

Nicotinamide induces liver regeneration and improves liver function by activating SIRT1

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Abstract. Nicotinamide (Nam) has recently been characterized as an agent for tissue regeneration due to the observed pro-proliferation effects. However, the effect of Nam on liver regeneration remains undetermined. In the present study, the potency of Nam as a regimen to promote liver regeneration and restore liver function was evaluated following partial hepatectomy (PH) on C57BL/6 mice. Ki-67 immunohistochemical and cell cycle analyses demonstrated that exogenous Nam supplementation promoted the proliferation of hepatocytes and accelerated the recovery of liver tissue. The addition of Nam protected liver function following PH, as evidenced by hematoxylin and eosin staining of liver tissue morphology and measurement of serum liver injury markers. Notably, immunoblotting results revealed that the expression and activity of NAD-dependent protein deacetylase sirtuin-1 (SIRT1) were significantly upregulated following treatment with Nam, suggesting that Nam may promote liver regeneration through activation of SIRT1. The present study demonstrated that Nam regulated the process of liver regeneration and improved liver function by activating SIRT1, suggesting that Nam has the potency to be used for promoting liver regeneration following surgical resection.

Introduction

Hepatocellular carcinoma (HCC) has been ranked the third most life-threatening cancer, causing approximately

700,000 mortalities each year globally (1). For patients with HCC at Barcelona Clinic Liver Cancer (BCLC) (2,3) stage 0 or A, surgical resection is the first-line treatment, which contributes to 60-80% of patients surviving for 5 years (4,5). Surgical resection is applicable in patients with HCC as the liver has a unique capacity to regenerate, following principal tissue loss (6). Notably, there are numerous previous studies that have reported that in patients with HCC beyond BCLC stage A, surgical resection was infrequently the best choice and improved the rate of survival (2,7,8). However, in order to remove the lesions thoroughly, extensive liver resection may be required, leaving an insufficient amount of residual tissue for the liver to recover to maintain vital function (9). On this occasion, a transient fatal form of hepatic failure may develop, leading to the untreatable small-for-size syndrome (9,10). Therefore, identifying a strategy to promote liver regeneration and liver function recovery following partial hepatectomy (PH) is urgently required.

Nicotinamide (Nam) is a form of vitamin B₃ that is absorbed from our daily diets (11). The therapeutic potential of Nam has been demonstrated in a diverse range of diseases, including skin disorders, diabetes and multiple sclerosis (12-14). Previous studies have revealed that Nam may accelerate the repair of human skin following injury, suggesting the potency of Nam in promoting the regeneration of vital tissues (15,16). It was additionally reported that Nam serves as a precursor of oxidized nicotinamide adenine dinucleotide (NAD⁺) during the metabolism of hepatocytes in liver tissue (17-20). However, the effect of Nam on liver regeneration remains unknown.

Sirtuins are class III histone deacetylases that consume one molecule of NAD⁺ during each deacetylation cycle (21). Accumulating evidence has demonstrated that NAD-dependent protein deacetylase sirtuin-1 (SIRT1), a member of the Sirtuin family, regulates numerous metabolic processes in the liver, including gluconeogenesis and lipid synthesis, by directly deacetylating target proteins (22,23). Furthermore, it has been demonstrated that downregulation of SIRT1 impairs liver regeneration (24). According to our previous study, the expression of SIRT1 in hepatocytes was significantly upregulated by Nam (25). Collectively, these results suggest that Nam may promote liver regeneration through the SIRT1-mediated pathways.

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In the present study, it was demonstrated that Nam induced the process of liver regeneration and improved liver function through activation of SIRT1, suggesting that Nam has the potency of becoming a regimen for promoting liver regeneration following surgical resection.

Materials and methods

Animals and treatment. In the present study, male C57BL/6 mice (6–8 weeks old; 15–20 g; $n=30$, 6 per group; Beijing HFK Bioscience Co., Ltd., Beijing, China) were raised in specific pathogen-free conditions ($23\pm1^\circ\text{C}$; relative humidity, 39–43%; 12 h light/dark cycle; free access to food and water). Animal care and experimental protocols were conducted in accordance with guidelines provided by the Institutional Animal Care and Use Committee of Sichuan University (Chengdu, China). Ethics approval was obtained from the Institutional Ethics Committee of Sichuan University. Prior to subjecting the mice to 70% PH, mice were anesthetized with a subcutaneous injection of pentobarbital at a dose of 80 mg/kg. For the study group, Nam (250 mg/kg per day) was administered 6 h prior to PH via intraperitoneal injection and once daily subsequently for a total of 3 days. Control animals were treated with the same frequency and volume of PBS as Nam. All of the study mice were sacrificed at the indicated time points (24, 36, 48 and 72 h) and the liver samples were harvested and stored in liquid nitrogen. Liver recovery rates were calculated as the ratio of the regenerating liver weight to the mean liver weight prior to PH. The serum samples (1 ml) were additionally collected from the angular veins of the mice. Blood glucose (Glu), triglyceride (TG), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using enzymatic methods (ADVIA 165; Siemens Healthineers, Erlangen, Germany).

