**Schisandra sphenanthera Extract Facilitates Liver Regeneration after Partial Hepatectomy in Mice**

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**ABSTRACT**

Liver regeneration after surgical liver resection is crucial for the restoration of liver mass and the recovery of liver function. *Schisandra sphenanthera* extract (Wuzhi tablet, WZ) is a preparation of an extract from the dried ripe fruit of *Schisandra sphenanthera* Rehd. et Wils, a traditional hepatoprotective herb. Previously, we found that WZ could induce liver regeneration-related genes against acetaminophen-induced liver injury. However, whether WZ can directly facilitate liver regeneration after liver resection remains unknown. We investigated whether WZ has potential in promoting liver regeneration after a partial hepatectomy (PHX) in mice. Remnant livers were collected 1, 1.5, 2, 3, 5, 7, and 10 days after PHX. Hepatocyte proliferation was assessed using the Ki-67 labeling index. Western blot analysis was performed on proteins known to be involved in liver regeneration. The results demonstrated that WZ significantly increased the liver-to-body weight ratio of mice after PHX but had no effect on that of mice after a sham operation. Additionally, the peak hepatocyte proliferation was observed at 1.5 days in PHX/WZ-treated mice but at 2 days in PHX/saline-treated mice, as evidenced by the Ki-67 positive ratio. Furthermore, WZ significantly increased the protein expression of ligand-induced phosphorylation of epidermal growth factor receptor and up-regulated cyclin D1, cyclin D-dependent kinase 4, phosphorylated retinoblastoma, and proliferating cell nuclear antigen protein expression and down-regulated the expression of cell cycle inhibitors p21 and p27 in the regenerative process after PHX. These results demonstrate that WZ significantly facilitates hepatocyte proliferation and liver regeneration after PHX.

**Introduction**

The liver has a tremendous capacity to regenerate after toxin-induced injury or surgical resection (Fausto et al., 2006). The remaining hepatocytes can quickly proliferate to restore the mass of the organ until the liver reaches its normal size and weight and ultimately regains normal liver structure and function (Fausto and Riehle, 2005). Thus, liver regeneration is technically a process of compensatory growth of the liver. A partial hepatectomy (PHX) is a curative treatment of hepatic carcinoma and end-stage liver disease in a clinical setting. It is also used for living donor liver transplantation (LDLT), which relies on the tremendous ability of residual liver tissue to regenerate (Belghiti et al., 2000; Schindl et al., 2005). For these reasons, therapeutic interventions that can promote liver growth after PHX are of clinical importance.

*Schisandra sphenanthera* (also known as “Nan wuweizhi”), which is derived from the dried ripe fruit of *Schisandra sphenanthera* Rehd. et Wils., is a well-known traditional Chinese herb that is widely used as a tonic or adjuvant drug in Asia (Panossian and Wikman, 2008). Numerous studies have demonstrated the potent, beneficial effect of *Schisandra sphenanthera* on immunity and heart and kidney function as well as for hepatoprotection against chemical hepatitis and various hepatotoxins (Zhu et al., 2000; Panossian and Wikman, 2008; Teraoka et al., 2012). Wuzhi tablet (WZ), an ethanol extract preparation of *Schisandra sphenanthera*, is commonly used for the treatment of chronic viral hepatitis and liver dysfunction in a clinical setting, and is indexed in the pharmacopoeia of the People’s Republic of China (Fan et al., 2014). The six main active components of WZ were identified in our previous study: Schisandrin A, Schisandrin B, Schisandrin C, Schisandrol A, Schisandrol B, and Schisantherin A (Qin et al., 2014).

Recently, we reported that WZ exerted significant hepatoprotection against acetaminophen-induced liver injury and may promote compensatory liver regeneration, as it induced the expression of hepatocyte proliferation-related genes (Bi et al., 2013; Fan et al., 2015). However, the effects of WZ on liver regeneration after a two-thirds partial hepatectomy have not been determined. Therefore, we investigated whether WZ has the potential to promote liver regeneration after PHX in mice, and we explored the possible molecular mechanisms that are involved.

**Materials and Methods**

**Chemicals and Reagents.** Wuzhi tablets with 7.5 mg of Schisantherin A per tablet were supplied by Fanglue Pharmaceutical Company (Guangxi, People’s Republic of China). Primary antibodies including Ki-67, epidermal growth factor receptor (EGFR), phosphorylated EGFR (p-EGFR), p27, proliferating cell nuclear antigen (PCNA), cyclin D1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were purchased from Cell Signaling Technology (Danvers, MA). All other antibodies including p21 were obtained from Santa Cruz Biotechnology (Burlingame, CA).

