To clarify the mechanisms through which platelets promote liver regeneration, we also investigated the STAT3, Akt, and ERK1/2 signaling pathways.   We also analyzed the dynamics of platelets infused via the portal vein before and after 70% hepatectomy in rats.

Methods

Animals

Seven-week-old male SD rats (Clea, Tokyo, Japan), weighing 180 to 200 g each were housed (4 per cage) in an animal room under a 12-h light/12-h dark cycle with free access to water and standard chow.   All animal experiments proceeded in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba.   These experiments also proceeded in accordance with the Regulations for Animal Experiments at our institution and with the Fundamental Guidelines for the Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Preparation of washed platelets

Blood was obtained from rats via the inferior vena cava under ether anesthesia. Platelet-rich plasma (PRP) was obtained by centrifuging anticoagulated blood in acid citrate dextrose (ACD) in a 1:4 volume ratio at $200 \times g$ and 24°C for 10 min.   The platelets were then washed twice by centrifugation at $1000 \times g$ and 4°C for 15 min, resuspended in citrate buffer (120 mM NaCl, 4.26 mM NaHPO4, 5.5 mM glucose, 4.77 mM sodium citrate, and 2.35 mM citric acid), adjusted to pH 6.5 and suspended in normal saline at a density of 1×10^9/mL.

Labeling platelets with rhodamine 6G

Platelets were isolated from the whole blood of syngenic rats and labeled with rhodamine-6G (0.05%; 50 μL/mL whole blood: R-4127; Sigma, St. Louis, MO, USA), as described[22, 24].   Briefly, blood was mixed with prostaglandin E1 and rhodamine-6G and diluted with buffer.   After two cycles of centrifugation, fluorescent platelets were resuspended in PBS. A total of 1×10^8 fluorescence-labeled platelets, representing about 1% of all circulating platelets in recipient rats, were injected via the portal vein before and after 70%

hepatectomy.

Surgical procedure for 70% hepatectomy and PRP infusion

Rats underwent 70% hepatectomy as described[25].   Briefly, the left lateral, left median and right median liver lobes were surgically removed after laparotomy.   Thereafter, the cecal vein was punctured using a 20-G needle and an elastic catheter was inserted into the portal vein. The rats were assigned to groups that were infused via the portal vein over a period of 1 min immediately after hepatectomy with 1 mL of either normal saline (NS group) or platelet-rich plasma (PRP group).   The amount of platelets corresponded to 10% of the total circulating platelets in each rat.   All surgical procedures were performed under ether anesthesia as approved by our institutional review board.   The rats were sacrificed and liver specimens were collected at 24 h after surgery to assess liver regeneration and at 15, 30, 60, 120 and 240 min after surgery to analyze signal transduction.   Blood samples were collected at 24 h after surgery to assess liver damage.

Plasma assays

Serum from blood samples collected at 24 h after hepatectomy was obtained by centrifugation at 3000 rpm for 10 min, and stored at -70°C.   Serum levels of AST, ALT, LDH, ALP and total-bilirubin were measured in both groups.

Histological and immunohistological analysis

Liver specimens were fixed for histological analysis in 10% neutral-buffered formalin, embedded in paraffin, sliced into 4-μm sections and stained with hematoxylin-eosin.   Ki-67 was immunohistochemically stained according to the manufacturer's guidelines.   Briefly, liver specimens were embedded in Optimal Cutting T (OCT) compound (Tissue-Tek; Sakura Fine Technical, Tokyo, Japan) and then frozen sections (10 μm) were fixed in 10% formalin for 1 min.   Endogenous peroxidase was inactivated by immersion in 0.3% hydrogen peroxide in absolute methanol for 5 min, and then the sections were washed with PBS.   The sections were incubated with 3% bovine serum albumin for 30 min, followed by Ki-67 antibody (YLEM,

Rome, Italy) at room temperature for 60 min.   Primary antibody reactions were enhanced using horseradish peroxidase (HRP)-conjugated antibody, EnVision (Dako, Carpinteria, CA, USA) and immunoreactivity was visualized using 0.05% 3,3-diaminobenzidine (DAB). Specimens were washed in distilled water, counterstained with hematoxylin for 2 min and then mounted.   The Ki-67 labeling indices of hepatocytes were determined from random evaluations of over 2000 hepatocytes and are expressed as ratios (%).