Protein isolation and western blotting. To obtain the total protein, the liver tissues were frozen in liquid nitrogen, washed twice with PBS, and homogenized in radioimmunoprecipitation assay lysis buffer (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The samples were incubated on ice for 30 min prior to centrifugation at 4°C for 15 min at a speed of $12,000 \times g$. Subsequent to centrifugation, the supernatant was divided into small fractions and stored at -80°C . The protein content of each supernatant was quantified using the Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA), according to the manufacturer's protocol. Western blot analysis was performed as described previously (26). Cyclin D1 (1:1,000; rabbit monoclonal; cat. no. ab134175), PCNA (1:1,000; mouse monoclonal; cat. no. ab29), and SIRT1 (1:1,000; mouse monoclonal; cat. no. ab110304) primary antibodies were obtained from Abcam (Cambridge, UK); β -actin (1:1,000; mouse monoclonal; cat. no. sc-69879), as well as the secondary antibodies [horseradish peroxidase (HRP)-conjugated goat anti-mouse and goat anti-rabbit; both 1:5,000; cat. no. sc-2005 and sc-2004] were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Densitometric analysis was performed using ImageJ software (version 1.48; National Institutes of Health, Bethesda, MD, USA).

Histology and immunohistochemistry. For immunohistochemical analysis and morphological examination

[hematoxylin and eosin (H&E) staining], formalin-fixed (10% buffered formalin at 4°C overnight), paraffin embedded liver tissues were sectioned to $5 \mu\text{m}$. H&E staining was performed as described previously (27).

For immunohistochemical analysis, sections were blocked with 5% bovine serum albumin (Biowest, Nuaille, France) diluted in PBS (pH 7.4) for 1 h at 37°C . Proliferation marker protein Ki-67 (Ki-67) staining was performed with an anti-Ki-67 antibody (1:4,000; rabbit polyclonal; cat. no. ab15580; Abcam) at 4°C overnight. Next, slides were incubated with the HRP-conjugated goat anti-rabbit antibody at 37°C for 1 h. Liver sections examined at each time-point were from at least three individual animals in the treatment and control groups. Images were captured with the AX10 imager A2 fluorescence microscope (magnification, $\times 40$; Carl Zeiss AG, Oberkochen, Germany). Percentage Ki-67 expression was evaluated in five random fields from each immunostained HCC section. Quantification of Ki-67 staining was performed using Image-Pro Plus version 6.0 (National Institutes of Health, Bethesda, MD, USA).

Bioinformatics analysis. The Kyoto Encyclopedia of Genes and Genomes database (<https://www.kegg.jp>) was used to explore the potential genes regulated by SIRT1.

Acetylation assays. To detect the deacetylation of peroxisome proliferator-activated receptor γ coactivator 1- α (PGC-1 α) and oxysterols receptor liver X receptor- α (LXR α) by SIRT1, lysine acetylation was analyzed by immunoprecipitation and western blotting, using an acetyl-lysine antibody. Primary antibodies against PGC-1 α (1:1,000; rabbit polyclonal; cat. no. ab54481) LXR α (1:1,000; mouse monoclonal; cat. no. ab41902) and acetyl-lysine (1:1,000; rabbit polyclonal; cat. no. ab21623) were purchased from Abcam. HRP-conjugated goat anti-mouse and goat anti-rabbit secondary antibodies were used, as described above, and the western blotting procedure was the same as aforementioned (26). An immunoprecipitation assay was performed as previously described (28,29) using the Pierce™ Classic Immunoprecipitation kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Statistical analysis. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA). A Student's *t*-test was performed to compare continuous values between the treatment and control groups. Results are presented as the mean \pm standard deviation of three independent experiments. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Nam promotes liver proliferation following PH. To investigate the effect of Nam on the process of liver regeneration, 70% PH was conducted on the treatment and control groups. As demonstrated in Fig. 1A, the Ki-67 immunohistochemistry staining was performed to validate hepatic cell proliferation following PH. It was observed that when compared with the control group, increased active proliferation was observed in the treatment group at 24–72 h post-operation. Furthermore, quantification of the Ki-67 staining revealed a significant restoration of hepatocyte proliferation in the treatment group

