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***Wnt* Agonist Attenuates Liver Injury and Improves Survival after Hepatic Ischemia/Reperfusion**

Michael Kuncewitch^{*}, Weng-Lang Yang^{*,†}, Ernesto Molmenti^{*}, Jeffrey Nicastro^{*}, Gene F. Coppa^{*}, and Ping Wang^{*,†}

^{*}Department of Surgery, Hofstra North Shore-LIJ School of Medicine, Manhasset, New York

[†]The Feinstein Institute for Medical Research, Manhasset, New York

Abstract

The *Wnt*/ β -catenin signaling pathway is well characterized in stem cell biology and plays a critical role in liver development, regeneration, and homeostasis. We hypothesized that pharmacological activation of *Wnt* signaling protects against hepatic ischemia/reperfusion (I/R) injury through its known proliferative and anti-apoptotic properties. Sprague-Dawley rats underwent 70% hepatic ischemia by microvascular clamping of the hilum of the left and median lobes of the liver for 90 min, followed by reperfusion. *Wnt* agonist (2-amino-4-[3,4-(methylenedioxy)benzylamino]-6-(3-methoxyphenyl)pyrimidine, 5 mg/kg BW) or vehicle (20% DMSO in saline) in 0.5 ml was injected intraperitoneally (i.p.) 1 h prior to ischemia or infused intravenously over 30 min right after ischemia. Blood and tissue samples from the pre-treated groups were collected 24 h after reperfusion, and a survival study was performed. Hepatic expression of β -catenin and its downstream target gene *Axin2* were decreased after I/R while *Wnt* agonist restored their expression to sham levels. *Wnt* agonist blunted I/R-induced elevations of AST, ALT, and LDH and significantly improved the microarchitecture of the liver. The cell proliferation determined by Ki67 immunostaining significantly increased with *Wnt* agonist treatment and inflammatory cascades were dampened in *Wnt* agonist-treated animals, as demonstrated by attenuations in IL-6, myeloperoxidase, iNOS and nitrotyrosine. *Wnt* agonist also significantly decreased the amount of apoptosis, as evidenced by decreases in both TUNEL staining as well as caspase-3 activity levels. Finally, the 10-day survival rate was increased from 27% in the vehicle group to 73% in the pre-treated *Wnt* agonist group and 55% in the *Wnt* agonist post-ischemia treatment group. Thus, we propose that direct *Wnt*/ β -catenin stimulation may represent a novel therapeutic approach in the treatment of hepatic I/R.

Keywords

Wnt/ β -catenin; hepatic ischemia/reperfusion; proliferation; inflammation; nitrosative stress; apoptosis

Please address correspondence, proofs, and reprint requests to: Ping Wang, MD, Professor and Vice Chairman for Research, Department of Surgery, Hofstra North Shore-LIJ School of Medicine, 350 Community Drive, Manhasset, NY 11030, Tel: (516) 562-3411, Fax: (516) 562-1022, pwang@nshs.edu.

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INTRODUCTION

Liver damage induced by hepatic ischemia/reperfusion (I/R) is a key contributing factor to the morbidity associated with several surgical conditions and interventions including orthotopic liver transplantation, oncologic resection, trauma, and prolonged shock states. In these clinical settings, hepatocellular injury is in part due to the anoxic cell death incurred during the ischemic period. However, with the return of blood flow comes an accumulation of inflammatory cells and mediators, reactive oxygen species (ROS) and reactive nitrogen species (RNS), and the subsequent biochemical derangements in intracellular homeostasis that all work to induce further cell death from inflammation, apoptosis and necrosis (1). These events may lead to delayed graft function in the case of transplant recipients, or increase in complications, length of hospital stay, and cost of care for those experiencing I/R injury. Currently, ischemic preconditioning is the only technique proven to provide a benefit (2), and this can only be used as an intraoperative preventative measure. Accordingly, many attempts have been made to discover pharmacologic treatments to alleviate hepatic I/R injury, however none have yet proven successful, stressing the importance of developing modalities that limit I/R injury and improve patient outcomes.

The liver differs from other visceral organs in its innate ability for short-term regeneration (3). Therefore, therapy aimed at stimulating pathways involved in the proliferation of new hepatocytes to replace dead/damaged cells, or at conditioning cells to respond differently to an ischemic insult, might prove particularly beneficial in cases of hepatic I/R. One crucial pathway in cell replication and regeneration is the *Wnt*/ β -catenin signaling axis. Canonical *Wnt* signaling has been identified as central to embryonic development, progenitor cell differentiation, and proliferation of cells arising from all three germ layers (4-6). Moreover, *Wnt*/ β -catenin signaling has been shown to play a key role specifically in liver development, prevention of apoptosis, and protection from metabolic stress (7). *In utero* deletion of hepatoblast β -catenin has been shown to lead to underdeveloped livers and embryonic lethality (8). In an animal model of chemical injury with partial hepatectomy, adult hepatic stem cells, or oval cells, were also found to replicate following insult in a *Wnt*/ β -catenin-dependent manner (9). These findings suggest that perhaps pharmacologic manipulation of *Wnt* signaling plays an important role in the treatment of liver injury.

The modified pyrimidine compound 2-amino-4-[3,4-(methylenedioxy)benzylamino]-6-(3-methoxyphenyl)pyrimidine (*Wnt* agonist) was recently identified as a small-molecule agonist of the *Wnt* signaling pathway (10). Its use has to date been limited to *in vitro* and embryologic studies, where it has demonstrated the ability to upregulate the β -catenin/T-cell factor (Tcf) dependent target genes that drive mitosis (11). The aim of the present study was therefore to test the hypothesis that administration of *Wnt* agonist reduces tissue damage and apoptosis, and promotes hepatocyte regeneration and proliferation in an established animal model of hepatic I/R.

MATERIALS AND METHODS

Experimental animals

Adult male Sprague-Dawley rats (250-275 g; Charles River Laboratories, Wilmington, MA) were housed in a temperature-controlled room on a 12-hour light/dark cycle and fed a standard Purina rat chow diet. Animals were fasted overnight before undergoing surgery but allowed water *ad libitum*. All experiments were performed in accordance with the National Institutes of Health guidelines for use of experimental animals, and this study was approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research.