**ABBREVIATIONS:** CDK, cyclin D-dependent kinase 4; EGFR, epidermal growth factor receptor; LDLT, living donor liver transplantation; PCNA, proliferating cell nuclear antigen; PHX, partial hepatectomy; p-Rb, phosphorylated retinoblastoma; WZ, Wuzhi tablet.
Biotechnology (Santa Cruz, CA). Phosphorylated retinoblastoma (p-Rb) was purchased from Shanghai Sangon Biotech (Shanghai, People’s Republic of China). All secondary antibodies were provided by Cell Signaling Technology.

**Animals.** Male C57BL/6J mice (8–9 weeks, 22–24 g) were purchased from the Guangdong Animal Experimental Centre (Guangzhou, People’s Republic of China) and bred in standard animal laboratories. All mice were housed on a 12-hour light/dark cycle with free access to standard food and water. All animal experimental protocols were approved by the Ethics Committee on the Animal Care and Use of Laboratory Animals at Sun Yat-sen University (ref. no 2014-156XS).

**Partial Hepatectomy.** A two-thirds hepatectomy (PHX) in mice has become one of the most popular models to understand the underlying molecular mechanisms involved during the process of liver regeneration. We performed the two-thirds PHXs according to standard procedures as previously described elsewhere (Hu et al., 2014). The mice were anesthetized by inhalation of ethyl ether for approximately 10 to 30 seconds and then were fixed to the operating board. The abdominal area was sterilized with 75% ethanol, and the peritoneum and outer skin were retracted using 7.5 × 1.75 mm Michel suture clips to close the incision, and the mice were kept on a heating pad before being transferred back into the cages. The partial hepatectomy procedures were completed within 5 to 7 minutes. The animals fully awakened 1 to 3 minutes after the partial hepatectomy.

**Treatment and Sample Preparation.** All mice were randomly divided into four groups: 1) sham/saline group, 2) sham/WZ (350 mg/kg) group, 3) PHX/saline group, and 4) PHX/WZ (350 mg/kg) group. The WZ solution was prepared as described previously elsewhere (Qin et al., 2010). The mice were given WZ (350 mg/kg) solution or saline by gavage twice a day (700 mg/kg/d) from 5 minutes after surgery until the end of the study. Mice were sacrificed at 1, 1.5, 2, 3, 5, 7, and 10 days after PHX, and the liver tissues were harvested. The liver and body weights at the time the animals were killed were used to calculate the liver-to-body-weight ratios. The middle part of the right posterior liver lobe was immediately fixed in 10% neutral buffered formalin for histologic sections, and the same portion was used for all formalin fixing. The remaining tissues were flash frozen in liquid nitrogen and kept at −80°C until further use.

**Immunohistochemistry.** Liver tissues fixed in neutral buffered formalin were embedded in paraffin and further processed to obtain liver sections. The expression of Ki-67 was detected by immunostaining with primary Ki-67 antibody according to a standard protocol to monitor hepatocyte proliferation (Schwabe et al., 2001). The number of proliferating hepatocytes was determined by counting the Ki-67-positive hepatocytes in at least five microscope fields (magnification, 20×) for each sample. The H&E-stained liver sections were examined via an Olympus BX41 microscope (Olympus, Tokyo, Japan).

**Western Blot Analysis.** Western blot analysis was performed as reported elsewhere (Chen et al., 2014). Briefly, frozen liver samples were lysed using radioimmunoprecipitation assay lysis buffer according to the manufacturer’s instructions. Protein concentrations were determined by a BCA protein assay kit (Thermo Scientific, Rockford, IL). We separated 60 μg of total protein per sample by SDS-PAGE and then electrophoretically transferred the samples to polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% bovine serum albumin or 5% nonfat milk in Tris-buffered saline and were incubated with different primary antibodies at 4°C overnight. Specific protein bands were detected with an electrochemiluminescence Western blot substrates (Enzyme Research, South Bend, IN) and the same portion was used for all formalin fixing. The intensity of protein bands was assayed using ImageJ software (http://imagej.nih.gov/ij/).

**Statistical Analysis.** All values are expressed as mean ± S.E.M. The statistical analysis was performed using an unpaired Student’s t test or one-way analysis of variance followed by Dunnett’s multiple comparison post hoc test. GraphPad Prism 5 (GraphPad Software, San Diego, CA) was used for the analyses. P < 0.05 was considered statistically significant.