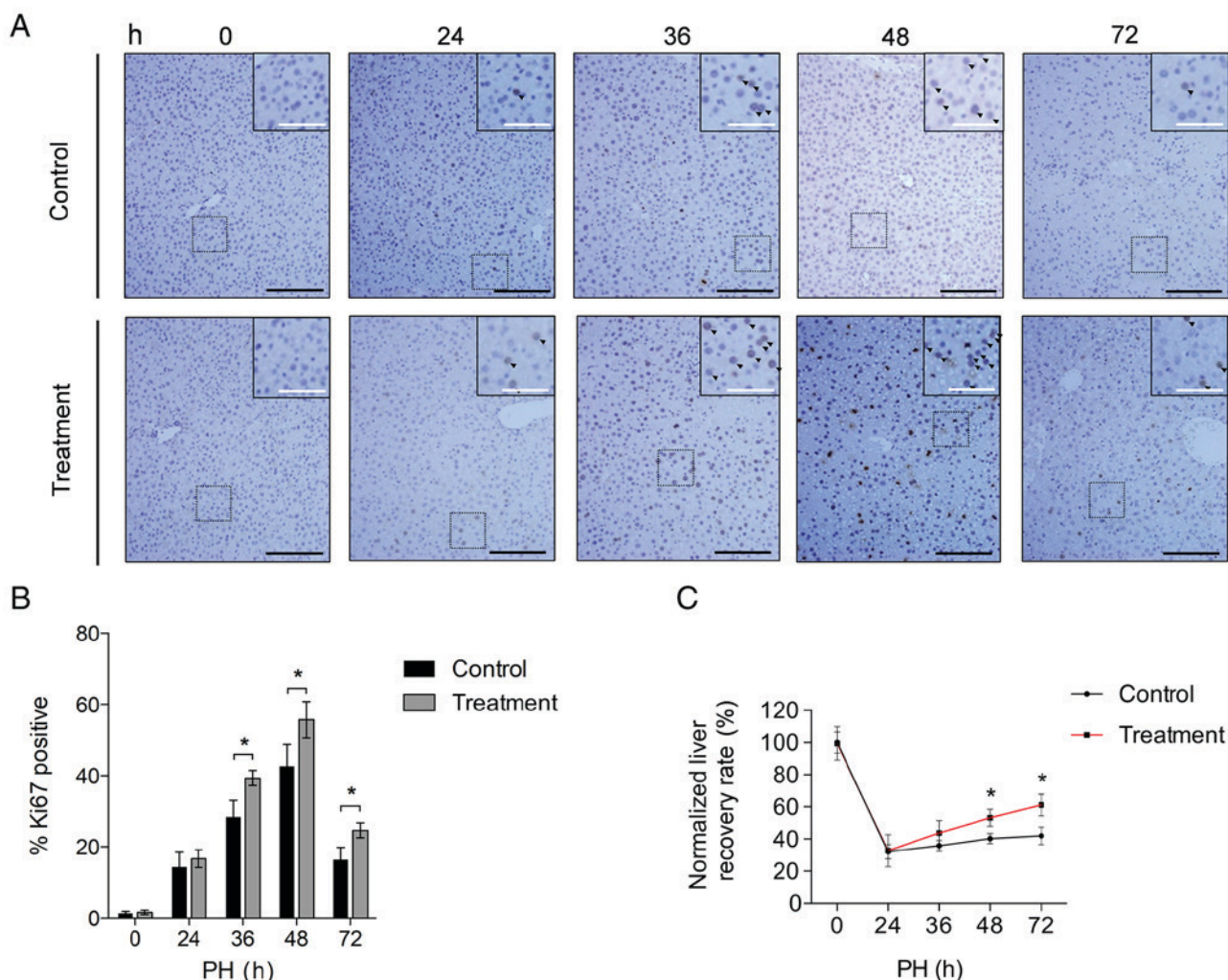


Figure 1. Exogenous supplementation of nicotinamide promotes liver regeneration. (A) Immunohistochemistry for Ki-67 at the indicated time points; the positive cells are marked with arrows. White scale bars, 50 μ m; Black scale bars, 200 μ m. (B) Quantification of Ki-67-positive hepatocytes at the indicated time points. (C) Normalized liver recovery rates are demonstrated as the ratio of the weight of regenerating livers to their mean weight prior to PH. All data are presented as the mean \pm standard deviation (n=3-5). *P<0.05, as indicated. Ki-67, proliferation marker protein Ki-67; PH, partial hepatectomy.

