

Fructus polygoni orentalis extract inhibited liver regeneration and proliferation of bone marrow cells of rat after partial hepatectomy

J.Y. Yang¹, J.S. Wang¹ and H.B. Liu²

¹College of Physical Education, Henan Normal University, Xinxiang, Henan Province, China ²College of Life Science, Henan Normal University, Xinxiang, Henan Province, China

Corresponding author: J.Y. Yang E-mail: 041146@htu.cn

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ABSTRACT. To study the effect of fructus polygoni orentalis extract (EFPO) on liver regeneration and proliferation of bone marrow cells on rat model of partial hepatectomy, EFPO was extracted, and 60 adult male Wistar rats were divided randomly into 6 experimental groups. Rats were treated with intergastric administration (*ig*) with EFPO daily. All rats were euthanized 7 days after administration, and the livers and bone marrow cells were collected. The levels of taxifolin and quercetin in EFPO were 1.238 and 0.381 mg/g, respectively. EFPO decreased the proliferating cell nuclear antigen expression of the regenerating liver. Obvious tissue damage was observed in the EFPO groups, such as a widened hepatic sinusoid cavity, several enlarged nuclei, slightly ballooning degeneration, and spotty and focal necrosis as compared to the control group. Additionally, 1.8 and 3.6 g/kg EFPO significantly inhibited proliferating cell nuclear antigen expression in bone marrows cells (P < 0.05), and induced gathering of these cells during the G_0/G_1 phases (P < 0.05). The karyocyte and myelosis of bone marrows cells clearly

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decreased, and mature erythrocytes increased (P < 0.05) in the EFPO groups. Additionally, 3.6 g/kg EFPO induced active proliferation, while the sham operation and control groups showed apparent active myeloproliferation. The maximum dosage of mice *ig* EFPO was 148.8 g/kg. Our results indicate that EFPO inhibits rat liver regeneration and bone marrow cell proliferation in regenerating rat liver.

Key words: Bone marrow cells proliferation; Fructus polygoni orientalis; Hepatocyte proliferation; Liver regeneration; Rat

INTRODUCTION

Fructus polygoni orientalis, named in *Dian Nan Ben Cao* in 1436 A.D. and recorded in the Pharmacopoeia of China 1997 edition, is a well-known herbal drug in Traditional Chinese Medicine (TCM). It is the fruit of the *Polygonum orientale L*. [(*P. orientale*), (Polygonaceae)] and has been used to treat various diseases including cirrhosis, hepatitis B, and liver cancer (The Editorial Committee of "Zhong Hua Ben Cao", 1999) in folk medicine in China. The major antitumor components, texifolin and quercetin (Kuroyanagi and Fukushima, 1982; Xie et al., 2005), are flavonoids that can scavenge free radicals and have antimicrobial, antithrombotic, and anticarcinogenic activities (Bautista-Ortín et al., 2007; Makris et al., 2007; Tu et al., 2007). Although fructus polygoni orientalis is thought to be effective for treating liver diseases in Chinese folk medicine, only a few preliminary studies have examined this herb, and studies on using this herb to treat liver diseases have not been reported. In this study, we explored the effect of fructus polygoni orientalis extract on liver regeneration and the proliferation of bone marrow cells in a rat model of partial hepatectomy (PH).

MATERIAL AND METHODS

Animals and administration

Adult male Wistar rats weighing 250-300 g were purchased from the Experimental Animal Center of Henan Normal University and provided with a diet of pellets and water *ad libitum*. The experiments involving rats were conducted according to the guidelines of the Animal Ethics Committee, Henan Province Animal Institute, China. Sixty Wistar rats were randomly divided into 6 experimental groups (N = 10 mice in each group), including a sham operation (SO) group and 2/3 PH group as described by Higgins et al. (1931), and then divided into a control group, cyclophosphamide (CTX) group, and 0.9, 1.8, and 3.6 g/kg EFPO groups. Excluding the SO and control groups, rats were administrated intergastrically (*ig*) with 0.9% NaCl, and CTX and EFPO groups *ip* with 0.03 g/kg CTX and *ig* 0.9, 1.8, and 3.6 g/kg EFPO daily, respectively. The rats were euthanized, and the liver and thighbone were placed on ice for further experiments after 7 days.

