doi.org/10.36721/PJPS.2020.33.5.REG.1955-1960.1

Investigating the anti-angiogenic effects of Fufang Kushen Injection in combination with cisplatin using a zebrafish model

Liwen Han[1,3#], Wenxian Zhang[2#], Xiaobin Li[3], Qiuxia He[3], Jian Han[3], Yun Zhang[3], Chen Sun[3], Hong Zhou[2] and Kechun Liu*[3]

[1]Institute of Materia Medica, Shandong First Medical University & Shandong Academy of Medical Sciences, Shandong, China
[2]Department of TCM Orthopedics & Traumatology, Gansu province hospital of TCM, Lanzhou, China
[3]Biology Institute, Qilu University of Technology (Shandong Academy of Sciences), Jinan, China

Abstract: The Traditional Chinese Medicine formula Fufang Kushen Injection (FKI) has demonstrated potential to enhance the efficacy and reduce the toxicity of the chemotherapeutic drug cisplatin. However, there is insufficient evidence to determine whether the combination of matrine and cisplatin were linked to the angiogenesis pathway. In this study, we selected two zebrafish lines, AB and Tg (*vegfr2*: GFP), as *in vivo* models to rapidly assess the anti-angiogenesis effects. KFI and cisplatin had no obvious effects when used individually, but combined KFI (5 and 10 μL/mL) and cisplatin (50μg/mL) significantly inhibited the zebrafish intersegmental vessel (ISV) formation and growth. Matrine at 50 μg/mL also showed synergetic anti-angiogenesis activity with cisplatin (50μg/mL) in 48hpf zebrafish larvae. This study has shown the potential of FKI to enhance cisplatin efficacy and reduce its toxicity by inhibiting angiogenesis. These results contribute to the scientific evidence supporting the use of KFI in combination with cisplatin to treat cancer in the clinic.

Keywords: Fufang Kushen Injection (FKI), zebrafish, angiogenesis, cisplatin.

INTRODUCTION

First-line chemotherapy drugs currently used in the clinic, such as cisplatin, usually suffer from poor targeting and low specificity for cancer cells, which cause side effects that limit patient obedience and reduce treatment efficacy(Karasawa *et al.*, 2015; Cathomas *et al.*, 2015). Traditional Chinese Medicine (TCM) has a long history in the prevention and treatment of cancer in China and many other Asian countries and regions. TCM has attracted increasing attention from researchers and clinicians for its ability to enhance the therapeutic effects and reduce the toxicity of chemotherapy drugs (Chen *et al.*, 2012; Zhang *et al.*, 2013; Jin *et al.*, 2017). Thus, it is important to identify the effects and mechanism of TCM in combination with chemotherapy drugs to contribute to the clinical use of TCM.

Plentiful research has shown that numerous TCM formulations possess synergistic anti-tumor effects in combination with chemotherapeutic agents. The Chinese herbal formulations Zuo Jin Wan Formula and Bu-Zhong-Yi-Qi Decoction induce the mitochondrial apoptosis of cisplatin-resistant gastric cancer cells and enhance cisplatin cytotoxicity in A549 lung cancer cells, respectively (Tang *et al.*, 2016; Yu *et al.*, 2017). Extracts obtained from *Stephania tetrandra*, *Ginseng*, *Astragalus polysaccharide* and *Hypericum japonicum Thunb.* also enhanced the efficacy and reduced the toxicity of cancer chemotherapeutics (Jin *et al.*, 2017; Pariyani *et al.*, 2017; Zhuang *et al.*, 2017). The mechanism through which TCM and cisplatin synergize might involve enhanced chemosensitivity mediated through a modulated immune response (Chuang *et al.*, 2017), the regulation of cytotoxicity, apoptosis-associated pathways (Kiartivich *et al.*, 2017; Yu *et al.*, 2017), and the promotion of antioxidant activities to protect against cisplatin-induced hepatic injury (Gao *et al.*, 2017). Fufang Kushen Injection (FKI) is a famous marketed TCM prescription injection composed of sophorae flavescentis radix (the root of deuto shrub *Sophora flavescens Ait.* of the family *Leguminosae*) and rhizome heterosmilacis (the root and stem of *Heterosmilax japonica.* Kunth of *Liliaceae*) (Liu *et al.*, 2011; Zhou *et al.*, 2012). In addition to the growth inhibition and apoptosis induction of tumor cells (MG-63 and OS732 human osteosarcoma cells and MCF-7 breast cancer cells) (Zhao *et al.*, 2011; Wang *et al.*, 2015; Qu *et al.*, 2016), FKI was able to reduce and stabilize the tumor body in combination with cisplatin (Xu *et al.*, 2011). However, little is known about the molecular mechanisms underlying angiogenesis upon the combined use of FKI with cisplatin.

