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Schisandra sphenanthera extract facilitates liver regeneration after partial hepatectomy in mice

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Abbreviations
APAP, acetaminophen; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CDK4, cyclin D-dependent kinase 4; EGFR, epidermal growth factor receptor; PCNA, proliferating cell nuclear antigen; PHX, partial hepatectomy; p-Rb, phosphorylated retinoblastoma; WZ, Wuzhi tablet.
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 hepato-protective herb. Previously, we found WZ could induce liver regeneration-related genes against acetaminophen-induced liver injury. However, whether WZ can directly facilitate liver regeneration following liver resection remains unknown. Therefore, this study aimed to investigate 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 blotting 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 (EGFR), and up-regulated cyclin D1, cyclin D-dependent kinase 4 (CDK4), phosphorylated retinoblastoma (p-Rb), and proliferating cell nuclear antigen (PCNA) 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 for hepatic carcinoma and end-stage liver disease in a clinical setting. It is also used for living donor liver transplantation (LDLT), depending on the tremendous ability of residual liver tissue to regenerate (Belghiti et al., 2000; Schindl et al., 2005). Therefore, therapeutic interventions that can promote liver growth after PHX are of clinical importance.

Schisandra sphenanthera (also known as “Nan wuweizhi”) is derived from the dried ripe fruit of Schisandra sphenanthera Rehd. et Wils., and 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 and beneficial effect of Schisandra sphenanthera on the improvement of immunity, heart and kidney function, and hepatoprotection against chemical hepatitis and various hepato-toxins (Zhu et al., 2000; Panossian and Wikman, 2008; Teraoka et al., 2012). Wuzhi tablet (WZ), a preparation of ethanol extract 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 China (Fan et al., 2014). The six main active components of WZ were identified in our previous study; these include 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, the aims of this study were to investigate whether WZ has potential in promoting liver regeneration after PHX in mice and to explore the possible molecular mechanisms that are involved.
Materials and Methods

Chemicals and reagents

Wuzhi tablets with 7.5 mg Schisantherin A per tablet, were supplied by Fanglue Pharmaceutical Company (Guangxi, China). Primary antibodies including Ki-67, p-EGFR, epidermal growth factor receptor (EGFR), p27, proliferating cell nuclear antigen (PCNA), cyclin D1, and GAPDH were purchased from Cell Signaling Technology (Danvers, MA, USA). All other antibodies including p21 were obtained from SantaCruz Biotechnology (Santa Cruz, CA, USA), while phosphorylated retinoblastoma (p-Rb) was purchased from Shanghai Sangon Biotech (Shanghai, 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, 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. Two-thirds PHXs were performed according to standard procedures as previously described (Hu et al., 2014). Mice were anaesthetized by inhalation of ethyl ether for approximately 10-30 seconds and then fixed to the operating board. The abdominal area was sterilized with 75% ethanol and the peritoneum was cut approximately 1-2 cm. Next, the left anterior lobe was suture-ligated and resected. Subsequently, the right anterior lobe and the left posterior lobe were suture-ligated and resected between the gall bladder and suprahepatic vena.
Following the surgery, the peritoneum and outer skin were attached together using 7.5x1.75 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 5-7 minutes. Animals fully woke up 1-3 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 (Qin et al., 2010). Mice were administered WZ (350 mg/kg) solution or saline by gavage (i.g.) twice a day (700 mg/kg/day) from 5 min 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 liver tissues were harvested. The liver and body weights at the time of animal sacrifice 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 histological sections, and the same portion was used for all formalin fixation. 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. Ki-67 expression was detected by immunostaining with primary Ki-67 antibody according to a standard protocol to monitor hepatocyte proliferation as previously described (Schwabe et al., 2001). The number of proliferating hepatocytes was determined by counting the Ki-67-positive hepatocytes in at least 5 microscope fields (20 x magnification) for each sample. H&E-stained liver sections were examined using an Olympus BX41 microscope.

**Western blot analysis**
Western blot analysis was performed as reported previously (Chen et al., 2014). Briefly, frozen liver samples were lysed using RIPA lysis buffer according to the manufacturer’s instructions. Protein concentrations were determined by a BCA protein assay kit (Thermo Scientific, Rockford, IL). Sixty micrograms of total protein per sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred to polyvinylidene fluoride membranes (Millipore, Bedford, USA). The membranes were blocked with 5% BSA or 5% non-fat milk in Tri-buffered saline, and incubated with different primary antibodies at 4°C overnight. Specific protein bands were detected with an electrochemiluminescence (ECL) Western blot substrates (Engreen Biosystem, Beijing, China) were visualized on X-ray film (GE Healthcare, Piscataway, NJ, USA). The intensity of protein bands was assayed using ImageJ software.

