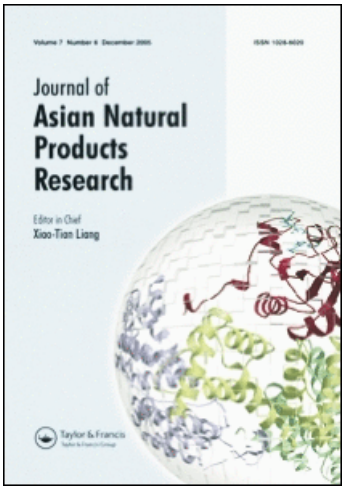

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Jing-Yuan Ma[a]; Wei-Wen Jiang[a]; Zeng-Tong Zhou[a]; Jing-Min Li[b]; Hai-Yan Wang[a]
[a] Department of Oral Medicine, School of Stomatology, Shanghai Research Institute of Stomatology, Shanghai Jiao Tong University, Affiliated The Ninth People's Hospital, Shanghai, China [b] Hangzhou Stomatology Hospital, Hangzhou, Zhejiang, China

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The promoting angiogenesis and anti-inflammation effect of scutellarin on polyglycolic acid scaffold of balb/c mice model

Jing-Yuan Ma[a1], Wei-Wen Jiang[a1], Zeng-Tong Zhou[a]*, Jing-Min Li[b] and Hai-Yan Wang[a]

[a]*Department of Oral Medicine, School of Stomatology, Shanghai Research Institute of Stomatology, Shanghai Jiao Tong University, Affiliated The Ninth People's Hospital, Shanghai, China;* [b]*Hangzhou Stomatology Hospital, Hangzhou, Zhejiang, China*

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Engineering artificial implantable tissues require rapid induction of angiogenesis to meet the significant oxygen and nutrient demands of the cell during tissue repair. In this study, we investigated the role of a Chinese medicine, scutellarin (**1**), in the tissue engineering scaffold of balb/c mice model. The trial groups of balb/c mice were given an intraperitoneal injection of **1** at 20, 60, and 100 mg/kg per ml, respectively. The implanted samples were retrieved at 1 week and 1 month post transplantation. Angiogenesis response in polyglycolic acid (PGA) scaffold was evaluated by histopathological and immunohistochemical methods. The expression levels of vascular endothelial growth factor (VEGF) were determined by RT-PCR. The results showed that the density of neocapillaries in the PGA was enhanced by basic fibroblast growth factor (bFGF) and **1** at 1 week. At 1 month, only 60 and 100 mg/kg per ml **1** groups continuingly kept significant neocapillaries. The inflammatory cells were significantly less in the 100 mg/kg per ml **1** group in comparison with bFGF group and negative group at 1 week and 1 month. Our results indicated that **1** not only promoted angiogenesis in the PGA scaffold, but also inhibited the host inflammatory response to the xenogenic materials.

Keywords: scutellarin; angiogenesis; inflammation; polyglycolic acid

1. Introduction

The success of tissue-engineered scaffolds should be biocompatible, and also supports angiogenesis and neovascularization [1]. The blood vessels are required to supply oxygen and nutrients, and to remove waste products from living tissues [2]. Angiogenesis is regulated by angiogenic factors in a complex multistep biological process [3]. Angiogenic factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) have been incorporated to stimulate angiogenesis in tissue-engineered extracellular matrices. Though those angiogenic factors have strong stimulating effects on vascularization, the biological activity of protein-type growth factors can not last long *in vivo* because of their poor stability [4]. If it is systemic administration in large doses, angiogenic factors can cause harmful side effects.

Scutellarin (**1**), a flavonoid, is the major active ingredient extracted from *Erigeron breviscapus* Hand Mazz, and used as a drug in clinic. Studies have demonstrated the protective effects of **1** on brain injury induced by cerebral ischemia/reperfusion through interaction with a variety of targets because of its anti-oxidative and anti-inflammatory actions, and its ability to attenuate neuronal damage

*Corresponding author. Email: zhouzengtong@hotmail.com
[1]Both of the authors contributed equally to this work.