36-72 h following surgery (Fig. 1B; P<0.05). The treatment group additionally exhibited higher liver recovery rates compared with the control group (Fig. 1C). These results suggested that the proliferation of primary hepatocytes was enhanced in the presence of Nam.

Cell cycle progression serves a vital role during liver regeneration (30). To examine the cell cycle of proliferating hepatocytes following PH, the expression of G₁/S-specific cyclin D1 and proliferating cell nuclear antigen (PCNA), two key regulators of the cell cycle, was analyzed by western blotting (Fig. 2A). It was identified that the expression levels of cyclin D1 and PCNA reached a peak at 72 h in the livers of mice from the treatment and control groups. Additionally, the livers of mice injected with Nam exhibited significantly increased expression of cyclin D1 at 24, 36, 48 and 72 h and PCNA at 0 and 24 h when compared with the control (Fig. 2B and C; P<0.05). These results suggested that Nam may accelerate liver regeneration following PH.

Damage caused by PH is repaired in the Nam-treated group. A previous study reported that severe liver damage may occur in short time periods following PH (31). To assess whether Nam may

reduce liver damage following PH, H&E staining was conducted on liver tissue sections. The results of the H&E staining revealed that the liver tissues of the control group exhibited evident swelling, fatty degeneration and nuclear condensation of hepatocytes 48 h following PH, along with severe sinusoidal narrowing (Fig. 3A). By contrast, sections from the treatment group demonstrated patent sinusoids, and better preservation of the cytoplasm and nuclear morphology, suggesting that treatment with Nam may repair liver damage following PH.

ALT and AST are markers of liver injury following PH. According to a previous study, these two markers increase markedly following PH (31). To further determine whether Nam has the potential to reduce liver injury following PH, the serum content of ALT and AST was analyzed (Fig. 3B and C). It was observed that the serum levels of ALT and AST were significantly decreased following PH in the treatment group compared with the control group (Fig. 3B and C; P<0.001), suggesting that Nam may protect liver function following trauma caused by PH.

Nam upregulates the expression of SIRT1. Our previous study demonstrated that the addition of Nam promoted the expression