Western blotting

Samples were boiled in 125 mM Tris-HCL buffer (pH 6.8), containing 5% glycerol, 2% sodium dodecyl sulfate (SDS) and 1% 2-mercaptoethanol for 5 min, resolved by 10% SDS-PAGE and then transferred to nitrocellulose membranes.   Samples were blotted against phospho-STAT3, phospho-Akt, phospho-ERK1/2, and β-actin primary antibodies (Cell Signaling, Beverly, MA, USA).

Platelet dynamics

We analyzed the dynamics of infused platelets before and after hepatectomy by intravital fluorescence microscopy using a modified microscope (BX30 FKA-SP; Olympus, Tokyo, Japan) with a 100-W mercury lamp attached to a filter block.   Hepatic microcirculation was recorded using a charge-coupled device (CCD) camera (C5810 NTSC; Sony, Tokyo, Japan) for offline evaluation.   Magnification of 325× to 650× was achieved on the video screen using water-immersion objective lenses (W10 ×/0.3 to W20 ×/0.7; Olympus, Tokyo, Japan). Microcirculatory platelet-endothelial cell interactions were analyzed using videotaped images. Rhodamine-6G labeled platelets were infused via the portal vein before or after hepatectomy, and 7-10 randomly selected acini were visualized as described[22].

Statistical analysis

Results are expressed as means ± S.D.   Data were statistically analyzed using Student's t test.   Values of $p < 0.05$ were considered statistically significant for all findings.

Results

Platelet counts

We counted platelets in the peripheral blood before and after platelet infusion via the portal vein to determine their effects on the blood circulation.   The counts did not significantly differ before and after platelet infusion (data not shown).

Liver regeneration

We measured the ratios of residual liver to body weight at 24 h after 70% hepatectomy (partial hepatectomy, PH) to determine the effect of platelet infusion on liver mass recovery (Fig. 1). Each group comprised 9 rats.   The liver/body weight ratio at 24 h after PH was significantly higher in the PRP, than in the NS group ($p < 0.05$).

Histological analysis

Light microscopy of liver tissues at 1, 24 and 48 hours after portal infusion did not uncover any serious changes (data not shown).

Immunohistochemical staining of Ki-67

We evaluated hepatocyte proliferation by immunohistochemically staining liver specimens for Ki-67.   Each group comprised 6 rats. The Ki-67 labeling index was significantly higher in the PRP, than in the NS group as shown in Figures 2 and 3 ($p < 0.05$).

Cellular signal transduction

We examined the activation of Akt, ERK1/2, and STAT3 signal transduction to understand the effect of PRP infusion on cellular signaling pathways of the regenerating liver. These factors are closely associated with liver regeneration.   The data in Figure 4 shows that Akt was phosphorylated earlier in the PRP, than in the NS group, and that although ERK1/2 was simultaneously phosphorylated in both groups, it persisted after PH in the PRP group. The phosphorylation of STAT3 did not apparently differ between the two groups (Fig. 4).

Platelet accumulation after hepatectomy visualized using CCD camera

Significantly more platelets adhered after, than before hepatectomy in the PRP group (Fig. 5).

Plasma liver function

We assayed plasma liver function in rats infused with PRP or NS for 24 h after hepatectomy to determine liver injury due to PRP infusion via the portal vein.   Each group comprised 10 rats.   The data in Table 1 shows that liver function did not significantly differ between the two groups (p < 0.05).

Discussion

Platelets are activated at the site of injury to intensify the coagulation process[6].   We previously reported that thrombocytosis promotes liver regeneration after partial hepatectomy[21, 23] and HGF and IGF-1 are essential for hepatocyte proliferation[9].   Thrombopoietin or splenectomy are often used to increase platelet counts both experimentally and clinically. However, the effect of thrombopoietin lasts for a specific period and that of splenectomy is essentially permanent.   This might cause thrombocytosis and various organs to become dysfunctional.   We postulated that a local and single administration of platelets during the critical period for liver regeneration would help to resolve these issues.   We examined the maximal dose of platelets that could be safely infused via the portal vein without causing thrombosis and liver dysfunction in preliminary study. We confirmed that infusing 10% of the total amount of platelets did not cause either thrombosis or liver damage.   In addition, we examined the critical timing for platelet administration by visualizing platelet dynamics before and after hepatectomy using intravital fluorescence microscopy. We confirmed that platelets infused via the portal vein accumulated in the sinusoids immediately after hepatectomy and speculated that platelet infusion could be applicable as liver regeneration therapy. The present findings indicated that a single administration of platelets via the portal vein obviously promoted liver regeneration after 70% hepatectomy.