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[5,6]. It was found that **1** has protective effects on cerebral injury through regulating the expression of NOS isoforms and angiogenic molecules [7]. It is, therefore, speculated that **1** may be able to stimulate angiogenesis and reduce inflammation, which could be beneficial to the survival of the scaffold for tissue regeneration. This study firstly investigated the angiogenesis and anti-inflammation actions of **1** in the polyglycolic acid (PGA) scaffold of the balb/c mice model.

2. Results and discussion

2.1 Histological and immunohistochemical findings

The PGA polymer remnants were visible in all sections, either at 1 week (data not shown) or 1 month (Figure 1). At 1 week, microvessel density (MVD; Figure 2(A)) in the PGA induced by bFGF, 100 or 60 mg/kg per ml of **1** were significantly greater than the negative control or 20 mg/kg per ml of **1** (17.5 ± 1.94, 16.5 ± 2.1, and 16.0 ± 1.87 versus 9.75 ± 1.49 and 10.5 ± 1.7, $p < 0.05$).

At 1 month, quantification of vascularization in each study group revealed a statistically significant increase of MVD in the high or medium concentration of **1** group in comparison with that in the negative group (24 ± 2.42 and 23.5 ± 2.25 versus 15.25 ± 1.75, $p < 0.05$; Figure 2(A)). The density of neovascularies observed in the high concentration of **1** group at 1 month was significantly greater than that of their counterparts observed at 1 week post-operatively (24 ± 2.42 versus 17.5 ± 1.94,