Animal model of hepatic I/R

On the day of surgery, rats were premedicated with intraperitoneal injection of either 0.5 ml of *Wnt* agonist (5mg/kg BW, EMD Biosciences, San Diego, CA) or vehicle (20% dimethyl sulfoxide in normal saline). One hour later, animals underwent induction of anesthesia with inhalational isoflurane, after which the ventral abdomen was shaved and cleansed with 10% povidone-iodine wash. A 3-cm midline incision was performed and the hilum of the liver was exposed, allowing for identification of the hepatic artery and portal vein. A microvascular clip was placed across the hilum of the left-lateral and median lobes in order to produce 70% hepatic ischemia. The clip was removed after 90 min to allow reperfusion, the abdomen closed, and the anesthesia withdrawn. Sham-operated animals underwent midline laparotomy alone, without hepatic ischemia or administration of treatment. Core body temperature was maintained between 35.5-37°C throughout the entirety of the operation by use of an indwelling rectal thermometer and a heating pad placed below the animals. Blood and liver samples were collected 24 h following clip removal and stored at -80°C prior to use.

Survival study

Following the process of hepatic I/R described in the animal model, the non-ischematized 30% of the liver was resected at the onset of perfusion. The animals were monitored for 10 days to record survival. Three experimental groups were created. In group 1, the rats were administered intraperitoneal *Wnt* agonist (5mg/kg BW) 1 h prior to hepatic ischemia. In group 2, the rats were administered intravenous *Wnt* agonist (5mg/kg BW) over 30 min, beginning at the onset of reperfusion. In group 3, rats were administered intraperitoneal vehicle (20% dimethyl sulfoxide in normal saline) 1 h prior to hepatic ischemia.

Western blotting analysis

Liver samples (100 mg) were lysed and homogenized in 300 μ l lysis buffer (10 mM Tris-HCl pH 7.5, 120 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) containing a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) using a sonic dismembrator on ice. Samples were centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant collected. Following measurement of sample protein concentration by Pierce BCA protein assay kit (Pierce Biotechnology, Rockford, IL), 50 μ g samples were separated on 4-12% Bis-Tris gels and transferred to nitrocellulose membranes. Membranes were incubated with primary antibody against β -catenin, iNOS, nitrotyrosine, or β -actin (Santa Cruz BioTechnologies, Santa Cruz, CA). All protein bands were detected by species-specific infrared fluorescence secondary antibodies (1:10,000) and analyzed by the LI-COR Odyssey Fc Imager (LI-COR, Lincoln, NE).

Determination of serum liver enzymes and IL-6

Whole blood samples were centrifuged at 4,000 rpm for 12 min to collect serum, which was then stored at -80°C prior to use. The activities of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were determined by commercial assay kits from Pointe Scientific (Lincoln Park, MI). Serum IL-6 levels were determined by an enzyme-linked immunosorbent assay kit specific for rat IL-6 (BD Biosciences, San Diego, CA). The assays were carried out according to the instructions provided by the manufacturer.

Real-time RT-PCR analysis

Total RNA was extracted from the liver by "TRIzol reagent" (Invitrogen, Carlsbad, CA). Real-time PCR was carried out on cDNA samples which were reversely transcribed from 2 μ g RNA using murine leukemia virus reverse-transcriptase (Applied Biosystems, Foster

City, CA). A PCR reaction was carried out in a 24 μ l final volume containing 0.08 μ mol of each forward and reverse primer, 2 μ l cDNA, 9.2 μ l H₂O and 12 μ l SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA). Amplification was conducted in an Applied Biosystems 7300 real-time PCR machine under the thermal profile of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The level of mouse β -actin mRNA was used for normalization and each specific mRNA was conducted in duplicate. Relative expression of mRNA was calculated by the $2^{-\Delta\Delta C_t}$ method and results were expressed as fold change in comparison to control group. The sequence of primers for this study is listed as follow. Rat Axin2, 5'-GAC CGA CGA TTC CAT GTC C-3' (forward) and 5'-CCA GCT CCA GTT TCA GCT TC-3' (reverse). Rat IL-6, 5'-AGG GAG ATC TTG GAA ATG AGA AAA-3' (forward) and 5'-CAT CAT CGC TGT TCA TAC AAT CAG-3' (reverse). Rat β -actin, 5'-CGT GAA AAG ATG ACC CAG ACT A-3' (forward) and 5'-TGG TAC GAC CAG AGG CAT ACA G-3' (reverse).

Hepatic myeloperoxidase assessment

Liver tissue (100 mg) was homogenized in 1 ml of KPO₄ buffer containing 0.5% hexadecyltrimethyl-ammonium bromide by sonication and incubated at 60°C for 2 h. Samples were centrifuged to collect the supernatant, and then measured for protein concentration. The reaction was carried out in a 96-well plate by adding samples into phosphate buffer containing o-dianisidine hydrochloride and H₂O₂. Light absorbance was read at 460 nm over a period of 5 minutes. MPO activity (1 unit was equal to the change in absorbance per min) was expressed as units per gram of protein.

Histologic evaluation of liver injury

Liver biopsies were taken from the median lobe following 24 h of reperfusion and stored in 10% formalin before being fixed in paraffin. Biopsies were then sectioned to 4 μ m cuts and stained with hematoxylin-eosin. Liver parenchymal injury was then assessed in a blinded fashion using a semi-quantitative light microscopy evaluation. The histologic injury score for each sample was expressed as the sum of the individual scores given for 6 different parameters: cytoplasmic color fading, vacuolization, nuclear condensation, nuclear fragmentation, nuclear fading, and erythrocyte stasis (12). Scores for each finding ranged from 0 (0%), to 1 (1-10%), 2 (10-50%), or 3 (>50%), with a highest possible score of 18. Each sample score was then averaged over 10 microscopic fields.