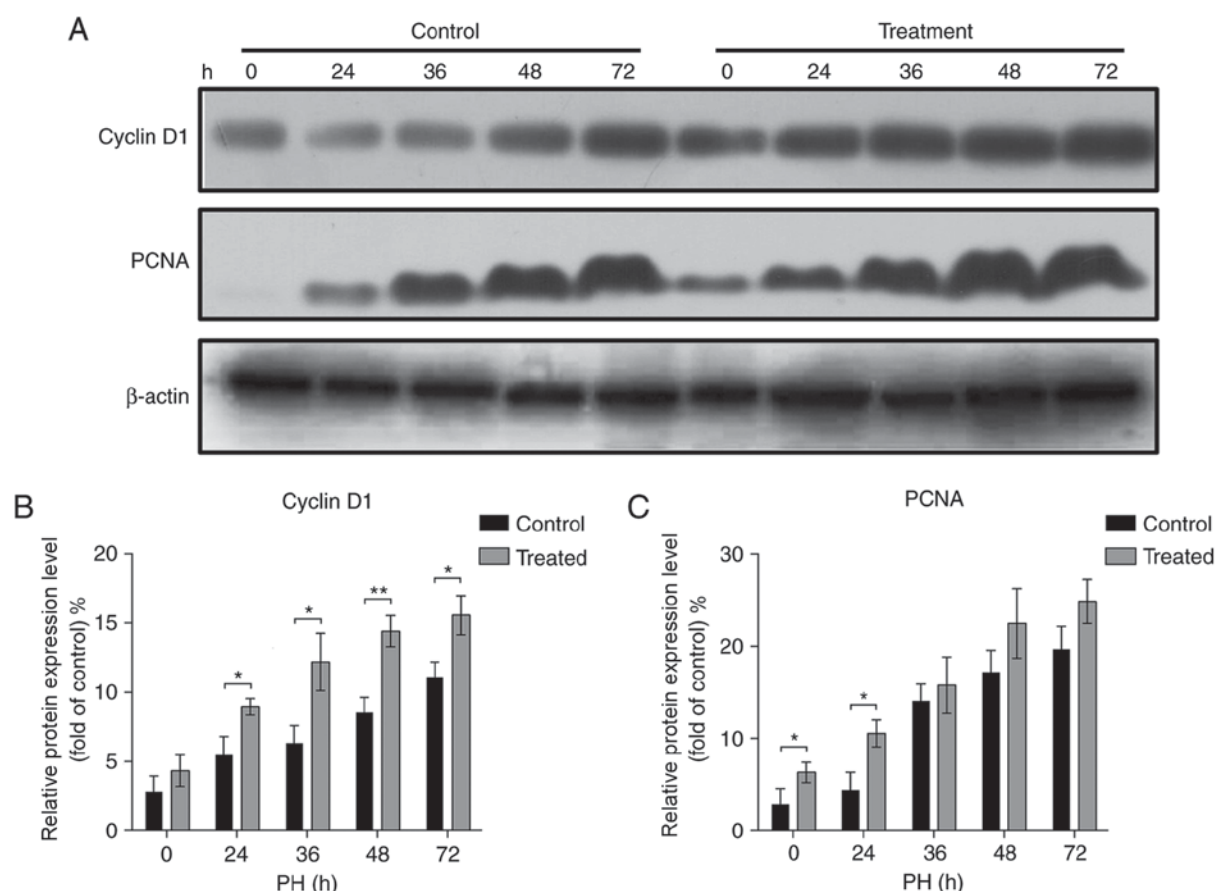


Figure 2. Expression of cyclin D1 and PCNA is increased in mice treated with nicotinamide compared with the control mice. (A) Western blots of the expressions of cyclin D1 and PCNA in the remnant livers following PH. Quantification of the protein expression levels of (B) cyclin D1 and (C) PCNA. Densitometry quantification of the band intensities was conducted using ImageJ software and is presented as a percentage of relative densitometry normalized to β -actin. Data are presented as the mean \pm standard deviation of three independent experiments ($n=3-5$). * $P<0.05$ and ** $P<0.01$, as indicated. Cyclin D1, G₁/S-specific cyclin-D1; PCNA, proliferating cell nuclear antigen; PH, partial hepatectomy.

of SIRT1, a key regulator of liver regeneration, suggesting that Nam may promote liver regeneration by upregulating the expression of SIRT1 (25). To verify the mechanisms of Nam in promoting liver regeneration, western blotting analysis was conducted to determine the expression of SIRT1 in the treatment and control groups. As demonstrated in Fig. 4A and B, although the expression of SIRT1 following PH increased in the two groups in a time-dependent manner, the addition of Nam further facilitated the significantly increased expressions of SIRT1 24-72 h following PH (Fig. 4A and B; $P<0.05$).

Glu and TG metabolisms have been reported to be a prerequisite for liver regeneration (23,32). Previous studies have demonstrated that the levels of Glu and TG are modulated by SIRT1 during the process of liver regeneration (24,33,34). To further elucidate the mechanisms of Nam in promoting liver regeneration, the levels of TG and Glu from serum samples were analyzed. Compared with the control group, the levels of Glu and TG were restored faster in the treatment group (Fig. 4C and D), which was consistent with the result that the expression of SIRT1 was upregulated by Nam. These results suggest that Nam may elevate the levels of Glu and TG by promoting the expression of SIRT1, thereby accelerating the process of liver regeneration.

Deacetylating ability of SIRT1 is enhanced following treatment with Nam. To further investigate the mechanism

underlying SIRT1-mediated metabolic processes following treatment with Nam, bioinformatics analysis was conducted (data not shown), which identified two substrates of SIRT1, PGC-1 α and LXR α , which may be deacetylated by SIRT1 and have been previously implicated in the process of glucogenesis and lipid synthesis, respectively (35,36). As demonstrated in Fig. 5A and C, compared with the control group, mice injected with Nam demonstrated a significant decrease in the lysine acetylation level of PGC-1 α ($P<0.01$), suggesting that Nam may regulate Glu levels through the SIRT1-PGC-1 α signaling pathways. Likewise, it was additionally observed that there was a significant decrease in the lysine acetylation level of LXR α in the treated group (Fig. 5B and D; $P<0.01$), suggesting that Nam may regulate lipid levels through the SIRT1-LXR α signaling pathways.