We assessed liver regeneration by comparing liver/body weight ratios at 24 h after hepatectomy and measured the Ki-67 labeling index as an indicator of cell proliferation in the PRP and NS groups. Both values were significantly higher in the PRP, than in the NS group. These results indicated that the administration of platelets induces prompt signal transduction for the proliferation of residual liver regeneration after massive hepatectomy.

The Akt and ERK1/2 pathways are important signaling processes that are activated in the liver by platelets[7, 8]. Hepatocytes cultured with platelets immediately activate both the Akt and ERK1/2 pathways and hepatocytes start to proliferate[8]. Inhibitors of PI-3K such as LY-294002 inhibit the proliferative effect of platelets on hepatocytes[8]. The present study found that the Akt pathway was activated earlier in the PRP, than in the NS group and that ERK1/2 pathway activation persisted in the PRP, compared with the NS group. These results support our previous findings that both the Akt and ERK1/2 pathways essentially contribute to liver regeneration. On the other hand, STAT-3 was activated at the same time in each group and more STAT-3 was expressed in the controls. The liver is a key vital organ in which the mechanisms of regeneration are highly orchestrated. Many processes play roles in the total number of mechanisms that function during liver regeneration. Platelets comprise one important factor involved in liver regeneration[7, 8], and other mechanisms such as the Il-6 to STAT-3 pathway and substrates released from Kupffer cells as well as sinusoidal endothelial cells also play very important roles in liver regeneration[26, 27]. These facts suggest that mechanisms via STAT-3 pathway compensated in the NS group for other mechanisms involved in platelet signaling.

Only a few reports have described the movement of platelets in vivo[28, 29]. Platelets accumulate in the liver in response to ischemic reperfusion[22]. We investigated the dynamics of rhodamine-6G labeled platelets before and after hepatectomy, using an intra-vital video microscopy system. Platelets flowed faster and fewer of them rolled and aggregated in the sinusoidal endothelium before hepatectomy. In contrast, many platelets accumulated in the sinusoid, the flow rate decreased, and the platelets rolled and aggregated in the sinusoid after massive hepatectomy (see Video, Supplemental Digital Content 1 and 2 which demonstrate the dynamics of infused platelets in the sinusoid before and after hepatectomy). Although

the mechanisms of platelet accumulation remain unclear, the present results indicated that platelets actually accumulate at the sinusoid of the residual liver after hepatectomy.

The safety of PRP infusion is critical in the clinical setting.   Portal injection of platelets would have a maximal effect on regeneration of the residual liver. However, thrombosis and liver damage arising during infusion due to the inherent nature of platelets are critical concerns. Therefore, we evaluated serum liver markers and histological findings to determine liver injury after PRP infusion.   The results did not indicate any increase in liver damage in the PRP group.   In addition, neither embolism nor necrosis of the residual liver was recognized in the PRP group (data not shown).   These findings indicated that PRP infusion promotes liver regeneration after massive hepatectomy without liver damage.

This is the first report to describe the clinical applicability of PRP infusion for liver regeneration therapy.   We believe that platelet infusion could offer a novel therapeutic strategy for liver regeneration after extended hepatectomy, liver damage or after small grafts in liver transplantation in the near future.

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Legends for illustrations

Fig. 1.   Liver/body weight ratio.

Liver regeneration at 24 h after 70% hepatectomy is significantly higher in PRP than NS (control) group.   Columns and bars represent means ± SD. *p < 0.05 versus NS group (n = 9 per group).

Fig. 2.   Immunohistochemistry for Ki-67.

Immunohistochemical staining of Ki-67 of PRP (A) and NS (B) samples at 24 h after 70% hepatectomy.   Ki-67 is remarkably stained in PRP compared with NS (control) group.

Fig. 3.   Ki-67 labeling index.

Ki-67 labeling index is significantly higher in PRP than NS (control) group. Columns and bars represent means ± SD. *p < 0.05 vs. NS group (n = 6 per group).

Fig. 4.   Cellular signals.