Immunostaining of Ki67 and TUNEL

To determine the proliferative status of hepatocytes in our study, we stained liver tissues for Ki67, which is a nuclear protein strictly present in proliferating cells and absent from resting/G₀ cells (13). Paraffin-embedded sections were dewaxed in xylene and rehydrated in a graded series of ethanol. For Ki67 staining, slides were incubated in 0.92% citric acid buffer (Vector Laboratories, Burlingame, CA) at 95°C for 15 min. After cooling to room temperature, the slides were incubated with 2% H₂O₂ in 60% methanol and blocked in 2% normal rabbit serum/TBS, after which they were incubated with goat anti-Ki67 antibody (1:50, Santa Cruz Biotechnologies) in 1% normal rabbit serum/TBS with 0.02% Triton X-100 at 4°C overnight. The detection was carried out as per the instructions provided by a commercially available immunohistochemistry kit with NovaRED substrate (Vector Laboratories). For TUNEL staining, fluorescence staining was performed using a commercially available In Situ Cell Death Detection Kit (Roche). The assay was conducted according to the manufacturer's instructions. The nucleus was stained with propidium iodide. Results were expressed as the average number of Ki67 or TUNEL positive staining cells per 10 microscopic fields.

Determination of tissue caspase-3 activity

Liver tissue was homogenized in a lysis buffer consisting of 10 mM Hepes (pH 7.4), 5 mM MgCl₂, 1 mM DTT, 1% Triton-X 100, 2 mM EGTA, 2mM EDTA, and a protease inhibitor cocktail by sonication. After centrifugation, the supernatant was collected and measured for protein concentration. Cell lysate and a caspase-3 substrate peptide, Ac-DEVD-AMC (BD Biosciences, San Diego, CA), was added to the assay buffer (20 mM Hepes (pH 7.4), 5 mM DTT, 2 mM EDTA, and 0.1% CHAPS) and incubated in the dark at 37°C for 75 min, after which a fluorometric reader was used to determine the cleaved product at the excitation (370 nm) and emission (450 nm). Results were divided by corresponding protein concentrations, and caspase-3 activity was expressed as relative fold change to the sham group.

Statistical analysis

All data are expressed as a mean \pm SE (n=4-6/group) and compared by one-way analysis of variance (ANOVA) and the Student-Newman-Keuls (SNK) test. Survival rate was determined by the Kaplan-Meier estimator and compared by a log-rank test (n=15-20/group). Differences in values were considered significant if $P < 0.05$.

RESULTS

Wnt agonist upregulates β -catenin signaling after hepatic I/R

To investigate whether *in vivo* Wnt agonist administration had any effect on β -catenin signaling, we measured hepatic protein levels of β -catenin and its downstream target gene 24 h after hepatic I/R. Compared to sham-operated animals, the vehicle-treated group had a 53% decrease of β -catenin protein levels in liver tissue (Fig. 1A). Hepatic β -catenin protein levels were restored to 88% of sham levels with administration of Wnt agonist (Fig. 1A). In a similar fashion, hepatic I/R resulted in a 49% decrease in gene transcription of *Axin2*, a known target gene of Wnt/ β -catenin signaling (14), compared to sham-operated animals, which was restored to 91% in the Wnt agonist-treated group (Fig. 1B). These findings suggest that administration of Wnt agonist effectively upregulates Wnt/ β -catenin signaling in the liver which is suppressed under I/R stress.

Wnt agonist attenuates liver tissue injury after hepatic I/R

Hepatocellular damage was surveyed by measuring serum AST, ALT, and LDH levels, which increased by 97-, 83-, and 10-fold, respectively, 24 h after hepatic I/R (Fig. 2). Contrastingly, treatment with Wnt agonist prior to I/R significantly reduced injury levels by 63%, 57%, and 76%, respectively (Fig. 2). This data correlated with the alterations in tissue architecture observed histologically. At 24 h after reperfusion, vehicle-treated livers demonstrated severe architectural abnormalities, most notably diffuse cellular edema and necrosis, micro-hemorrhage, and leukocyte infiltration (Fig. 3B). In contrast, liver tissue architecture was remarkably preserved in Wnt agonist treated animals, with moderate hepatocellular edema being the predominant difference when compared to the livers of sham-operated animals (Figs. 3A and C). As quantified in Fig. 3D, animals undergoing I/R with vehicle treatment exhibited a significant increase in the liver histologic injury score when compared to sham-operated animals, which was reduced by 63% with administration of Wnt agonist. Together, these results demonstrate that Wnt agonist administration confers a significant protection against liver injury from hepatic I/R.

Wnt agonist promotes hepatocyte proliferation after hepatic I/R

In order to determine the effect of Wnt agonist administration on hepatocyte proliferation after I/R injury, we performed immunohistochemical staining against Ki67. As shown in Figs. 4A-C, minimal immunostaining of Ki67 was seen in liver tissue sections of either the

sham or vehicle groups, but was markedly increased after hepatic I/R with *Wnt* agonist treatment. With quantification by counting, I/R with vehicle administration resulted in a 66% reduction in Ki67 positive staining cells, compared to sham-operated animals (Fig. 4D). However, the number of Ki67 positive staining cells was increased 11.5-fold when *Wnt* agonist was given (Fig. 4D). These results indicate that upregulation of *Wnt*/ β -catenin signaling was able to promote cellular proliferation despite the presence of physiologic insult.

Wnt agonist lowers nitrosative stress after hepatic I/R

In order to determine the effect of *Wnt* agonist on nitrosative burden following I/R, hepatic tissue levels of iNOS and nitrotyrosine were evaluated 24 h after I/R. Compared to the sham group, we observed a 23-fold increase in iNOS protein expression in vehicle-treated I/R animals, which was reduced by 83% with administration of *Wnt* agonist (Fig. 5A). There was an associated 2.5 fold increase in nitrotyrosine levels in vehicle-treated I/R animals when compared to sham-operated animals. This was decreased by 37% in animals treated with *Wnt* agonist (Fig. 5B).