Angiogenesis is necessary for the vascularization of a tumor, providing essential nourishment for tumor growth and the progression and metastasis of cancer cells (Folkman, 2002). Zebrafish (*Danio rerio*) is an ideal *in vivo* animal model for angiogenesis research (Delvecchio *et al.*, 2011; Santoro, 2014). The embryonic angiogenesis of zebrafish is very similar to that of mammals (Ungos *et*

**Corresponding author:* e-mail: hliukch@sdas.org

#Equal contributions to this study

al., 2007). Its intersegmental vessels (ISVs) sprout from the dorsal aorta and cardinal vein from 22-24hpf and develop fully at 48 hpf (Ungos *et al.*, 2007). The generation of collateral vessels separated from the dorsal artery can be easily observed under a microscope, and thus animal sacrifice is not necessary. It is thus easy to study the independent effects of compounds on angiogenesis free from the interference of other pathological factors using zebrafish models.

Therefore, we chose zebrafish as an *in vivo* animal model for investigating the angiogenesis inhibition of FKI with and without cisplatin. The experimental results provide evidence of enhanced bioactivity for combined FKI and cisplatin.

MATERIALS AND METHODS

Chemicals and reagents

Fufang Kushen Injection (FKI) was produced by Shanxi Zhendong Pharmaceutical Corporation with batch number 20100502. Oxymatrine (batch number110780-201007, purity > 98%) and matrine (batch number: 110805-200508, purity > 98%) were purchased from Food and Drug Verification Research Institute, China. Cisplatin was purchased from Perfemiker Company, China. Matrine, oxymatrine, and cisplatin were dissolved in DMSO and prepared as 50 mg/mL stock solutions. These stocks were then diluted into different concentrations for zebrafish assays. FKI was directly decanted from ampoules and stored as a stock solution.

Zebrafish breeding and embryo isolation

The AB line and the Tg (*vegfr2*: GFP) transgenic zebrafish line, provided by the Zebrafish Drug Screening Platform of the Shandong Academy of Sciences, were raised in accordance with standard breeding methods (Westerfield M,1995). For experiments, healthy adult zebrafish were placed in a mating cylinder in 1:1 or 1:2 male to female ratios, and fertilized eggs were collected the next day from 9:00- 10:00. After disinfection and cleaning, the fertilized eggs were moved into zebrafish embryo cultivating water at 28°C.

Drug treatment

Healthy zebrafish embryos were selected 24hpf and placed in 24-well plates with 8-10 embryos per well. Different concentrations of test drugs were added to wells in a total volume of 2 ml of water. Then, the sealed sample plate was placed in a temperature-controllable illumination incubator (produced by Shanghai Jixing Biological Technology Co. Ltd., China) at 28°C. DMSO used as cosolvent was limited to 0.5% in wells.

Microscopic imaging

After 24 h of drug treatment, zebrafish embryos were analyzed under a microscope to observe the death and development of ISVs in each group. For AB zebrafish, a TH4-200 microscope with a CCD (Olympus) was used to observe and count the number of ISVs with blood flow; these were considered functional ISVs. The embryos in each group were then fixed with 4% PFA and stained with alkaline phosphatase. For transgenic zebrafish, an SZX16 fluorescence microscope (Olympus) was used to observe the fish in profile, images were acquired using the DP2 - BSW photograph image collection system (Olympus). Using these images, the ISVs of each zebrafish were counted, and their lengths were measured using the Image-Pro-plus 6.0 software.

Ethical approval

Zebrafish experiments were approved by the Animal Ethics Committee in the Biology Institute of Shandong Academy of Sciences.

STATISTICAL ANALYSIS

Statistical analysis was performed using SPSS 18.0 for Windows and the values were expressed as the means ± S.D. The statistical significance of differences between the means was analyzed using one-way analysis of variance (ANOVA) to compare different treatment groups. Statistical significance was set at $p<0.05$.

RESULTS

Tolerance of FKI in zebrafish model

The survival rates of zebrafish embryos after treatment with different concentrations of FKI for several days were assessed. FKI showed limited toxicity in zebrafish embryos; only slight toxicity was seen at 15 µL/mL for 72 h (fig. 1).