Cellular signals were analyzed before and after portal infusions of PRP and normal saline.   A. Akt was more rapidly activated in PRP than NS group. B. Activation of ERK1/2 pathway persisted longer in PRP than in NS group.   C. STAT-3 pathway activation is similar in both groups.   D. Equalized loaded protein confirmed by amount of β-actin at any time point in each group.

Fig. 5.   Intravital-videomicroscopy.

Platelet dynamics were analyzed before and after hepatectomy in PRP group.   Rhodamine 6G-labelled platelets were infused via portal vein before and after hepatectomy.   Intravital video microscopy shows fewer platelets in sinusoid before (A), than immediately after (B) hepatectomy.

List of abbreviations:

ERK, extra cellular signal- regulated protein kinase; STAT3, signal transducer and activator of

transcription factor kB.

Legends for Supplemental Digital Contents

Supplemental Digital Content 1.

Platelets flowed faster and fewer of them rolled and aggregated in the sinusoidal endothelium before hepatectomy.

Supplemental Digital Content 2.

Many platelets accumulated in the sinusoid, the flow rate decreased, and the platelets rolled and aggregated in the sinusoid after massive hepatectomy.

List of Supplemental Digital Contents

Supplemental Digital Content 1.   Video that demonstrates the dynamics of infused platelets in the sinusoid before hepatectomy.   wmv

Supplemental Digital Content 2.   Video that demonstrates the dynamics of infused platelets in the sinusoid after hepatectomy.   wmv