## Results

**Effect of WZ on Liver Regrowth after PHX.** To determine the effect of WZ on PHX-induced liver regeneration, we subjected all mice to sham or partial hepatectomy operations before administering the saline solution or WZ treatment. Mice were euthanized after PHX during the period of hepatocyte proliferation (days 1, 1.5, 2, or 3) and liver mass restoration (days 5, 7, or 10).

The liver-to-body-weight ratios are shown in Fig. 1 to indicate the liver regrowth. At days 1, 1.5, 7, and 10 after PHX, the liver-to-body-weight ratio analysis in PHX/saline-treated and PHX/WZ-treated mice suggested that there was no difference in the rate of liver regrowth. However, the PHX/WZ mice showed a significant increase in the liver-to-body-weight ratio at days 2, 3, and 5 after PHX (1.4-fold, 1.2-fold, and 1.7-fold higher, respectively, than that of the PHX/saline mice). WZ treatment caused no difference in the liver-to-body-weight ratio of the sham-operated mice over the time course of 0 to 10 days (Fig. 1B). These data indicate that WZ may promote liver regrowth in response to PHX but has no significant effect on the normal liver of sham operated mice.

**Effect of WZ on Hepatocyte Proliferation after PHX.** To determine the effect of WZ on hepatic cell proliferation after PHX, we immunostained liver sections with a primary Ki-67 antibody to monitor hepatocyte proliferation (Fig. 2). Hepatocytes of PHX/WZ mice reached a peak of cell proliferation earlier than hepatocytes from PHX/saline mice. The PHX/WZ mice displayed a significant increase in Ki-67-positive hepatocytes at days 1 and 1.5 after PHX compared with the PHX/saline mice. In the PHX/WZ mice, the peak of cell proliferation was observed at day 1.5 after PHX; in the PHX/saline mice, the peak was delayed to day 2. The number of Ki-67-positive hepatocytes in PHX/WZ mice returned to baseline by day 5. A similar decreasing trend was observed in the PHX mice without WZ treatment, which also returned to baseline by day 5. As expected, there were no Ki-67-positive hepatocytes in the sham/saline mice or sham/WZ mice over the time course of 0 to 10 days. These findings demonstrate that WZ can induce an early initiation and peak of cell proliferation in response to PHX.

**Fig. 1.** Liver-to-body weight ratio in saline-treated mice and WZ-treated mice subjected to PHX (A) or sham operation (B) over a time course of 10 days. Data are the mean ± S.E.M. (n = 8). *P < 0.05, and **P < 0.01 versus PHX/saline mice.
Effect of WZ on Epidermal Growth Factor Receptor after PHX.

Epidermal growth factor receptor (EGFR), is one of the primary mitogens crucial for proper liver regrowth (Kang et al., 2012). In this study, the dynamic changes of EGFR protein expression were determined in mice following PHX (Fig. 3). PHX/WZ mice exhibited strongly increased phosphorylation of EGFR protein expression at day 1, followed by a slight reduction. The expression of p-EGFR in PHX/WZ mice was not significantly higher than in PHX/saline-treated mice at later time points. Additionally, WZ alone exhibited no significant effect on EGFR phosphorylation following a sham operation. These findings reveal that WZ may stimulate the liver to regenerate in part by increasing phosphorylated EGFR protein levels.

Effect of WZ on Cell Cycle Proteins after PHX. To further determine the molecular mechanism underlying the increase in cell proliferation observed in PHX mice treated with WZ, we measured the expression of core cell cycle proteins including cyclin D1, cyclin D-dependent kinase 4 (CDK4), p-Rb, PCNA, p21, and p27 (Fig. 4). Western blot analysis revealed that the levels of cyclin D1 and PCNA in PHX/WZ mice were significantly increased, with a peak at day 1.5; this was followed by a time-dependent decline to normal levels at day 7.

The expression of both cyclin D1 and PCNA in the PHX/saline mice exerted a similar pattern but with a peak level at day 2. The expression of CDK4, the catalytic partner of cyclin D1, exhibited a peak level at day 1 in PHX/WZ mice, and this was delayed until day 2 in PHX/saline mice. The expression of p-Rb showed a similar pattern to both cyclin D1 and CDK4, which form the active complex required for phosphorylation of Rb.