**Statistical Analysis**

All values are expressed as the mean±S.E.M. Statistical analysis was performed using the unpaired Student’s t-test or one-way ANOVA followed by Dunnett’s multiple comparison post hoc test, which was carried out using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). P values less than 0.05 were considered statistically significant.
Results

Effect of WZ on liver regrowth following PHX

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

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

Effect of WZ on hepatocyte proliferation following PHX

To determine the effect of WZ on hepatic cell proliferation following PHX, liver sections were immunostained 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. PHX/WZ mice displayed a significant increase in Ki-67 positive hepatocytes at day 1 and day 1.5 after PHX compared to PHX/saline mice. In PHX/WZ mice, the peak of cell proliferation was observed at day 1.5 after PHX, whereas the peak was delayed to day 2 in the PHX/saline mice. The number of Ki-67 positive hepatocyte in PHX/WZ mice returned to baseline by day 5. A similar decreasing trend was observed in the PHX mice without WZ treatment, also returning to baseline by day 5. As expected, there were no Ki-67 positive hepatocytes in the sham/saline mice and sham/WZ mice over the time course of 0-10 days. These findings demonstrate that WZ can induce an early initiation and peak of cell proliferation in response to PHX.
Effect of WZ on epidermal growth factor receptor after PHX

Epidermal growth factor receptor (EGFR), 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 following 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 cyclinD1, 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 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 1-3 days and 1-2 days, respectively. Furthermore, the levels of p21 and p27 at day 1 and day 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.
following 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 pre-condition 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 various 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 hepato-protective drug. Recently, we reported that WZ exerts significant hepato-protective effects against APAP-induced acute liver injury (Bi et al., 2013; Fan et al., 2015). This hepato-protection, 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 following liver resection and what related mechanisms are involved remain unknown. Therefore, this study aimed to investigate whether WZ has potential in promoting liver regeneration after PHX in mice. Male mice are typically chosen for hepatectomy 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-Simoes Mde et al., 2009; Umeda et al., 2015). Thus, male mice were used in the current study. The dosage of WZ used in the current study was calculated based on a dose of 80 mg/kg, which is used in clinical practice, with a correction for the body surfacedifferences between humans and mice. Furthermore, in our previous study, it was found that 350 mg/kg WZ possessed a therapeutic potential on APAP hepatotoxicity through promoting liver regeneration following acute liver injury (Fan et al., 2015). Thus, a dose of 350 mg/kg WZ was chosen 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 mice after sham
operations. Furthermore, hepatocyte proliferation peaked at day 1.5 in 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 Figure 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 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). p21 and p27, known as cell cycle inhibitors, inhibit the cyclin D1-CDK4 complex and binds 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 above proteins using the PHX model in mice.

In the 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/WZmice 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, 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 IL-6, c-fos, c-jun, HNF-4α, 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 in PHX/WZ-treated mice, respectively. Additionally, WZ significantly down-regulated Il-6 in the regenerative process after PHX (Supplemental Figure 2). Typically, these transcripts have significant changes during the first 24 h 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 2/3 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 following 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; therefore, a 1.5 day time point was specifically added 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 2/3 PHX in mice, in which the liver regenerates to its full size after approximately 10 days; this process takes approximately 3-6 months in human (Michalopoulos, 2007). In fact, a higher proportion of liver tissue in patients with liver disease are resected in clinical practice. The ability of remaining hepatocytes to regenerate determines survival after surgery. In the absence of regeneration, delayed or decelerated liver regeneration results in various complications, such as postoperative liver failure, which is a serious complication and 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 following 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. Thus, WZ may represent a promising intervention to facilitate liver recovery after undergoing PHX or liver transplantation.

In summary, this 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, PCNA and down-regulation of cell cycle inhibitors p21 and p27 following 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.

Conducted experiments: X. Li, D.S. Li, X.Z. Zeng, H. Zeng, Wang, Zhou, Chen.

Performed data analysis: X. Li, Fan

Wrote or contributed to the writing of the manuscript: Bi, X. Li, Fan
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Footnotes

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Figure legends

Figure 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, **P<0.01 versus PHX/saline mice.

Figure 2. Hepatocyte proliferation during liver regeneration induced by PHX in saline-treated mice and WZ-treated mice over a time course of 10 days. (A) Liver sections following Ki-67 immunostaining from PHX/saline mice and PHX/WZ mice at each time point. (B) The percentage of Ki-67 positive hepatocytes. Data are the mean±S.E.M (n=4). *P<0.05, **P<0.01, ***P<0.001 versus PHX/saline mice.

Figure 3. Effect of WZ on epidermal growth factor receptor following PHX. (A) EGFR and phosphorylated EGFR protein expression detected by western blot in livers from sham/saline mice, sham/WZ mice, PHX/saline mice, and PHX/WZ mice. (B) Densitometric analysis of western blot. Data are the mean±S.E.M (n=3). *P<0.05, **P<0.01, ***P<0.001 versus sham/saline mice, #P<0.05, ##P<0.01 versus PHX/saline mice.

Figure 4. Effect of WZ on cell cycle proteins during liver regeneration. (A) p21, p27, cyclin D1, CDK4, p-Rb, and PCNA protein expression detected by western blot in livers from sham/saline mice, sham/WZ mice PHX/saline mice, PHX/WZ mice. (B) Densitometric analysis of western blots. Data are the mean±S.E.M (n=3). *P<0.05, **P<0.01, ***P<0.001 versus sham/saline mouse, *P<0.05, **P<0.01 versus PHX/saline mice.
Figure 1

A

B

Liver/body weight ratio (%)

2.0 2.5
3.0 3.5
4.0 4.5
5.0

1 d 1.5 d 2 d 3 d 5 d 7 d 10 d

PHX/saline

PHX/WZ

Sham/saline

Sham/WZ

1 d 1.5 d 2 d 3 d 5 d 7 d 10 d
Figure 2

A

B

Ki-67 % positive hepatocytes

PHX/saline

PHX/WZ

0 20 40 60 80 100

1 d 1.5 d 2 d 3 d 5 d 7 d 10 d
Figure 3

A

![Western blot analysis of p-EGFR and EGFR levels with or without WZ treatment in sham and PHX models at different time points.](image)

B

![Bar graph showing relative protein expression levels of p-EGFR/EGFR with statistical comparisons.](image)