Figure 1   **Liver / body weight ratio**

n=9, p<0.05 vs. NS
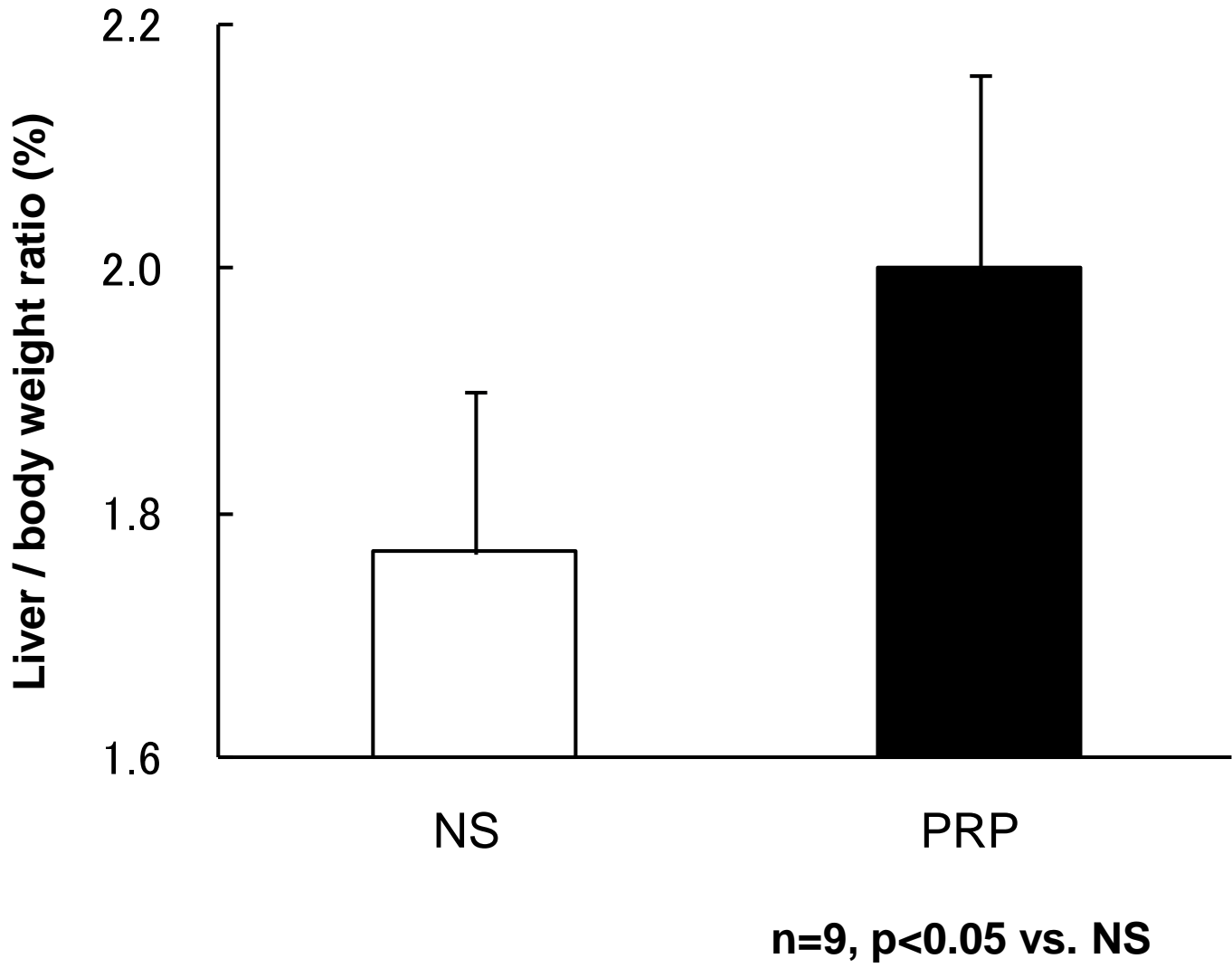