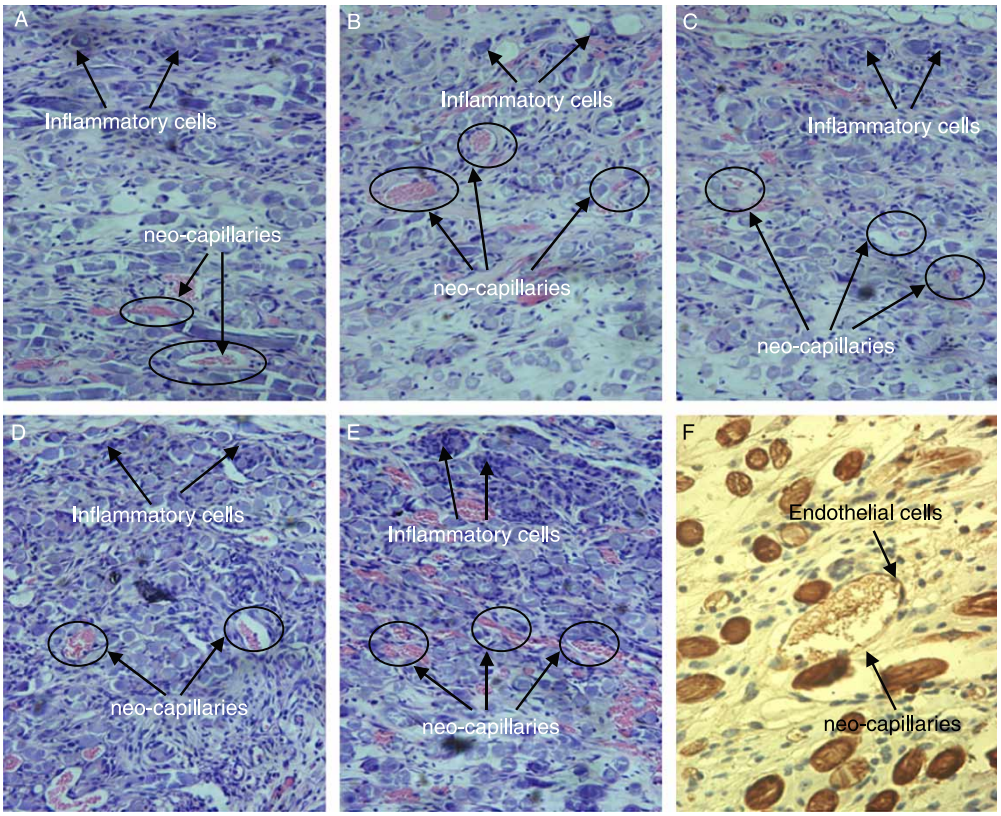

Figure 1. H&E and immunohistochemical analysis at 1 month post transplantation. (A) negative group, (B) bFGF group, (C) 20 mg/kg per ml scutellarin group, (D) 60 mg/kg per ml scutellarin group, (E) 100 mg/kg per ml scutellarin group (200 × magnification), and (F) immunohistochemical staining of VEGF in 100 mg/kg per ml scutellarin group retrieved at 1 week post-operatively (400 × magnification).

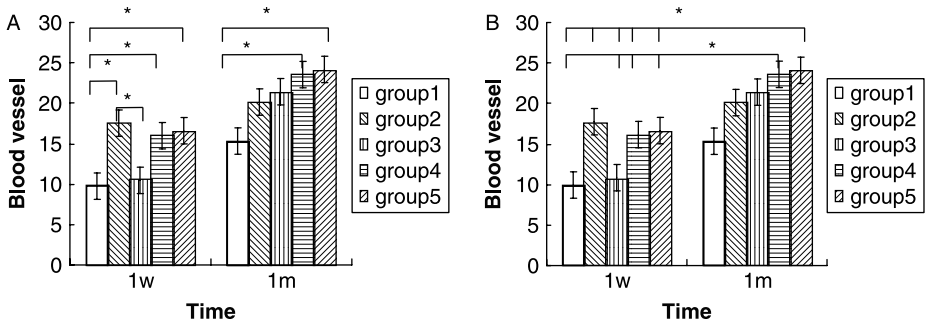

Figure 2. Quantitative assessment of the MVD after implantation into the dorsal skinfold chamber of balb/c mice at 1 week and 1 month. *Statistical significance (LSD test, $p < 0.05$).

16.5 ± 2.1, 10.5 ± 1.7, 16.0 ± 1.87, and 9.75 ± 1.49, $p < 0.05$), whereas those seen in the bFGF group stayed approximately the same (20 ± 1.58 versus 17.5 ± 1.94, $p > 0.05$; Figure 2(B)).

To see whether the regenerated capillary-like structures express VEGF, the immunohistochemical analysis was performed. The expression of VEGF in the endothelial cells was increased slightly in implanted PGA with the increasing concentration of **1**. Figure 1(F) shows VEGF positive staining in 100 mg/kg per ml **1** group.

To find whether there was any difference of regeneration of connective tissue among control group and trail groups at a different time point, we used Masson's trichrome staining. We found there were minimal neo-connective-tissue fibrils in all the groups at 1 week (Figure 3(A) and (B)). In contrast, neocapillaries together with neo-connective-tissue fibrils and fibroblasts were found to fill the pores in all the groups at 1 month. There was no significant difference of regeneration of connective tissue fibrils among each group (Figure 3(C) and (D)).

2.2 *Effect on mRNA level of VEGF in PGA*

Compared with other groups, the mRNA level of VEGF in the bFGF group was higher than that in other groups at 1 week (1.063 ± 0.12, $p < 0.05$; Figure 4(A) and (B)). The VEGF mRNA level in the high or medium concentration of **1** group was higher than that in the negative group and low concentration of **1** group, but the difference was not statistically significant (1.023 ± 0.013 and 1.022 ± 0.011 versus 1.017 ± 0.011 and 1.016 ± 0.18, $p > 0.05$). Surprisingly, VEGF expression in the high or medium concentration of **1** group was higher than that in the negative group and bFGF group at 1 month, but the difference was not statistically significant (1.081 ± 0.036 and 1.077 ± 0.023 versus 1.026 ± 0.046 and 1.067 ± 0.014, $p > 0.05$).