Plant material extraction and sample preparation

Fructus polygoni orientalis was purchased from a drugstore and authenticated by Hu Benxiang, Pharmacognosy Professor at the of College of Pharmacy, Shaanxi University of

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Chinese Medicine, and was consistent with the standard of China Pharmacopoeia. After the powder of dried fruits was immersed in water for 30 min, the sample was extracted by heating and refluxing in the water bath thrice for 2 h each time. The content of texifolin and quercetin was determined by high-performance liquid chromtography on an Agilent TC-C₁₈ column (4.6 x 250 mm, 5- μ m) (Santa Clara, CA, USA), and eluted with a gradient elution of methanol-water containing 0.1% phosphoric acid. The flow rate was 1 mL/min, and the column temperature was set at 25°C. The extract was concentrated using a rotary evaporator to 1 mg/mL, and then stored at 4°C. EFPO concentrations of 0.9, 1.8, and 3.6 g/kg were obtained using gradient dilution prior to use.

Histopathological analysis and immunohistochemistry

Liver tissue samples were collected and fixed in 4% formaldehyde, processed routinely, and paraffin-embedded. Serial sections were stained with hematoxylin and eosin and immunohistochemistry (IHC) was used for histological analysis. For IHC, after paraffin removal and microwave-based antigen retrieval, endogenous peroxides' activity was eliminated, and non-specific binding was blocked. The slides were incubated with proliferating cell nuclear antigen (PCNA) primary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; dilution, 1:500) at 4°C overnight, and then with goat anti-mouse secondary antibody (Zhongshan Goldbridge, Nanjing, China). The specimens were then incubated with diaminobenzidine peroxidase substrate (Beijing Solarbio. Co. Ltd., Beijing, China) for brown staining and then subsequently counterstained with hematoxylin. All sections were observed under a microscope (Nikon eclipse, E100, Tokyo, Japan).

Liver index

Following treatment as described above, the body weight and livers were weighed and then used to calculate the liver index.

Bone marrow biopsy and proliferation

Following treatment as described above, the thighbone was removed, and marrow cavity was opened. The red marrow was exposed and imprinted on a clean glass slide. The imprints were dried in air, stained with Giemsa-Wright's solution for 20 min, and examined by microscopy.

Isolation of bone marrow cells (BMCs) and IHC

Following treatment as described above, the thighbone was removed, and the marrow cavity was flushed with phosphate-buffered saline (PBS) on ice. The single cell suspension with RPMI-1640 containing 1% fetal serum was smeared on clean glass slips, and PCNA expression was detected.

Cell cycle of bone marrow mononuclear cells analysis by fluorescence correlation microscopy

Bone marrow mononuclear cells were isolated by density gradient centrifugation and

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then analyzed by fluorescence correlation microscopy. Briefly, BMCs were centrifuged on 60% Percoll (Pharmacia Biotech, Uppsala, Sweden) at 1500 rpm for 20 min. Bone marrow mononuclear cells were washed in 4°C PBS, pH 7.4, and cell viability was tested by trypan blue staining. The bone marrow mononuclear cells were fixed on ice with 100% alcohol and stained with propidium iodide (Sigma, St. Louis, MO, USA) solution containing 50 mg/L propidium iodide and 50 mg/L RNase in PBS at room temperature in the dark for 30 min. The samples were analyzed by flow cytometry (BD FACSCalibur, Franklin Lakes, NJ, USA) and analyzed using the Multicycle software.

Acute toxicity experiment

After administration *ig* of the disposable maximum dosage to 10 Kunming healthy mice, their movement, excrement, water, and food consumption as well as body weight were observed and recorded for 7 days.

Data analysis

The 2-tailed Student *t*-test was employed to assess the significance of the data. The data are reported as the means \pm standard deviation. Values of P < 0.05 were considered to be statistically significant.

RESULTS

Active ingredients

EFPO was extracted by heating and refluxing in a water bath, and the contents of texifolin and quercetin were found to be 1.238 and 0.381.1 mg/g, respectively.

EFPO inhibited liver cell proliferation

The IHC results showed that PCNA expression in the control group (76.56 ± 14.50) was higher than that in the SO group $(66.24 \pm 9.37, P < 0.05)$, and the expression in 3.6 g/ kg EFPO (67.41 ± 10.06) and CTX (62.88 ± 9.45) groups were lower than that in the control group (P < 0.05, P < 0.01, respectively), while 0.9 g/kg EFPO (74.48 ± 12.27) and 1.8 g/kg (72.70 ± 5.97) showed no clear differences. This indicated that PH activated liver regeneration, and EFPO could inhibit liver cell proliferation.