Figure 2   **Immunohistochemistry (Ki-67)**
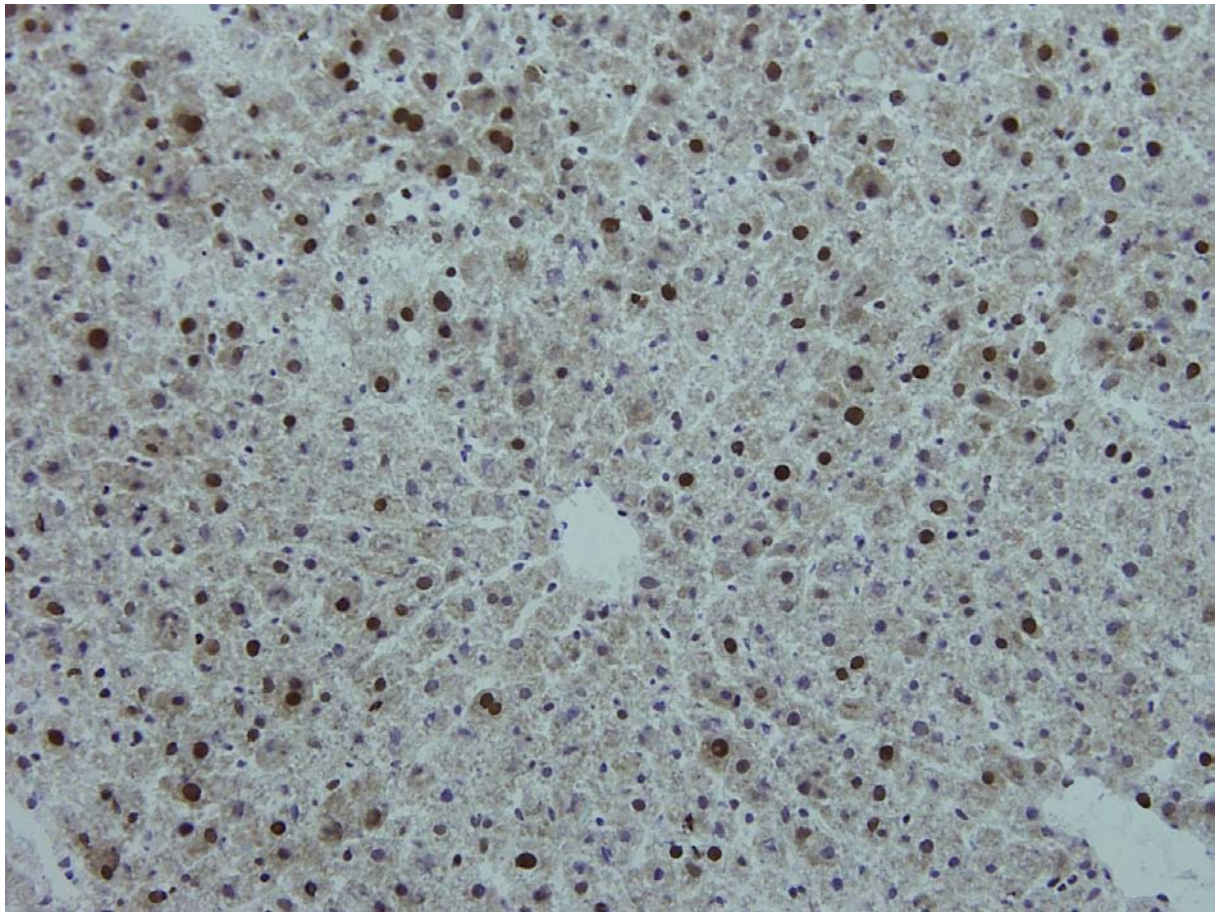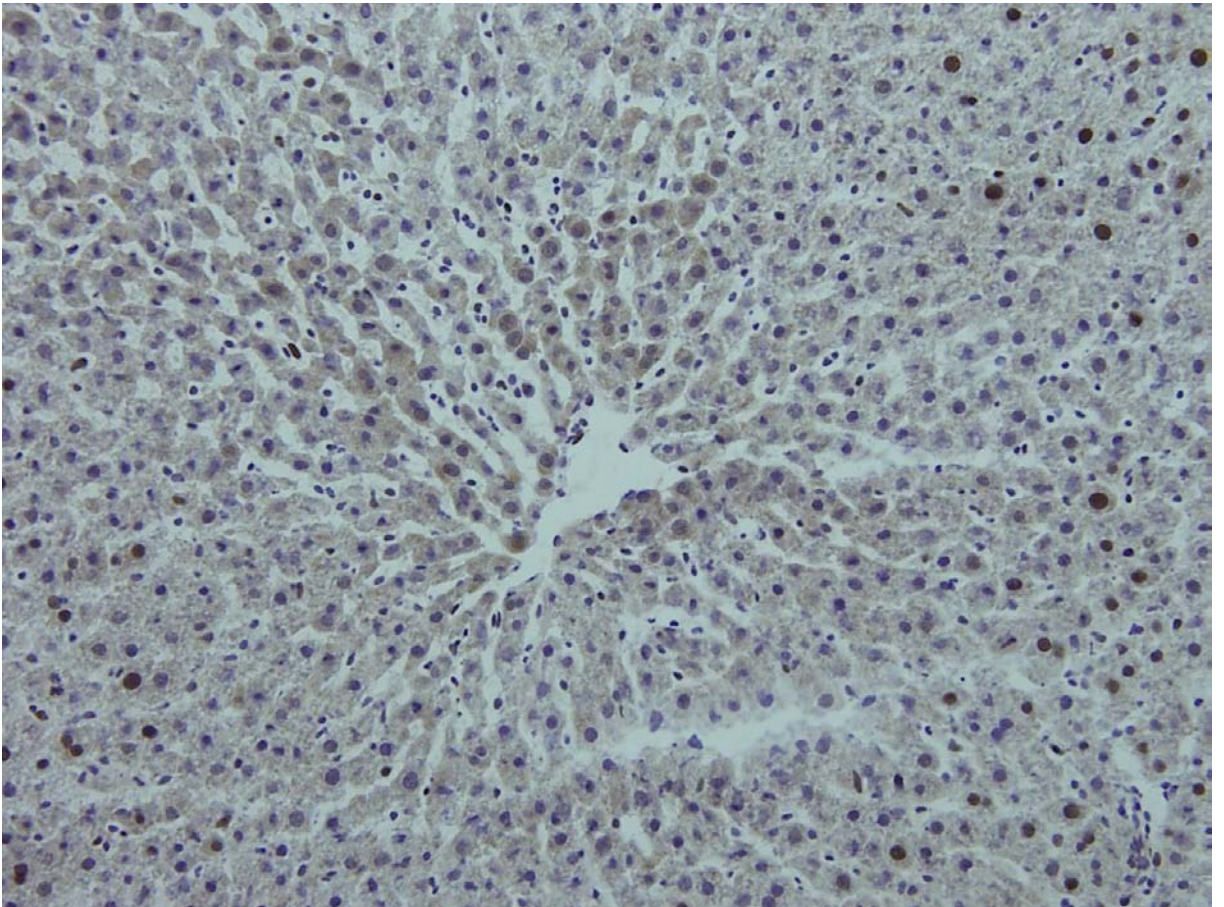