2.3 *Assessment of scutellarin on inflammatory response*

To assess the difference of inflammatory response, the inflammatory cells were counted in all the groups. Although the inflammatory cells in the high concentration of **1** group were less than those in the bFGF group or negative group (Table 1) at 1 week (431.25 ± 14.52 versus 484.25 ± 17.2 and 485.75 ± 11.19, $p < 0.05$), there were less inflammatory cells in the neocapillaries and neo-connective tissue fibrils at 1 month in all the groups (Figure 2). Furthermore, the high concentration of **1** group kept less inflammation in the tissue-engineered region compared with the negative group and bFGF group at 1 month (349.75 ± 19.69 versus 415.75 ± 20.02 and 424 ± 22.49, $p < 0.05$).

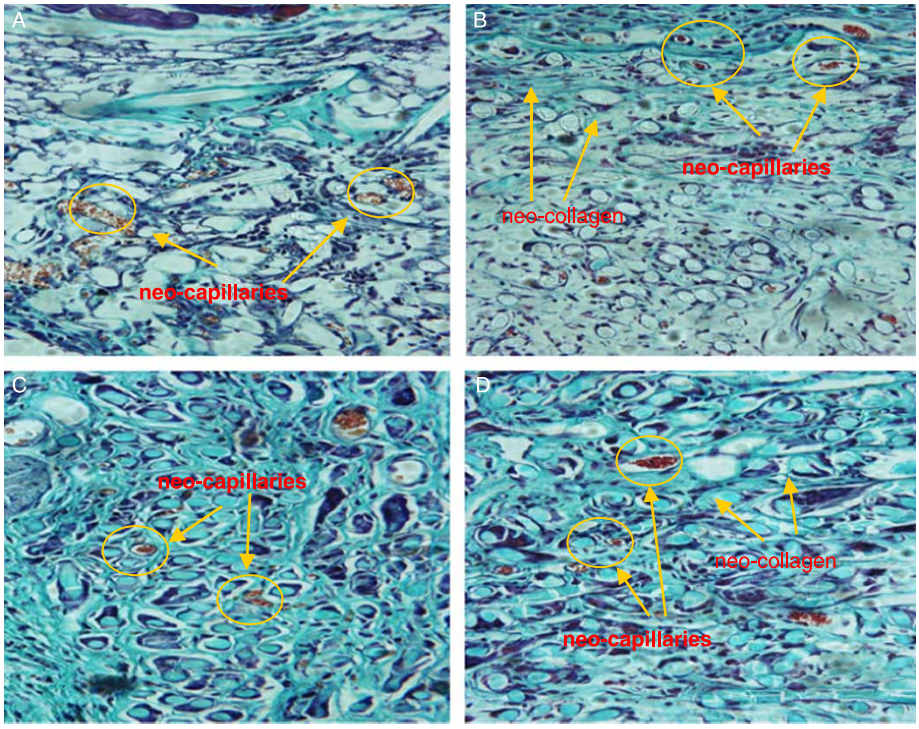

Figure 3. Masson's trichrome staining for histological evaluation. (A) negative group at 1 week, (B) 100 mg/kg per ml scutellarin group at 1 week, (C) negative group at 1 month, (D) 100 mg/kg per ml scutellarin group at 1 month (200 × magnification).

2.4 Discussion

It is considered that **1** has the effect of promoting blood circulation and removing stasis. Most of the studies about **1**, however, are focused on its effects on cerebrovascular disorders and there are few data about biological function in tissue engineering scaffold. Enhancement of the angiogenic potential of implantable biomaterials has received much attention [8]. Because the angiogenic potential of most synthetic and natural materials is insufficient, many attempts