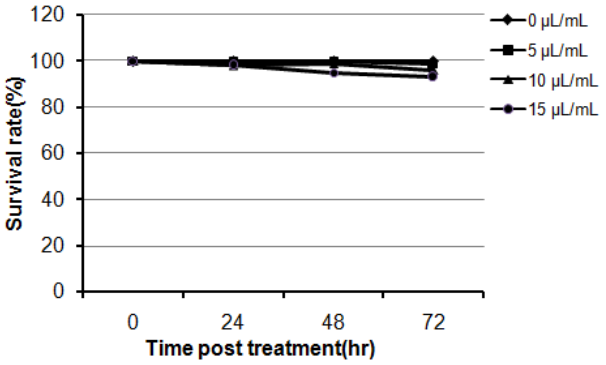

Fig. 1: Survival rates of zebrafish embryos after treatment with different doses of FKI (30 embryos per experiment)

Anti-angiogenesis effects of FKI in zebrafish model.

Angiogenesis is a complex process involving vascular endothelial cell activation, proliferation, migration, and further reconstruction. Only structurally sound intact blood vessels can function appropriately in normal blood transport. In the somites of normal zebrafish at 48hpf, blood cells can be observed moving though the ISVs

under the microscope. Thus, this method is a relatively rapid and sensitive early indicator of ISV formation. When treated with FKI at 3 and 6μL/mL, the numbers of functional ISVs with migrant blood cells showed no clear decline. Upon increasing the FKI dose to 12μL/mL, the number of functional ISVs decreased significantly compared with the normal control group ($p<0.05$) (fig. 2D).The results of alkaline phosphatase staining also showed that FKI decreased the formation of functional ISVs in 48hpf zebrafish (fig. 2B,C).

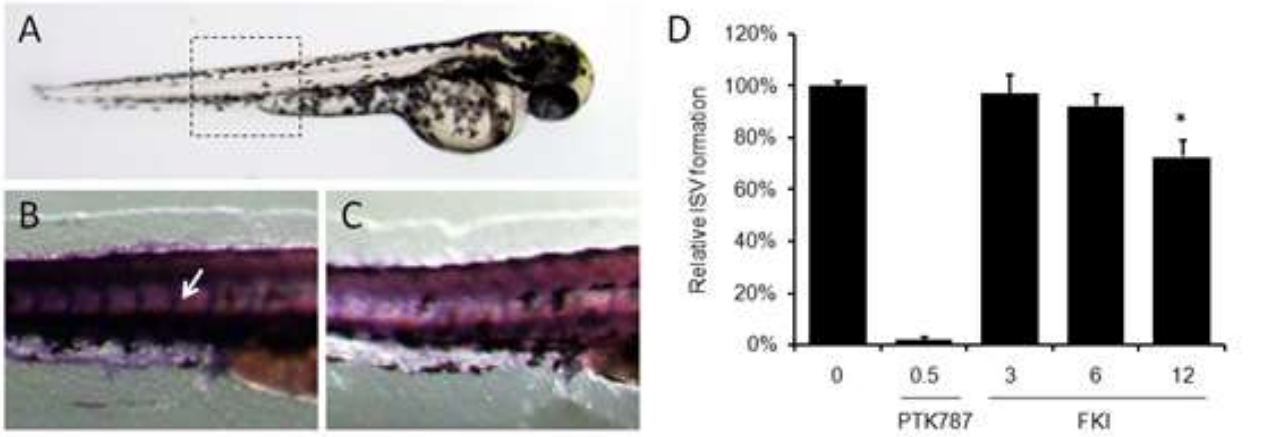

Fig. 2: The inhibition of functional ISV formation in zebrafish after treatment with FKI. A) Intact normal zebrafish larva at 48hpf. B) Magnified region of the dotted box in A showing the trunk stained with alkaline phosphatase. C) Stained zebrafish trunk in the FKI group (12 μL/mL). D) The inhibition of functional ISV formation by different KFI doses. *$p<0.05$ compared with DMSO control group. The white arrow indicates functional ISVs.