We also examined the levels of cell cycle inhibitors, such as p21 and p27, which are known to inhibit cyclin D1 and CDK4 complex activity resulting in decreased phosphorylation of Rb. In PHX/WZ mice, the expression of p21 and p27 remained at a significantly low level over a time course of 1 to 3 days and 1 to 2 days, respectively. Furthermore, the levels of p21 and p27 at days 1 and 1.5 were significantly lower than in PHX/saline mice.

Additionally, in sham/WZ mice, there was no significant increase in cyclin D1 and PCNA, but a slight increase in CDK4 and p-Rb were observed. Taken together, these data suggest that WZ treatment promotes the initiation of cell cycle progression and facilitates liver regeneration after PHX, at least in part through activating core cell cycle proteins, such as cyclin D1, CDK4, p-Rb, and PCNA, and inhibiting cell cycle inhibitors p21 and p27.

Discussion

In clinical settings, liver resection and LDLT are the most effective therapies for patients with hepatocellular carcinoma or malignancies liver disease. The regenerative ability of the liver is an essential precondition for the successful application of a partial hepatectomy or LDLT (Kellersmann et al., 2002; Topal et al., 2003). Nevertheless, in the absence of regeneration, delayed or decelerated liver regeneration results in complications such as postoperative liver failure, which remains the major cause of postoperative mortality, septic infections, bleeding, hepatic encephalopathy, and renal failure (Schneider, 2004; van den Broek et al., 2008; Lock et al., 2009). Therefore, therapeutic interventions that can facilitate liver regeneration after resection are of clinical importance.
In clinical practice, WZ is widely used for the treatment of chronic viral hepatitis and liver dysfunction as a hepatoprotective drug. Recently, we reported that WZ exerts significant hepatoprotective effects against acetaminophen-induced acute liver injury (Bi et al., 2013; Fan et al., 2015). This hepatoprotection, in large part, contributes to the ability of WZ to induce the expression of hepatocyte...
proliferation-related genes and may promote compensatory liver regeneration to prevent hepatic failure, suggesting that WZ may act as a therapy drug to limit liver injury (Fan et al., 2015). However, whether WZ can directly facilitate liver regeneration after liver resection and what related mechanisms are involved remain unknown. Thus, our study investigated whether WZ has potential in promoting liver regeneration after PHX in mice. Male mice are typically chosen for hepatocytology models to study liver regeneration because it has been reported that the estrogen levels in female mice have a significant effect on liver regeneration (Biondo-Simões et al., 2009; Umeda et al., 2015). Thus, male mice were used in our study as well.

The dosage of WZ that we used was calculated based on the dose of 80 mg/kg used in clinical practice with a correction for the body surface differences between humans and mice. Furthermore, our previous study found that 350 mg/kg WZ possessed therapeutic potential in acetaminophen hepatotoxicity through promoting liver regeneration after acute liver injury (Fan et al., 2015). Thus, we chose a dose of 350 mg/kg WZ to study the effect of WZ on liver regeneration after PHX.

The results demonstrated that WZ significantly increased the liver-to-body-weight ratio of mice after PHX but had no effect on the mice after sham operations. Furthermore, hepatocyte proliferation peaked at day 1.5 in the PHX/WZ mice but was delayed until day 2 in PHX/saline mice, as evidenced by a Ki-67 positive ratio. Additionally, we observed that WZ treatment successfully rescued liver hemorrhage, inflammation, and hepatocyte-disrupted architecture at 1 day after PHX, as evidenced by H&E staining (Supplemental Fig. 1).

The liver exerts a remarkable ability to regenerate, achieving this through a wide range of molecules and redundant signaling pathways (Michalopoulos, 2010). The mechanisms governing the process of liver regeneration are complicated and remain to be fully elucidated. The high levels of EGFR in the adult liver play a critical role in hepatic development, function, and regeneration (Carver et al., 2002). The absence of EGFR in the liver has been found to decrease survival and hepatocyte proliferation after PHX due to an impaired G1 to S phase transition in the cell cycle (Natarajan et al., 2007). Under a PHX-induced acute-phase response, hepatocytes transition from a quiescent state into the cell cycle and return to the quiescent state after one to two cycles of replication (Fausto et al., 2006).

In the initial phase, hepatic cells undergo a transition from G0 to G1, which is followed by the up-regulation of cyclin D1 and CDK4 to drive cells into the cell cycle (Nelsen et al., 2001). In the next phase, hepatic cells undergo the G1 to S transition, and p-Rb is activated by elevated levels of active cyclin D1/CDK4 and S phase PCNA (Sherr and Roberts, 2004). Both p21 and p27, known as cell cycle inhibitors, inhibit the cyclin D1-CDK4 complex and bind to PCNA, causing cell cycle arrest, and several studies have demonstrated the importance of p21 and p27 for liver regeneration (Hayashi et al., 2003; Choudhury et al., 2007; Lehmann et al., 2012). Therefore, we further explored the role of WZ in liver regeneration with a focus on the proteins described herein using the PHX model in mice.