Discussion

At present, one of the clinical concerns regarding liver regeneration is identifying effective regimens to accelerate liver regeneration following surgical resection (37,38). Conventionally, Nam has been regarded as an anti-inflammatory, anti-oxidant and anti-apoptotic agent (39). Previous studies have demonstrated that Nam may promote the expression of SIRT1, an important regulator in liver

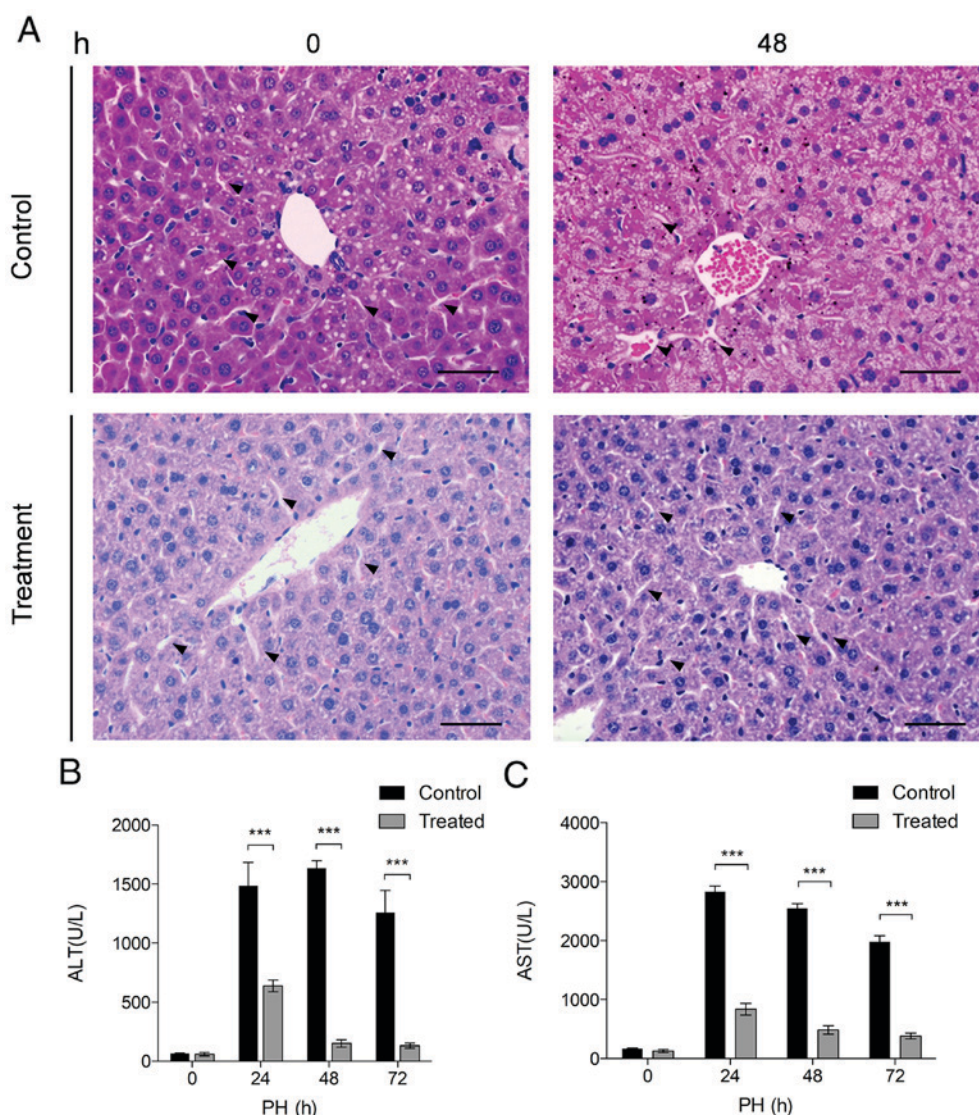


Figure 3. Damage caused by PH is repaired following treatment with Nam. (A) Hematoxylin and eosin staining demonstrated that Nam-treated livers exhibit patent sinusoids (marked with arrows) and less hepatocyte vacuolar alteration. Scale bar, 50 μ m. (B) ALT and (C) AST levels at the indicated time points following PH. All data are presented as the mean \pm standard deviation (n=3-5). ***P<0.001, as indicated. PH, partial hepatectomy; Nam, nicotinamide; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

regeneration in hepatocytes (25,33,34), indicating that Nam has the potential to promote liver regeneration. In the present study, the results demonstrated that the addition of Nam promoted hepatocyte proliferation and induced the activation of SIRT1. Furthermore, the present results are supported as Nam may accelerate skin tissue regeneration following physicochemical injuries (15,16), suggesting that Nam has the potential for application in vital tissue regeneration; however, this requires further examination.