Wnt agonist reduces the inflammatory response and neutrophil infiltration into the liver after hepatic I/R

IL-6 levels in both serum and the liver were measured to ascertain the ability of *Wnt*/ β -catenin activation the systemic and local inflammatory responses to hepatic I/R, respectively. At 24 h after reperfusion, vehicle-treated animals had circulating levels of IL-6 that were 16-fold greater than their sham-operated counterparts (Fig. 6A). This increase in serum IL-6 levels was decreased by 88% when animals were administered *Wnt* agonist (Fig. 6A). In the liver, hepatic I/R resulted in a 449-fold increase of IL-6 mRNA expression in comparison to sham, which was decreased by 97% when *Wnt* agonist was administered (Fig. 6B). An additional means by which we assessed the inflammation associated with I/R was measuring alterations in hepatic neutrophil infiltration 24 h after reperfusion. MPO activity was used as an indicator of neutrophil migration and subsequent proteolytic inflammation. When compared to the sham group, vehicle-treated animals showed a 25-fold increase in hepatic tissue levels of MPO (Fig. 6C). This was reduced by 91% with *Wnt* agonist administration (Fig. 6C). Together, these data suggest that *Wnt* agonist administration led to a downregulation of proinflammatory markers and the associated neutrophil recruitment.

Wnt agonist reduces apoptosis after hepatic I/R

To investigate whether *Wnt* agonist administration had any effect on apoptosis following hepatic I/R, we first conducted the TUNEL assay to detect the DNA fragmentation in the liver tissues of each group 24 h after reperfusion. The TUNEL positive cells (green spot) were sparsely observed in the sham animals, whereas there were many green spots in the vehicle-treated animals (Fig. 7A and B). With *Wnt* agonist administration, the TUNEL positive cells became hardly detectable in the liver (Fig. 7C). The number of countable apoptotic cells increased dramatically in hepatic I/R with vehicle in comparison to sham, and was returned to sham levels with administration of *Wnt* agonist (Fig. 7D). Furthermore, we measured the activity of caspase-3 in the liver. We observed a 91% increase in caspase-3 activity in the vehicle-treated I/R group in comparison to the sham-operated group, which was reduced by 39% with administration of *Wnt* agonist (Fig. 7E). Although this significant attenuation of apoptosis by *Wnt* agonist was observed, the number of apoptotic cells and magnitude of caspase-3 activation were not markedly elevated in the vehicle group. From the analysis of hematoxylin-eosin staining, the majority of hepatocytes died from necrosis. Thus, protection of hepatocytes from apoptosis may not be the primary activity of *Wnt* agonist in reducing hepatic I/R injury.

Wnt agonist improves survival following a lethal model of hepatic I/R

To determine the potential survival benefit of *Wnt* agonist treatment, we used a total hepatic I/R model as previously described (15). We observed that only 27% of vehicle-treated animals survived 10 days after undergoing I/R with partial hepatectomy. Contrastingly, the 10-day survival rate increased significantly to 73% in the group that was treated with *Wnt* agonist 1 h prior to the onset of ischemia and to 55% in those animals receiving *Wnt* agonist at the onset of reperfusion (Fig. 8), with no statistical difference between these two treatment groups ($P = 0.34$).

DISCUSSION

As advances in surgery and critical care allow for more aggressive interventions and successful resuscitations, and as the annual number of orthotopic liver transplants performed increases, so too does the number of patients at risk for hepatic I/R injury. For this reason, research aimed at the prevention and treatment of this pathology has garnered considerable attention. One molecular pathway that has demonstrated promise in animal models is *Wnt*/ β -catenin signaling. We herein report the effect of canonical *Wnt*/ β -catenin activation through a novel *Wnt* agonist on liver injury and survival in a rat model of hepatic I/R. In untreated animals, 90 min of 70% hepatic I/R resulted in dramatic increases in pro-inflammatory cytokines, apoptosis, and associated hepatocellular injury. Conversely, through pharmacologic manipulation of *Wnt* activation these damage indices were significantly attenuated and the mortality rate was significantly reduced in a lethal model of hepatic I/R.

In the *Wnt* signaling cascade, when endogenous *Wnt* ligands bind their G protein-coupled cell surface receptors, *frizzled*, cytoplasmic *dishevelled* is able to inactivate a β -catenin destruction complex that includes APC, Axin, and GSK-3 β . This complex works to phosphorylate β -catenin, effectively tagging it for proteasomal destruction. *Wnt* ligand signaling thus leads to stabilized cytoplasmic β -catenin that is free to translocate to the nucleus, where it communicates with the transcription factors T-cell factor/lymphoid enhancer factor (Tcf/Lef) to drive downstream target gene expression (16). These genes include c-myc, cyclin-d1, and axin2, which regulate cell cycle progression, amongst other actions (17). Our study proved consistent with these findings, as we observed that the *Wnt* agonist compound was able to reverse the downregulation in β -catenin protein and axin2 mRNA that occurred as a result of I/R injury, indeed restoring them to normal levels. The direct association between β -catenin and axin2 is supported by the findings in other studies that increased axin2 levels indicate an upregulation of *Wnt*/ β -catenin signaling (18). Axin2, also referred to as axil or conductin, acts as safety valve in *Wnt*/ β -catenin signaling through its ability to phosphorylate/ubiquitinate cytosolic β -catenin and thereby act as a negative-feedback regulator in the prevention of unregulated β -catenin gene transcription (19).

One of the striking phenomena observed in the present study is that despite the severe cellular insult caused by 90 min of warm hepatic I/R, animals treated with *Wnt* agonist had an abundance of hepatocytes staining with Ki67, indicating that these cells were proliferating instead of suffering from apoptosis or necrosis. Although the liver is unique amongst visceral organs in its ability to rapidly regenerate almost all of its mass after partial hepatectomy or metabolic stress (3), we did not observe this in vehicle-treated animals following I/R, which was likely due to the severity of our model. The presence of proliferating hepatocytes in our *Wnt* treatment group was consistent with other studies, in which activation of *Wnt*/ β -catenin signaling has demonstrated to be associated with proliferative and regenerative activities (20). For example, Terada and colleagues demonstrated that *Wnt*/ β -catenin signaling plays a key role in cellular regeneration after renal I/R injury by stimulating renal tubule cell cycle progression (21). Nejak-Bowen, et al, found that transgenic mice with overexpression of hepatic β -catenin levels showed more

hepatocytes in the S-phase following partial hepatectomy than in wild-type mice (22). They further confirmed that this phenomenon was depended on β -catenin by activating β -catenin signaling with administration of *Wnt-1* naked DNA in wild-type mice. Similarly, Lehwald, et al, observed that liver-specific β -catenin knock-down mice had increased hepatic injury following I/R, whereas transgenic *Wnt-1* overexpressing mice were resistant to hepatic I/R injury (23). The authors attributed this to an increase in hypoxia inducible factor-1 α (HIF-1 α) activation by β -catenin. The role of HIF-1 α in mediating *Wnt* agonist activity during hepatic I/R requires further investigation.