In our current study, the dynamic changes of phosphorylated EGFR and the G1 to S phase cell cycle proteins such as cyclin D1, CDK4, p-Rb, PCNA, p21, and p27 were determined. We observed that WZ led to increased phosphorylation of EGFR at day 1 after PHX. These changes in EGFR expression suggest that WZ stimulates the early initiation of regeneration through rapidly activating EGFR at an early stage of liver regeneration. Additionally, the results showed a significant down-regulation of p21 and p27 protein levels and up-regulation of downstream cell proliferation proteins, including cyclin D1, CDK4, p-Rb, and PCNA in PHX/WZ mice during the process of regeneration. When the termination of liver regeneration occurred at later time points, the levels of the cell cycle inhibitors p21 and p27 as well as the cell-proliferation proteins cyclin D1, CDK4, p-Rb, and PCNA were returned to the normal baseline, which is beneficial to the recovery of liver function.

These data suggest that the earlier up-regulation of cell cycle proteins after WZ treatment may be directly related to EGFR phosphorylation. These results were consistent with previous reports that EGFR can drive cell cycle progression by affecting the expression of proliferation-related proteins (Ortega et al., 2015).

Many important genes, such as interleukin-6, c-fos, c-jun, and hepatocyte nuclear factor 4α (HNF-4a), play a vital role in driving hepatocyte entry into cell cycle at the initiation step (Riehle et al., 2011; Jiao et al., 2015). We measured the mRNA levels of these transcripts and found that WZ had no effect on Hnf-4α expression but significantly up-regulated c-jun and c-fos at day 1 and day 2, respectively, in PHX/WZ-treated mice. In addition, WZ significantly down-regulated Hif-1 in the regenerative process after PHX (Supplemental Fig. 2). Typically, these transcripts have significant changes during the first 24 hours after PHX; however, in the current study we collected samples from day 1 after PHX. This may be the reason why we could not observe the typical and representative changes of these genes.

After a two-thirds partial hepatectomy, approximately 95% of the remaining hepatic cells, which are normally quiescent, rapidly re-enter the cell cycle. In the mouse liver, the rate of DNA synthesis in hepatocytes begins to increase at day 1 after surgery and peaks at around day 2. In our study, we considered whether WZ could promote hepatocyte proliferation in mice undergoing a partial hepatectomy and initiate DNA synthesis in advance; thus, we specifically added a time point at day 1.5, between day 1 and day 2. As expected, the peak of hepatocytes proliferation was observed at day 1.5 in mice treated with WZ after PHX but at day 2 in the PHX mice.

The most commonly used experimental model to study liver regeneration is a two-thirds PHX in mice, in which the liver regenerates to its full size after approximately 10 days; this process takes approximately 3 to 6 months in humans (Michalopoulos, 2007). In fact, a higher proportion of liver tissue in patients with liver disease is resected in clinical practice. The ability of the remaining hepatocytes to regenerate determines survival after surgery. In the absence of regeneration, delayed or decelerated liver regeneration results in serious complications such as postoperative liver failure, which remains an important clinical problem (Lock et al., 2009; Kawaguchi et al., 2013). Thus, timely onset of liver regeneration plays a crucial role in recovery and survival after liver resection. If hepatocellular regeneration is stimulated in a timely manner by a therapeutically compatible mechanism, it should be possible to prevent death. Therefore, it is of great value to develop effective and safe drugs for intervention strategies to promote liver regeneration after liver resection. WZ may represent a promising intervention to facilitate liver recovery after undergoing PHX or liver transplantation.

In summary, our study demonstrates that WZ promotes hepatocyte proliferation and liver regeneration after PHX, at least in part, due to the induction of EGFR phosphorylation, up-regulation of cell proliferation proteins including cyclin D1, CDK4, p-Rb, and PCNA, and down-regulation of cell cycle inhibitors p21 and p27 after WZ treatment. WZ may provide a beneficial and therapeutic intervention to facilitate liver recovery after undergoing PHX or liver transplantation through the previously mentioned signals.

Authorship Contributions
Participated in research design: Bi, Huang, X. Li.
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