EFPO inhibited liver repair

After administration for 7 days, in addition to the SO group, the liver index and body weight in the other groups decreased. Compared to the control group, the change in body weight and liver index in the 3.6 g/kg EFPO group significantly decreased (P < 0.01); however, there was no clear effect on the residual liver. The change in body weight in the 3.6 and 1.8 g/kg EFPO groups were lower than that in the control group (P < 0.05), and the change in body weight in 0.9 g/kg EFPO group was higher than that in the control group (P < 0.05). This indicates that EFPO delayed the liver repair of the PH rats, and 3.6 and 1.8 g/kg EFPO

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decreased the body weight of PH rats, whereas the 0.9 g/kg EFPO increased the body weight (Figure 1).



Figure 1. Effect of fructus polygoni orentalis extract (EFPO) on changes in body weight and liver index rats after partial hepatectomy (PH). EFPO delayed the liver repair of PH rats, while 3.6 and 1.8 g/kg EFPO reduced the body weight of rats with PH. A, B, C, D, E, and F represent the sham operation (SO), control, cyclophosphamide (CTX), and 0.9, 1.8, and 3.6 g/kg EFPO groups, respectively.

Histopathological analysis

Hepatocytes in the EFPO groups showed clear structures, arranged radially along the central vein, and the hepatic sinusoid cavity was wider than that in the PH group. The 3.6 g/kg EFPO group showed clearly widened hepatic sinusoid cavity, several enlarged nuclei, slightly ballooning degeneration of hepatocytes, and spotty and focal necrosis in portal area away from the central vein. Hepatic sinusoid cavities of 1.8 and 0.9 g/kg EFPO groups were narrower, and hepatocytes were arranged more loosely than that in the 3.6 g/kg EFPO group. The liver organization structure in the CTX group was quite damaged, showed a widened hepatic sinusoid cavity, significant ballooning degeneration of hepatocytes, and several anuclear hepatocytes (Figure 2).



Figure 2. Effect of fructus polygoni orentalis extract (EFPO) on liver tissue structure of rat (400X). Hepatocytes in the EFPO groups had clear structures, were arranged radially along the central vein, and the hepatic sinusoid cavity was wider than that in the partial hepatectomy (PH) group. A, B, C, D, E, and F represent the sham operation (SO), control, cyclophosphamide (CTX), and 0.9, 1.8, and 3.6 g/kg EFPO groups, respectively.

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BMC morphology and proliferation

Marrow cavity hyperemia and loosened and dark red tissue were observed in the 3.6 g/ kg EFPO and CTX groups, while a full marrow cavity and bright red tissue were observed in the SO and control groups. The 1.8 and 0.9 g/kg EFPO groups showed looser and lighter red tissue than the control group, which was fuller and darker than the CTX group (data not shown).

The results of G-R staining are shown in Figure 3. The BMC karyocyte clearly increased, and very few mature erythrocytes were observed in the control group. Karyocyte decreased, and mature erythrocytes increased in a dose-dependent manner (P < 0.05) in the EFPO groups. No obvious difference was observed between the SO and control groups. The control and SO groups exhibited myeloproliferative activity in the bone marrow. The EFPO groups inhibited myelosis in a dose-dependent manner (Figure 3).



Figure 3. Morphology of bone marrow cells (BMCs;1000X). Karyocyte decreased, and mature erythrocytes increased in a dose-dependent manner in the fructus polygoni orentalis extract (EFPO) groups observed by G-R (Giemsa-Wright) staining. **A**, **B**, **C**, **D**, **E**, and **F** represent the sham operation (SO), control, cyclophosphamide (CTX), and 0.9, 1.8, and 3.6 g/kg EFPO groups, respectively.

The expression of PCNA (Figure 4) in BMCs in the 1.8 and 3.6 g/kg EFPO groups was lower than that in the control group (P < 0.05). This implied that PH had no obvious impact on BMC proliferation (P > 0.01), and that 1.8 and 3.6 g/kg EFPO significantly inhibited the proliferation of BMCs compared with the PH and SO groups (P < 0.01).