In addition to conditioning hepatocytes to proliferate in the presence of I/R injury, *Wnt* agonist administration also decreased the cellular stress caused by I/R. Nitrosative stress is increasingly being recognized as playing an important role in the cellular damage associated with I/R injury (24). When iNOS is upregulated in I/R injury the excessive nitric oxide (NO) produced is free to react with superoxide anion (O₂⁻), creating peroxynitrite (ONOO⁻). Peroxynitrite then contributes to injury through lipid peroxidation, apoptosis, necrosis, and neutrophil recruitment by nitration of tyrosine residues on tissue proteins (25). Therapies aimed at iNOS inhibition have been suggested as therapeutic targets in hypoxia-induced injury settings in part because iNOS inhibition leads to decreased tissue caspase-3 activity, and therefore decreased apoptosis (26). Indeed, we observed that *Wnt* stimulation in hepatic I/R lead to decreased iNOS levels, which was associated with a decrease in caspase-3 activity and number of apoptotic cells. We believe this to be significant as previous studies have demonstrated that apoptosis is a key contributor to injury following hepatic I/R, and that therapies aimed at reducing apoptosis have proven to be hepatoprotective (27).

Inflammatory cascades also contribute to the tissue damage during I/R. We demonstrated that *Wnt* agonist administration significantly inhibited the production of proinflammatory cytokines at local and systemic levels after hepatic I/R. Activation of β -catenin signaling by other molecules has also demonstrated the anti-inflammatory properties of this pathway. R-spondin 1, a naturally secreted protein that shares similar receptor-binding properties with *Wnt* ligands, has demonstrated anti-inflammatory and osteoblastogenic properties in bone and joint disease and has been observed to decrease IL-6 and inflammation in an animal model of colitis (28). Moreover, inhibition of dickkopf1 protein, a *Wnt* signaling antagonist, has been associated with decreased inflammation, decreased apoptosis, and earlier recovery of gut mucosa in a separate animal model of colitis (29). This decrease in inflammation coincided with a greatly reduced level of MPO in liver tissue, suggesting the prevention of neutrophil infiltration into the liver following I/R with *Wnt* agonist treatment.

By dampening the severe tissue damage associated with hepatic I/R, *Wnt* agonist provided a significant survival advantage to treated animals. We found that pharmacologic activation of *Wnt* signaling lead to increased survival rates in animals treated either before or after the ischemic insult when partial hepatectomy was performed following hepatic I/R. To our knowledge, this represents the first study in which upregulation of *Wnt*/ β -catenin signaling has demonstrated an *in vivo* survival benefit when administered during the reperfusion phase of I/R. However, the activation of *Wnt*/ β -catenin signaling has been linked to tumorigenesis (30). Thus, it may generate a concern of promoting cancer when applying the *Wnt* agonist for long-term treatment. In this study with a single dose administration, we have observed a beneficial effect of *Wnt* agonist on the 10-day survival. Whether one-time activation of *Wnt*/ β -catenin signaling can lead to cancer requires a long-term follow-up.

In addition, while it is always a plus to have a detailed time-course study to follow through all the changes after I/R, in animal models of hepatic I/R injury, the tissue damage, serum levels of organ injury markers and inflammation can be well detected 24 hours after I/R. So we used this time point to do all the correlated measurements in this study. We observed a

significant increase of *Wnt* target gene expression and Ki67 staining, and a reduction of nitrosative stress and inflammation by the *Wnt* agonist treatment at 24 h, so we did not further pursue the effect of *Wnt* agonist on all these parameters at earlier time points, such as 6 hours.

There are several approaches to manipulating the canonical *Wnt* signaling pathway, including *frizzled* surface receptor binding, targeting the *Wnt*-axin relationship, GSK-3 β inhibition, and *Wnt* target gene regulation. In particular, pharmacologic inhibition of GSK-3 β to stabilize β -catenin has been applied to animal models of hepatic I/R, renal I/R, hemorrhage, and sepsis, and has shown protective effects (31-34). However, the serine/threonine protein kinase GSK-3 β has been implicated in multiple signaling pathways apart from *Wnt*/ β -catenin, ranging from metabolic pathways to structural proteins and cell-cycle transcription factors (35). Such non-specific effects of targeting GSK-3 β in order to regulate *Wnt* signaling will limit this approach on clinical use. The *Wnt* agonist that we used in this study has been well demonstrated to have no effect on GSK-3 β activity. Therefore, it reduces the off-target effect of activating *Wnt* signaling through GSK-3 β .

In summary, administration of *Wnt* agonist to animals undergoing hepatic I/R was able to increase hepatocyte proliferation, decrease inflammation, and decrease apoptosis and necrosis. In addition, it offered a distinct survival advantage in a lethal hepatic I/R model when it was administered before or after the ischemic insult. It did so through upregulating β -catenin gene transcription, decreasing inflammatory cascades, and decreasing iNOS-dependent nitrosative tissue damage. We therefore propose that the findings in our study using a novel compound as a *Wnt*-signaling agonist provide a new strategy in the prevention and treatment in hepatic I/R injury incurred in a variety of clinical settings.