Hepatic metabolism, including modulations in serum Glu and lipid, has been revealed to be an essential part of liver regeneration (26). Within hours of surgery, mice subjected to PH develop significant hypoglycemia when compared with the control group (40). Subsequently, adaptation by modulating the level of blood Glu occurs, including the induction of hepatic gluconeogenic machinery and the suppression of liver glycolytic activity (41). The regulation of lipid is additionally significant for liver regeneration (42). Lipid has always been considered to be a structural component of biological

membranes. Furthermore, lipid is involved in the regulation of the intermediate metabolism of different liver cell types, including hepatocytes, hepatic stellate cells and Kupffer cells (42). In the present study, it was demonstrated that Nam promoted the proliferation of hepatocytes, in addition to Glu and lipid metabolism, which was indicative of an association between metabolism and liver regeneration.

SIRT1 has been reported to serve an essential role in various metabolic processes of the liver. During the process of liver regeneration, SIRT1 has been recognized as an important regulator of metabolic circadian rhythms, which have been revealed to be closely associated with cellular proliferation (43-46). Furthermore, it was observed that SIRT1 serves a central role in maintaining proper circadian regulation of metabolic processes, including gluconeogenesis and lipid synthesis, thus affecting the process of liver regeneration (33,34). However, the detailed molecular mechanisms underlying SIRT1-mediated regulation of liver regeneration have not been fully elucidated. In the present

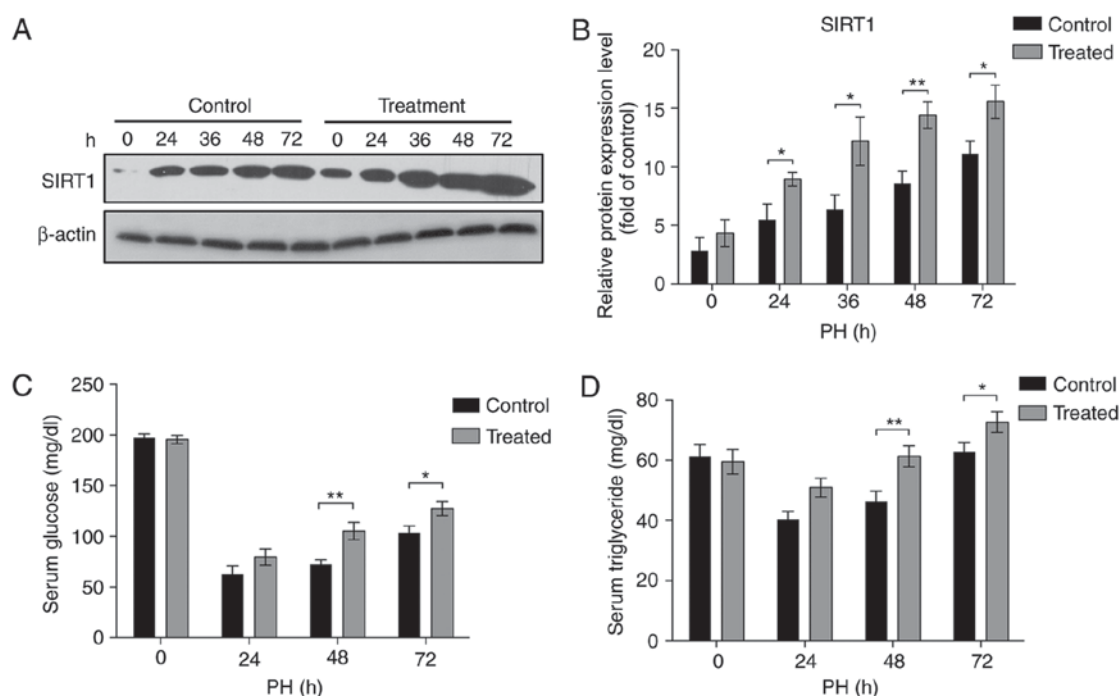


Figure 4. Expression of SIRT1 is higher in mice treated with nicotinamide compared with the control mice treated with PBS. (A) Western blots of the expressions of SIRT1 in the remnant livers following PH. (B) Quantification of SIRT1 protein expression levels. (C) Levels of glucose were determined at the indicated time points following PH. (D) Levels of triglyceride were determined at the indicated time points following PH. All data are presented as the mean \pm standard deviation (n=3-5). *P<0.05 and **P<0.01, as indicated. SIRT1, NAD-dependent protein deacetylase Sirtuin-1; PH, partial hepatectomy.