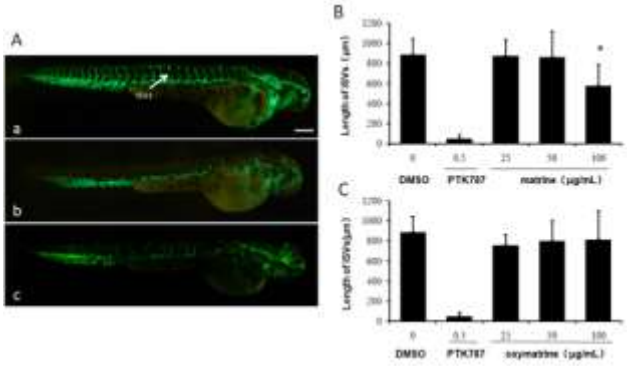

Fig. 3: The anti-angiogenic effects of matrine and oxymatrine from FKI. A) Zebrafish lateral images at 48hpf for normal group (DMSO)(a), PTK787-treated group(b), and matrine(100 μg/mL)-treated group(c). B) and C) Relationship between matrine and oxymatrine doses and anti-angiogenic effects. *$p<0.05$ compared with the control group. The white arrow indicates functional ISVs. The white bar in A indicates 200 μm.

Angiogenesis inhibition in zebrafish model using active ingredients from FKI.

PTK787 is a tyrosine kinase inhibitor that can inhibit angiogenesis in mammal and human cell models. Thus, we used PTK787 as a positive control to inhibit zebrafish angiogenesis; it significantly inhibited the expression of GFP-tagged vascular endothelial growth factor receptor 2 (VEGFR2)(fig. 3A,B).

Two known alkaloid compounds in *radix sophorae flavescentis*, matrine and oxymatrine, were selected to investigate angiogenesis inhibition effects. The transgenic zebrafish line Tg (*vegfr2*: GFP) was used to assess angiogenesis because the ISVs are marked with green fluorescence to simplify assessment of the accurate formation of ISVs. At low doses of 25 to 50μg/mL, matrine showed no obvious effects on angiogenesis. However, at 100 μg/mL, obvious ISV inhibition was seen ($p<0.05$) (fig 3B). Oxymatrine showed no apparent effects at the concentrations tested (fig. 3C).

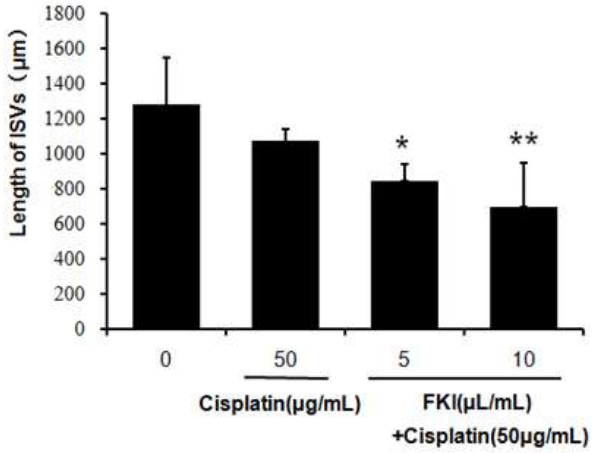

Fig. 4: The anti-angiogenic effects of FKI in combination with cisplatin. *$p<0.05$ compared with DMSO control group. **$p<0.01$ compared with DMSO control group.

The anti-angiogenic effects of FKI and cisplatin in zebrafish.

The results showed that cisplatin, a well-known

chemotherapeutic drug, had no significant anti-angiogenic activity in zebrafish when used alone at 50μg/mL. However, when cisplatin was combined with FKI, the formation of ISVs was obviously inhibited compared with the DMSO control group (p<0.05) (fig. 4). FKI alone showed no effect on angiogenesis at doses of 6μL/mL and below (fig. 2D), whereas 5μL/mL FKI in combination with cisplatin significantly inhibited ISV formation (fig. 4). When the FKI dose increased to 10μL/mL, the anti-angiogenesis effect showed a more marked difference compared with the cisplatin-alone group (p<0.01)(fig 4).

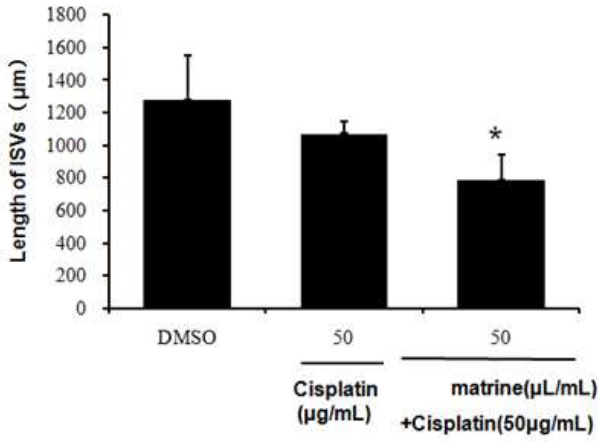

Fig. 5: The anti-angiogenic effects of matrine in combination with cisplatin. * p<0.05 compared with DMSO control group.