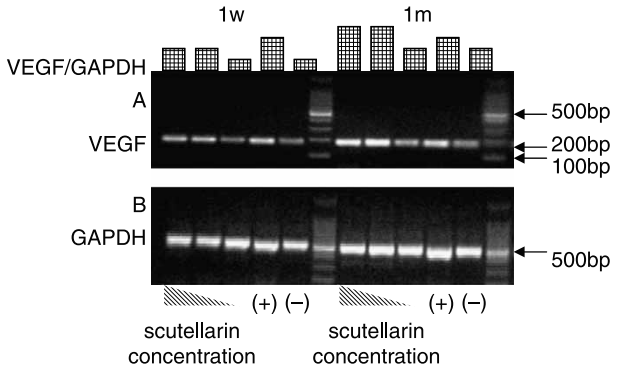

Figure 4. RT-PCR analyses for VEGF and GAPDH. (A) The expression of VEGF at 1 week (left) and 1 month (right). (B) The expression of GAPDH at 1 week (left) and 1 month (right).

Table 1. Effect of *scutellarin* on anti-inflammatory cells.

Inflammatory cells	Group 1	Group 2	Group 3	Group 4	Group 5
1 Week	484.25 ± 17.20*	485.75 ± 11.19*	473.50 ± 15.67	445.75 ± 18.17	431.25 ± 14.52
1 Month	415.75 ± 20.02*	424.00 ± 22.49*	399.00 ± 11.73	374.25 ± 16.72	349.75 ± 19.69

*$p < 0.05$ as compared with high-concentration *scutellarin* group (group 5). Data expressed as mean ± SD.

have been made to enhance angiogenic potential either by changing physicochemical parameters or by supplementation with angiogenic factors. Though those angiogenic factors have strong stimulating effects on vascularization, the biological activity of protein-type growth factors may not last long *in vivo* because of their poor stability (like short half-life) [4]. In our study, although bFGF can enhance angiogenesis at 1 week post transplantation, it is difficult to achieve long-term delivery of functional properties. Our results were in line with the previous study [9]. In contrast, **1** showed the long-term increases in angiogenic effect. The angiogenic response of **1** is confirmed by immunohistochemistry and histopathological measurements as well as by RT-PCR. These results indicated that **1** continued to be effective in enhancing angiogenesis within a relative long term. Using Chinese traditional medicine may help bring a new strategy in tissue engineering.

VEGF is the key used by oxygen-hungry cells to promote the growth of blood vessels. It binds to specialized receptors on the surfaces of endothelial cells and directs them to build new vessels. After receiving this message, the cells build specialized proteases to break through the basal lamina, and migrate into the oxygen-starved region. Once there, the cells multiply and form into tubes, creating a new path for blood to flow [10]. In our study, the expression of VEGF showed elevated tendency in the high or medium concentration of **1**-treated group compared with other groups. It suggested that angiogenesis function of **1** may be interpreted as a mechanism of VEGF-driven neovascularization in the harvested PGA.

In our study, the infiltration of PGA scaffolds with host inflammatory cells was found to be significantly lower in **1** (100 mg/kg per ml) mice compared with control mice, suggesting that **1** injection impairs the foreign body response, which may be necessary for the integration of implanted constructs into the host. Clinical practice for years has also proven that **1** can be used as a Ca^{2+}-channel-blocking agent in the treatment of ischemic cerebrovascular disease. It is known that calcium movement is an important factor in the activation of cells responsible for inflammation [11]. Calcium does this by releasing the inflammatory mediators [12] or by the activation of the plasma membrane or intracellular enzymes [13].

In conclusion, our results indicated that **1** not only promoted angiogenesis in the PGA scaffold of the balb/c mice model, but also eliminated the host inflammatory response to the xenogenic materials.

3. Experimental

3.1 Preparation of materials

1 (Figure 5) was supplied by Pharmacy College of Shanghai medical University (Shanghai, China). The purity of this compound was more than 95% and it was dissolved in 0.9% saline before use. PGA (25 mg, 10 × 10 mm) was obtained from Shanghai Tissue Engineering Center.