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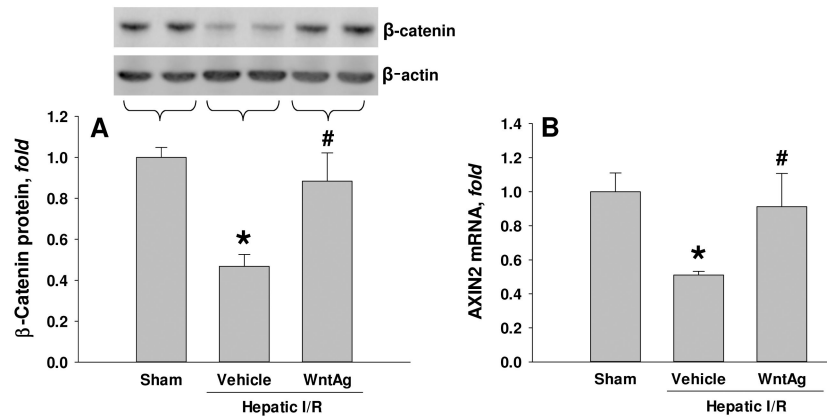


Fig. 1. Effect of *Wnt* agonist on β -catenin and its downstream gene expression after hepatic I/R Liver tissues were harvested 24 h after reperfusion from the sham, vehicle, and *Wnt* agonist-treated (*WntAg*) groups. (A) Total cell lysate were subjected to Western blotting. Representative blots against β -catenin and β -actin are shown. Blots were scanned and quantified with densitometry. Band intensity of β -catenin was normalized to the corresponding band intensity of β -actin. (B) Total RNA was isolated for determining *Axin2* mRNA levels by real-time RT-PCR analysis. *Axin2* expression levels were normalized to β -actin. The value in sham group is designated as 1 for comparison. Data presented as means \pm SE (n=4-6/group) and compared by one-way ANOVA and SNK method; * $P > 0.05$ vs. Sham; # $P > 0.05$ vs. Vehicle.

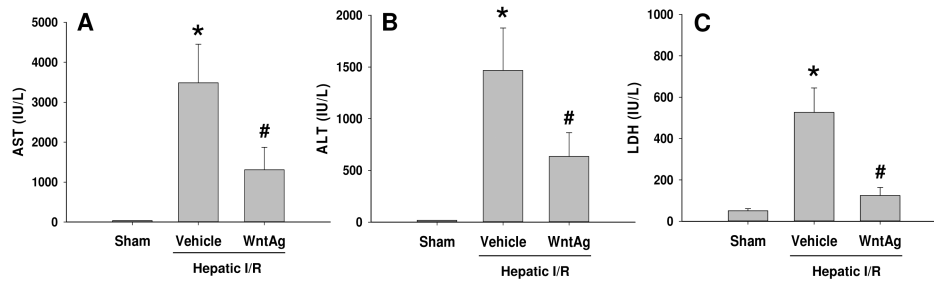


Fig. 2. Effect of *Wnt* agonist on liver tissue injury after hepatic I/R

Serum samples were collected 24 h after reperfusion from the sham, vehicle, and Wnt agonist-treated (WntAg) groups for measuring AST (A), ALT (B), and LDH (C). Data presented as means \pm SE (n=4-6/group) and compared by one-way ANOVA and SNK method; * $P > 0.05$ vs. Sham; # $P > 0.05$ vs. Vehicle.

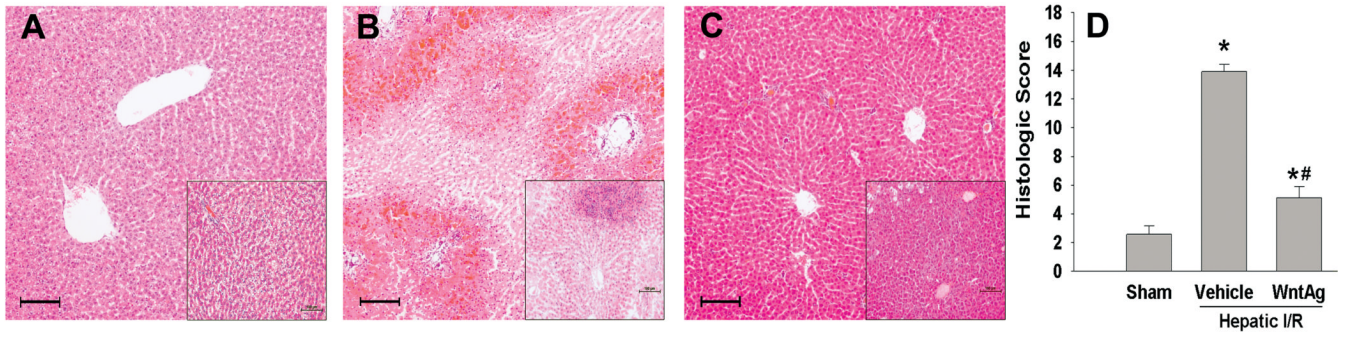


Fig. 3. Effect of *Wnt* agonist on tissue damage and cellular architecture in the liver after hepatic I/R

Histological findings of the liver in the sham (A), vehicle (B), and *Wnt* agonist-treated (C, WntAg) groups. Liver tissues were harvested 24 h after reperfusion, processed, and stained with hematoxylin-eosin. Representative photomicrographs at 100× magnification with 200× magnification insets. Bar length equal to 100 micrometers. (D) Semi-quantitative histologic injury score measuring differences in cytoplasmic vacuolization, cytoplasmic fading, nuclear condensation, nuclear fading, nuclear fragmentation, and erythrocyte stasis examined on standard hematoxylin-eosin staining as described in Materials and Methods. Data presented as means ± SE (n=4-6/group) and compared by one-way ANOVA and SNK method; * $P > 0.05$ vs. Sham; # $P > 0.05$ vs. Vehicle.