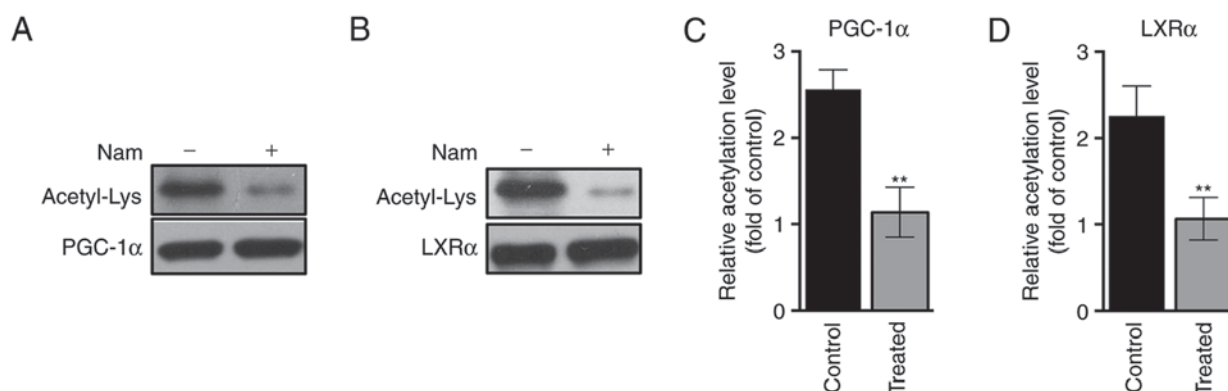


Figure 5. Acetylation levels of PGC-1 α and LXR α decrease in mice treated with Nam. Immunoprecipitation was performed to detect the acetylation of (A) PGC-1 α and (B) LXR α . Quantification of (C) PGC-1 α and (D) LXR α protein expression levels. All data are presented as the mean \pm standard deviation (n=3-5). **P<0.01 vs. control. PGC-1 α , peroxisome proliferator-activated receptor γ coactivator 1- α ; LXR α , oxysterols receptor liver X receptor- α ; Nam, nicotinamide; Acetyl-Lys, acetylated lysine.

study, it was observed that the acetylation levels of PGC-1 α and LXR α were significantly decreased upon treatment with Nam, suggesting that Nam-induced SIRT1 activation may promote liver regeneration by inducing the deacetylation of PGC-1 α and LXR α , thereby promoting gluconeogenesis and lipid synthesis during the process of liver regeneration.

The role of Nam in regulating the activation of SIRT1 remains controversial. Nam is generally accepted as the product of SIRT-catalyzed deacetylation reactions and was reported to inhibit the activity of SIRT1 (35,36). However, as Nam is the predominant precursor for cellular NAD⁺ biosynthesis via the salvage pathway (47,48), a number of previous studies have reported that Nam supplementation in cell culture increased intracellular NAD⁺ levels, leading to the activation

of SIRT1 (49,50). Our previous study demonstrated that Nam elevated the intracellular cyclic adenosine 3',5'-monophosphate (cAMP) expression level by suppressing phosphodiesterase activity, leading to downstream cAMP-dependent protein kinase and cAMP-response element binding activation, thereby inducing the upregulation of SIRT1 (25). By conducting PH in mouse models, the results of this previous study were validated; Nam may promote the expression of SIRT1 *in vivo*. The factors leading to these conflicted observations may derive from cell type, dosage and exposure duration. In the present study, the results revealed that Nam at a dose of 250 mg/kg promoted the expression of SIRT1 and activated its deacetylating ability in residual liver tissue. Based on relevant literature, it was hypothesized that SIRT1 may be upregulated by Nam at a low

dosage, and downregulated at a high dosage. However, since SIRT1-knocked-out mice could not be obtained, we were not able to clarify the specific mechanism under which Nam regulates the expression of SIRT1. Further studies are required to investigate the mechanism of how Nam modulates the expression of SIRT1 in regulating the metabolic response of the liver during regeneration.

In conclusion, the present study revealed that Nam may promote the proliferation of hepatocytes following PH and stimulate the activation of SIRT1 during liver regeneration. The present results provided insight into the potency of Nam in manipulating liver regeneration, which support a preclinical rationale to examine the clinical use of Nam for surgical resection recovery.

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Availability of data and materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Authors' contributions

H-FW, H-TL, J-XL and K-FY designed the experiments. H-FW, H-TL and LX performed the experiments. H-FW, M-HL and LL analyzed the data. H-FW, H-TL and K-FY wrote the manuscript. H-FW, K-FY and YZ revised the manuscript. YZ proposed the study concept and supervised the research. All authors reviewed the manuscript.

Ethics approval and consent to participate

Animal care and experimental protocols were in accordance with guidelines provided by the Institutional Animal Care and Use Committee of Sichuan University. Ethics approval was obtained from the Institutional Ethics Committee of Sichuan University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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