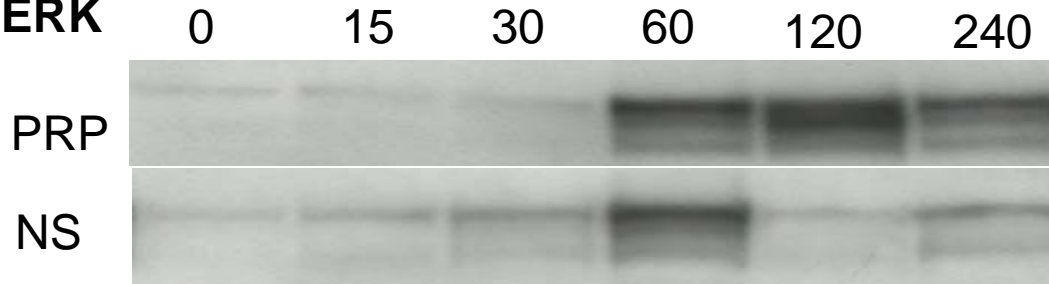**Figure 3** **Ki-67 labeling index**
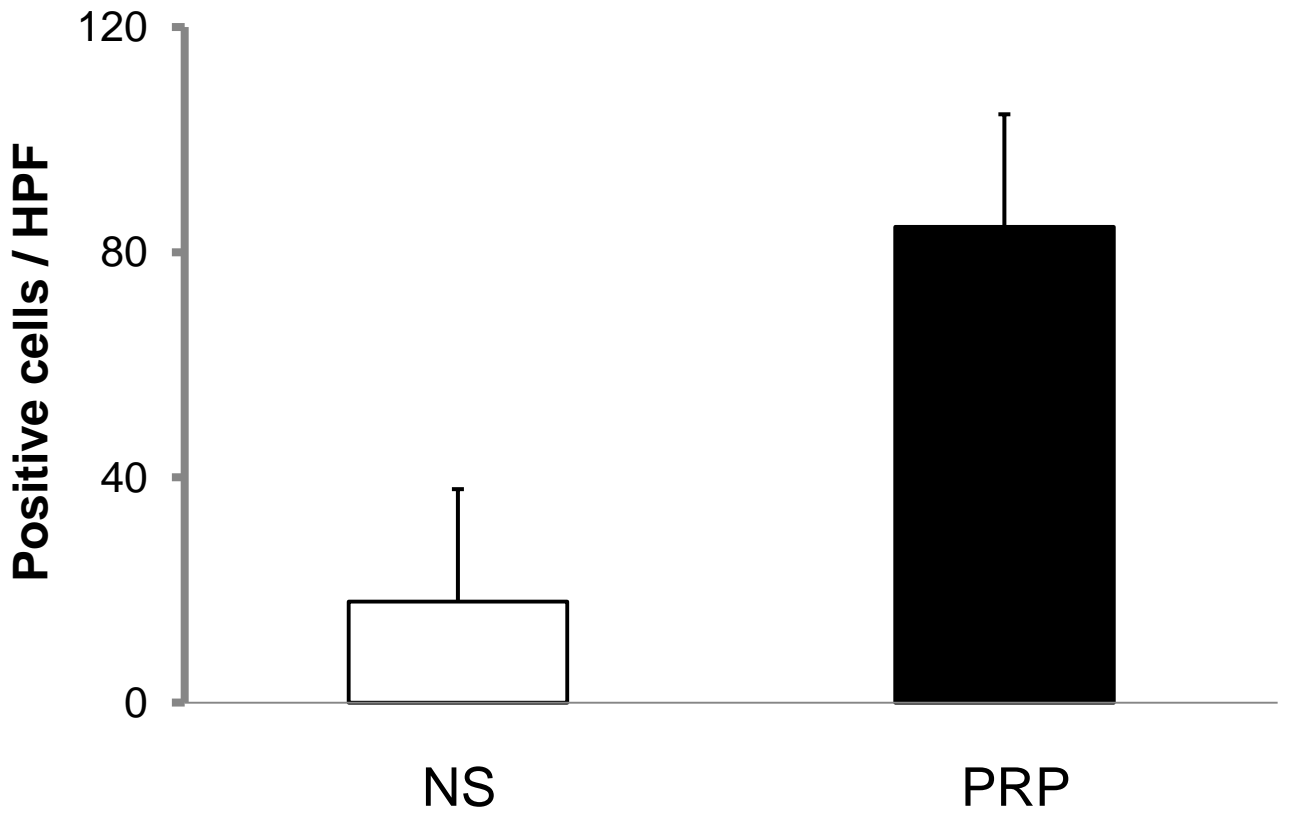
n=6, *P<0.05