Figure 4. Effect of fructus polygoni orentalis extract (EFPO) on bone marrow cell (BMC) proliferation in partial hepatectomy (PH) rats. PH had no obvious impact on the proliferation of BMCs, and 1.8 and 3.6 g/kg EFPO significantly inhibited the proliferation of BMCs. A, B, C, D, E, and F represents the sham operation (SO), control, cyclophosphamide (CTX), and 0.9, 1.8, and 3.6 g/kg EFPO groups, respectively.

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Figure 5. Effect of fructus polygoni orentalis extract (EFPO) on the cell cycle distribution of bone marrow mononuclear cells in partial hepatectomy (PH) rats. PH induced bone marrow mononuclear cell proliferation, while EFPO inhibited bone marrow mononuclear cell proliferation after PH. A, B, C, D, E, and F represent the sham operation (SO), control, cyclophosphamide (CTX), and 0.9, 1.8, and 3.6 g/kg EFPO groups, respectively.

Effect of EFPO on cell cycle distribution of BMC

Cell cycle experiments (Figure 5) showed that the proportion of G_2/M and S% bone marrow mononuclear cells in PH rats were higher than that in the SO group (P < 0.01). After administration for 7 days, the proportion of $G_0/G1\%$ in the CTX and EFPO groups were increased compared to that in the control group (P < 0.01).

Maximum dosage

Compared to control mice, clear postmortem differences were not observed in the EFPO groups. Thus, the maximum dosage of administration *ig* is 148.8 g/kg, which is 89.3-fold of the adult oral administration dosage per day.

DISCUSSION

Recently, plants used in folk medicine have been accepted as the main sources of drug discovery and development. In China, Traditional Chinese Medicines (TCMs) represent a very large reservoir of plants with tremendous diversity. Fructus polygoni orientalis has been used to treat liver disease in China, with a total cirrhosis effective rate of 90.3%. The negative conversion ratio of HBsAg was 59%, and 1-year survival rate for hepatocarcinogens was 60% in combination with other herbs as reported by TCM practitioners.

Advances in molecular techniques over the past few decades have resulted in the development of regenerative medicine methods. Liver regeneration in response to different types of liver injury such as drugs, toxins, resection, or acute viral diseases has been examined in numerous studies (Fausto, 2006; Riehle et al., 2011). A detailed understanding of cell proliferation in response to injury will be applicable to other organ dysfunctions (Yamazaki et al., 2011), and may also shed light on how cancer, cirrhosis, and other liver disease pathogenesis develops, can be treated, and is repaired after PH (Hixson et al., 2000). Thus, liver regeneration and PH rat models have been used extensively to explore regeneration mechanisms, liver disease pathogenesis, and drug screening. In our study, we explored the effect of EFPO extract

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on PH rats, and we found that EFPO inhibited liver cell proliferation in a concentration-dependent manner, increased body weight loss, and delayed liver repair. We provided a theoretical basis for using fructus polygoni orientalis to treat liver diseases.

With the increasing identification of TCM and development of technology, the toxicity and side effects of TCMs has caused widespread concern. Our preliminary experiments showed that EFPO inhibited mononuclear phagocyte activity and T- and B-lymphocyte transformation rate, as well as decreased the proportion of T-lymphocyte in the peripheral blood and suppressed the secretion of IgM in normal Kunming mice. To determine whether EFPO could also inhibit the immune function during liver regeneration in rats, we explored bone marrow function in PH rats. The results showed that EFPO inhibited PCNA expression of BMCs in PH rats, accumulated in the BM mononuclear cells during the G_0/G_1 phases, clearly decreased the karyocyte of BMCs, and increased mature erythrocytes. These results were in agreement with those of some studies examining immunosuppressive agents of TCMs (Kato et al., 1994; Nagy et al., 1997; Cho et al., 2000; Kang et al., 2000; Krakauer et al., 2001). Numerous TCMs have been applied to treat liver diseases and studied for their ability to limit the progression of liver diseases by improving immunological function (Agrawal et al., 2008; Lee et al., 2012).

In conclusion, EFPO inhibited liver regeneration and liver repair after PH in rats, and inhibited bone marrow function at the same dosage, which was slightly higher than CTX. Whether the suppression of bone marrow induced by EFPO can be prevented by TCMs should be further examined.

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