3.2 Treatment of animals

Four- to five-week-old male balb/c mice (Grade I, Certificate no. 2002-0041) were purchased from Slac (Shanghai, China). In this study, 40 balb/c mice were randomly divided into 5 groups as follows: Group 1 served as the negative group that received intraperitoneal injections of 0.2 ml of 0.9% saline. Group 2 was the positive control with 0.7 μg/ml bFGF (PeproTech, Rocky Hill, NJ

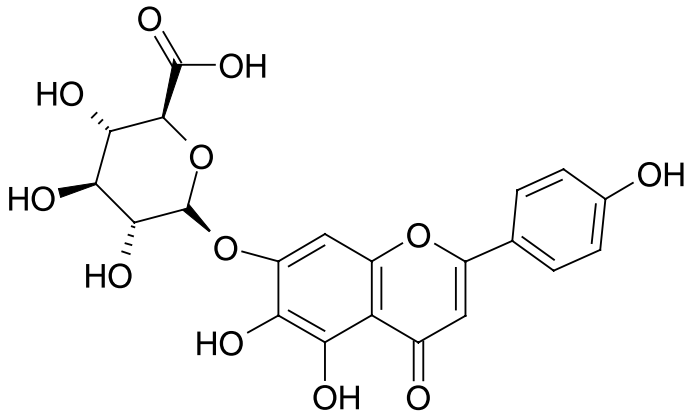

Figure 5. Chemical structure of **1**.

USA) [9,14]. The trial groups received an intraperitoneal injection of 0.2 ml **1** as 20, 60, and 100 mg/kg per ml, respectively.

3.3 Transplantation of the scaffolds into balb/c mice

A full-thickness incision was made down to the dorsal skin of mice to create a subcutaneous chamber. Then the prepared PGA was implanted. The implanted samples were retrieved at 1 week and 1 month ($n = 4$ at each time point) post transplantation.

3.4 MVD measurements

Serial slide sections were used for hematoxylin and eosin (H&E) and Masson's trichrome staining. The average number of capillaries observed on the sections of the experimental groups was compared. The sections were analyzed using the vascular hotspot technique to obtain MVD [15]. The number of inflammatory cells observed in each studied case was quantified with a computer-based image analysis system (Nikon Eclipse E600, Bioquant Osteo II version 8.00) at 200 × magnification [16].

3.5 Immunohistochemical analysis

VEGF protein was localized by immunostaining with the following method. After 10 min microwaving in a 0.01 M citrate buffer (pH 6), the sections were stained with an anti-VEGF rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) at 1:100 dilution. Then the sections were incubated with biotinylated swine antirabbit antibody (1:100) for 30 min, and streptavidin–biotin complex/horseradish peroxidase for 30 min. All of the sera and avidin–biotin reagents were obtained from Dako. Appropriate negative and positive controls were used throughout.

3.6 RT-PCR for VEGF

Total RNA was isolated from the samples using TriIzol reagent (Gibco BRL, Grand Island, NY, USA) and reverse-transcribed with a cDNA synthesis kit (Takara Biotechnology, Dalian, China) according to the manufacturers' protocols. PCR was performed with Taq DNA polymerase (Takara). The primer sequences are as follows: 5′-GGAGAGCAGAAGTCCCATGA-3′ (sense) and 5′-ACTCCAGGGCTTCATCGTTA-3′ (antisense; 189 bp) for mouse VEGF, 5′-GGTGAAGGTCGGTGTCAACG-3′ (sense) and 5′-CAAAGTTGTCATGGATGACC-3′ (antisense; 506 bp) for mouse GAPDH. PCR conditions were as follows: 95°C for 5 min, then 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 60°C, 30 s extension at 72°C, and a 5-min terminal extension at 72°C.

The RT-PCR products were subjected to electrophoresis on 2% agarose gels. The bands were scanned by a UV scanner, and the ratio of band densities between the target gene and GAPDH gene was used for quantitative evaluation.

3.7 Data analysis

Statistical analysis of data was carried out using one-way analysis of variance (ANOVA) followed by LSD's test. The data were expressed as mean ± SD. The whole statistical analysis was performed using the computer software SPSS. A value of $p < 0.05$ was considered to represent a significant difference.

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