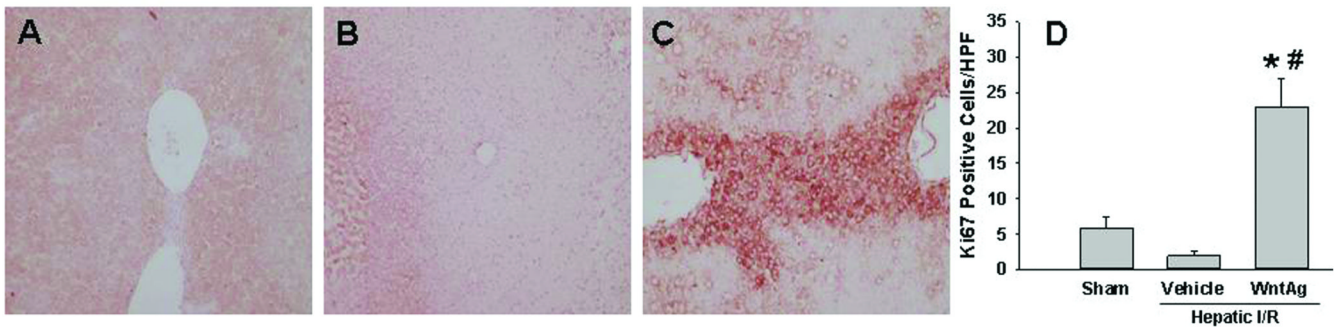


Fig. 4. Effect of *Wnt* agonist on hepatocyte proliferation after hepatic I/R

Liver tissues were harvested 24 h after reperfusion, processed, and subjected to immunohistochemical analysis against Ki67 with red staining. Representative images for sham (A), vehicle (B), and *Wnt* agonist-treated (C, WntAg) groups are shown at 100× magnification. (D) A graphical representation of Ki67 positive staining cells averaged over 10 microscopic fields/animal. Data presented as means ± SE (n=4-6/group) and compared by one-way ANOVA and SNK method; * $P > 0.05$ vs. Sham; # $P > 0.05$ vs. Vehicle.

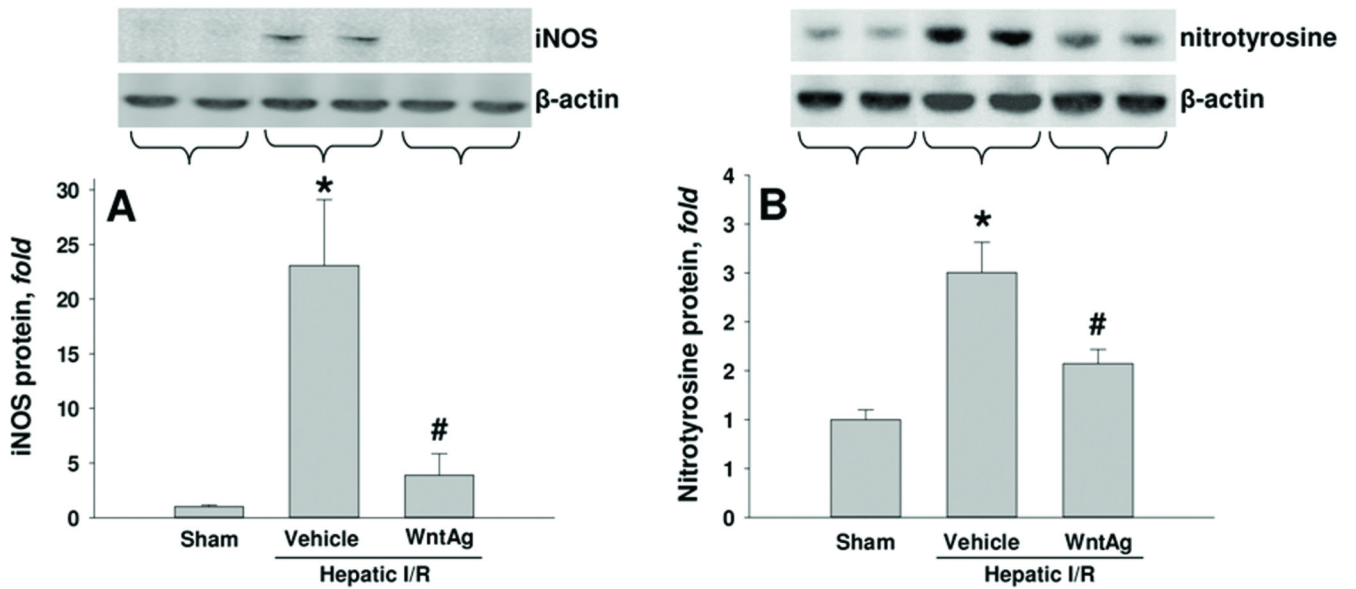


Fig. 5. Effect of *Wnt* agonist on iNOS and nitrotyrosine expression after hepatic I/R

Liver tissues were harvested 24 h after reperfusion from the sham, vehicle, and *Wnt* agonist-treated (*WntAg*) groups. Total cell lysate were subjected to Western blotting. Representative blots against (A) iNOS and corresponding β -actin (B) nitrotyrosine and corresponding β -actin are shown. Blots were scanned and quantified with densitometry. Band intensity of iNOS or nitrotyrosine was normalized to the corresponding band intensity of β -actin. Data presented as means \pm SE (n=4-6/group) and compared by one-way ANOVA and SNK method; * $P > 0.05$ vs. Sham; # $P > 0.05$ vs. Vehicle.

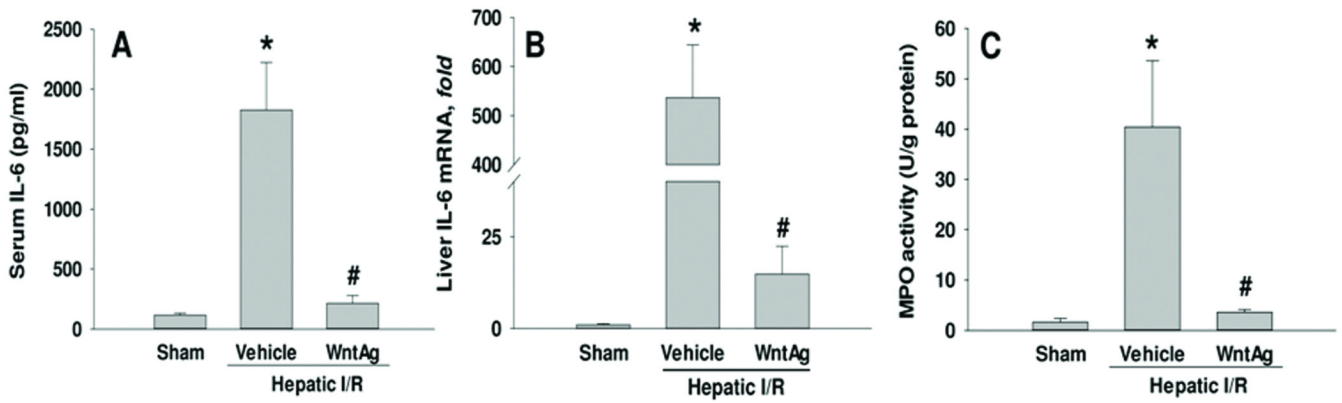


Fig. 6. Effect of *Wnt* agonist on the proinflammatory cytokine expression and neutrophil infiltration into the liver after hepatic I/R