Further research showed that matrine (50μg/mL) also has synergistic anti-angiogenic effects in combination with cisplatin (50μg/mL) (fig. 5).

DISCUSSION

The zebrafish genome shows a high level of conservation compared with the human genome. The similarity at the molecular level among the central nervous system, internal organs, blood and visual systems between zebrafish and humans is as high as 85% (Bugel *et al.*, 2014). For the purposes of drug research and development, although the protein homology between zebrafish and humans is less than 70%, the homology of certain protein domains (such as drug targets) is nearly 100%. Cell models and aortic rings are used as *in vitro* angiogenesis test models, but they do not reflect the overall effects of drugs on the body. Furthermore, mouse xenograft *in vivo* models do not allow direct observation with the naked eye. The transgenic zebrafish Tg (*vegfr2*: GFP) is a special line in which vascular endothelial growth factor receptor 2 is stably and heritably labeled by green fluorescent protein (GFP). GFP expression can thus be used to directly visualize the development of blood vessels. Due to these biological advantages, zebrafish have been widely used to screen drugs, monitor water quality, assess toxicity and reveal the mechanisms of development. Many angiogenesis inhibitors (such as PTK787, SU5416, etc.) show equivalent biological responses in mammalian and zebrafish model (Yoshizawa *et al.*, 2012). In this experiment, zebrafish were used to successfully evaluate the anti-angiogenic effects of TCM samples.

Research has shown that angiogenesis is a critical mechanism of some TCM agents to enhance the radioresponse of xenografts in mice (Cao *et al.*, 2017). FKI can inhibit the growth of MCF-7 stem-like cells in *in vitro* and *in vivo* analyses, which may also explain how FKI can attenuate the side effects of chemotherapy (Song *et al.*, 2006). In this study, we were surprised to find that the combination of FKI and cisplatin caused more powerful angiogenesis inhibition compared with individual treatments.

Many bioactive components, such as matrine, oxymatrine, sophocarpine and other alkaloids, have been discovered in FKI. Matrine and oxymatrine exhibit a variety of pharmacological activities, including anti-inflammatory, anti-allergic, anti-viral, anti-fibrotic and cardiovascular protective effects (Zhang *et al.*, 2013; Wang *et al.*, 2015). Matrine significantly inhibited the proliferation, migration, and p-ERK expression of HUVECs (Lu *et al.*, 2009). Recent research reported that matrine and cisplatin inhibit the proliferation of rhabdomyosarcoma RD cells and induce apoptosis, and the combined effect was significantly greater than either agent in isolation (Li *et al.*, 2016). However, there was not enough evidence to prove that the combination of matrine and cisplatin was linked to the angiogenesis pathway.

Our results suggested that combined FKI and chemotherapy drugs can inhibit angiogenesis. Further, angiogenesis might be relevant in the inhibition of vascular endothelial growth factor (VEGF) receptor expression. The matrine in FKI maybe partly responsible for these anti-angiogenesis effects.

This study also provides a new strategy for assessing the therapeutic effects of TCM combined with chemotherapy drugs. Because it involves complex mixtures of chemical components, it is difficult to study the validity and safety of TCM. Because of their integral drug metabolism and conjugation enzymes, *in vivo* animal models are generally considered superior for characterizing systematic biological activity, especially for multi-component system, compared with *in vitro* models (Zhang *et al.*, 2015). The zebrafish is a fully developed vertebrate *in vivo* model that has been used to reveal the mechanisms of potential drug-drug interactions (Pelkonen *et al.*, 2012).

CONCLUSION

This investigation demonstrates the value of a combination cancer therapy using TCM prescription FKI and cisplatin. However, further investigation is needed to

verify the angiogenesis effects in other models and with other active compounds in FKI.

ACKNOWLEDMENTS

This work was supported by the Shandong Provincial Key Research and Development Plan (2016GSF121009); Shandong Academy of Sciences Fundamental Research Plan(2015009); Natural Science Foundation of Shandong Province (ZR2015YL013, ZR2015YL009) and Open Foundation Traditional Chinese Medicine Quality Control Key Laboratory of Shandong Province.

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