Figure 4

A. Phospho-Akt

| | 0 | 15 | 30 | 60 | 120 | 240 |

B. Phospho-ERK

| | 0 | 15 | 30 | 60 | 120 | 240 |

C. Phospho-STAT3

| | 0 | 15 | 30 | 60 | 120 | 240 |

D. tubulin

| | 0 | 15 | 30 | 60 | 120 | 240 |

Figure 5

A

00-00 00:24:55:18

B

00-00 00:01:58:09
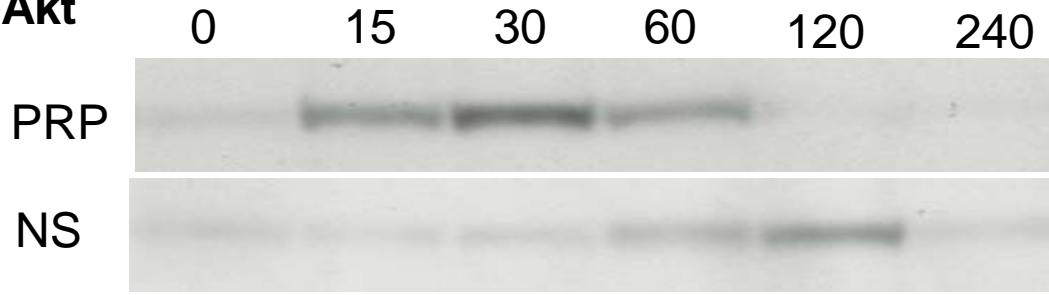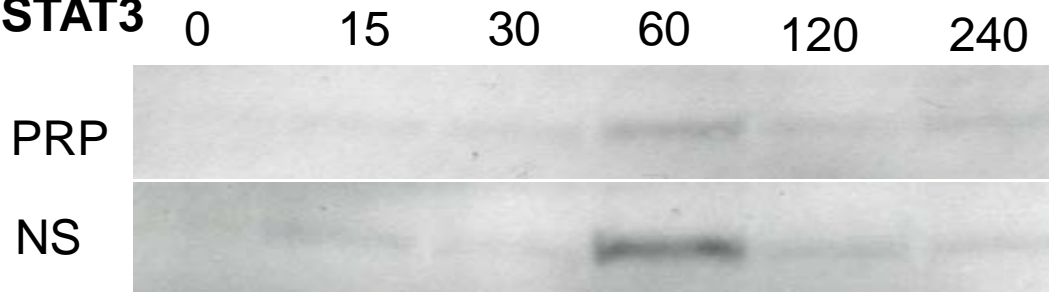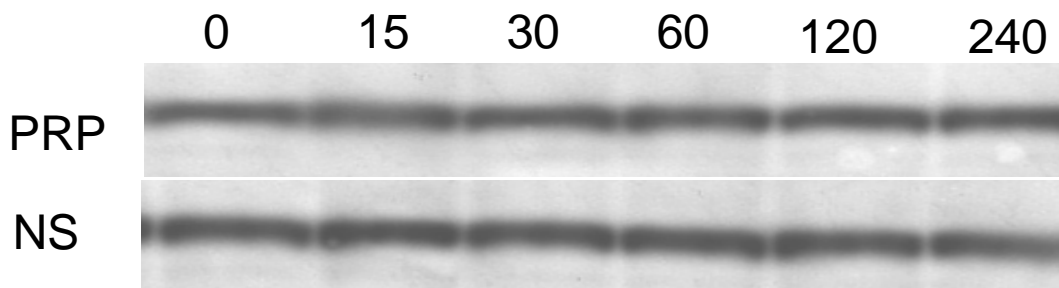
Table 1

group	Alb	AST	ALT	LDH	ALP	T-Bil
NS	2.16±0.36	225.0±68.3	71.5±21.2	224.1±133.0	170.5±155.5	0.12±0.04
PRP	2.10±0.15	182.5±18.4	55.6±4.7	228.5±77.4	257.8±116.4	0.11±0.03

n=10