Serum and liver tissues were harvested 24 h after reperfusion from the sham, vehicle, and *Wnt* agonist-treated (*WntAg*) groups. (A) Serum IL-6 levels were determined by ELISA. (B) Liver tissue IL-6 mRNA expression was determined by real time RT-PCR analysis. IL-6 expression levels were normalized to β -actin. The value in sham group is designated as 1 for comparison. (C) Liver tissue myeloperoxidase (MPO) activity, a marker for neutrophil infiltration, was determined spectrophotometrically. Data presented as means \pm SE (n=4-6/group) and compared by one-way ANOVA and SNK method; * $P > 0.05$ vs. Sham; # $P > 0.05$ vs. Vehicle.

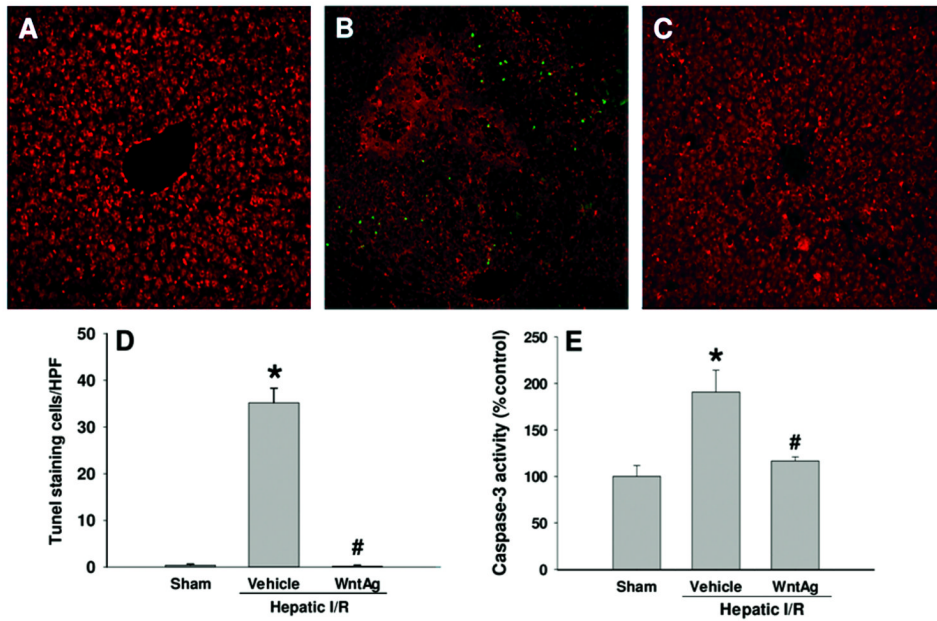


Fig. 7. Effect of *Wnt* agonist on induction of apoptosis after hepatic I/R

Liver tissues were harvested 24 h after reperfusion. (A-C) Liver sections were subjected to TUNEL assay to detect DNA fragmentation. Representative images for sham (A), vehicle (B), and *Wnt* agonist-treated (C, WntAg) groups. *Green*, TUNEL positive and *red*, propidium iodide staining for nucleus. (D) A graphical representation of TUNEL positive staining cells averaged over 10 microscopic fields/animal. (E) Liver tissue caspase-3 activity was measured by a fluorometric assay. Data presented as means \pm SE (n=4-6/group) and compared by one-way ANOVA and SNK method; * $P > 0.05$ vs. Sham; # $P > 0.05$ vs. Vehicle.

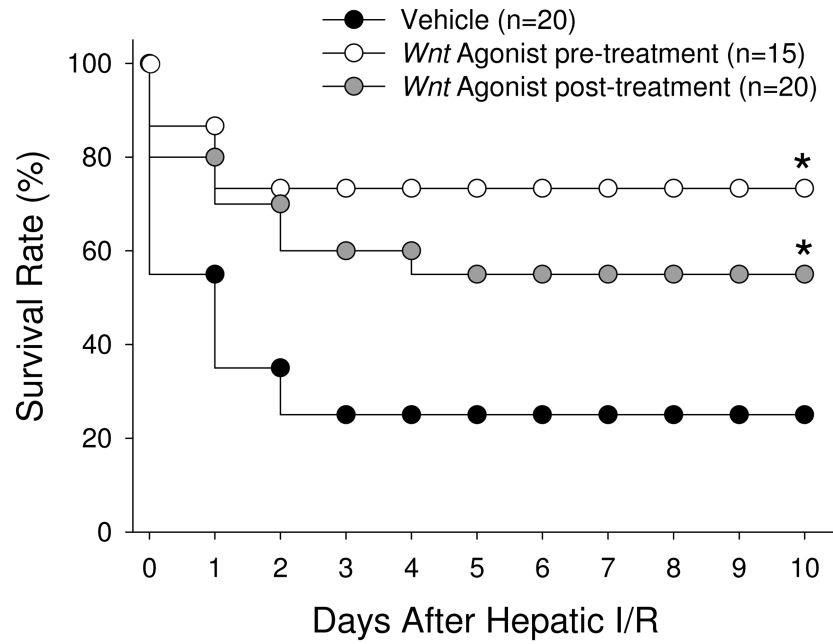


Fig. 8. Effect of pre- and post-administration of *Wnt* agonist on survival of a lethal model of hepatic I/R

Survival rates over a 10-day period following 70% hepatic I/R with resection of the remaining 30% of liver tissue are demonstrated for vehicle (black), pre-treatment (grey), and post-treatment (white) groups. Survival rates were analyzed by the Kaplan-Meier estimator using a log-rank test. * $P > 0.05$ vs. Vehicle.