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Original Article

Pro-angiogenic effects of Guo Min decoction in a zebrafish model

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Abstract

Objectives: Guo Min decoction (GMD) is a Chinese traditional medicine that can regulate allergy-related symptoms. Although GMD treatment was reported to treat allergy-associated symptoms by regulating the immune response, the rationale between GMD treatment and angiogenesis has not been reported yet. Our objective is to investigate the angiogenesis-modulating activity of GMD. **Materials and Methods:** In this study, we used fluorescence recording, alkaline phosphatase (AP) activity staining, and real-time polymerase chain reaction (PCR) experiments to examine the effects of GMD on angiogenesis in a zebrafish model. **Results:** GMD-treated zebrafish embryos exhibited more intercapillary spaces in the caudal vein plexus (Mock: 11.1 ± 1.8 [n = 20; n: numbers of embryos]; GMD-treated: 16.2 ± 1.9 [n = 20]). AP activity staining showed that treatment with GMD and liquorice (Gan Cao, a component of GMD) induced subintestinal vein outgrowth. However, glycyrrhizin (a component of Gan Cao) had no obvious pro-angiogenic effects on zebrafish. Furthermore, real-time PCR experiments indicated that GMD exposure might be through regulating angiogenesis-related genes (*cdh5*, *nrp1a*, and *flt1*) expressions. **Conclusion:** Based on these observations, we proposed that GMD had pro-angiogenic activity in a zebrafish model, and it might partially be contributed by one of the components, liquorice.

Keywords: *Angiogenesis, Guo Min decoction, Real-time polymerase chain reaction, Zebrafish*

Introduction

"Guo Min," in Chinese means allergy. An herbal formula (Guo Min decoction [GMD]) which was developed by a traditional Chinese Medicine physician, Zhu Shenyu, is often used for treating allergy associated symptoms [1]. GMD is a composite blend of herbs and is extensively employed in China for treating allergic conditions [2]. Previous research indicated that GMD treatment can attenuate asthma symptoms in mice by regulating different subsets of T cells within the spleen tissue [2]. This action involves the modulation of IL-4 and IL-13 expression levels [1]. Additionally, GMD treatment can attenuate PM2.5-induced symptoms in rats, such as trachea mucus hypersecretion and lung inflammation. These observations indicated that GMD is efficient in regulating immune function.

The immune response is a very complex physiological process involving many events. For example, the vascular endothelial cell would undergo proliferation, migration, and activation. Meanwhile, many pro-angiogenic factors would be released to recreate an appropriate vascular microenvironment to facilitate the immune response [3,4]. Thus, immune response and angiogenesis are highly associated in many respects. In general, angiogenesis was triggered by receiving some inductive signals (which might be produced by the nonvascular endothelial cells in the microenvironment), such as vascular endothelial growth factor (VEGF) or other pro-angiogenic factors, stimulating vascular endothelial cells to undergo VEGF-related signaling pathways, and then leading to vascular endothelial cell proliferation, sprouting, and outgrowth [5]. Although GMD treatment was reported to be associated with immune response, the rationale between GMD treatment and angiogenesis has not been reported yet.

We aimed to investigate the angiogenic effects of GMD in a zebrafish model. Because zebrafish have transparent embryos and well-defined developmental stages, those advantages allowed us to record angiogenesis phenotypic changes efficiently. We could also use a fluorescent transgenic fish

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(Tg*(fli1:egfp)*) which allows us to record the subtle changes of GMD effects on angiogenesis more efficiently [6].

Materials and methods

Fish care

Zebrafish (AB strain and Tg*(fli1:egfp)*) were maintained following the regular protocols as previously described [6,7]. In brief, all fish were kept in a 28.5°C incubator under a daily periodic light cycle with 10 h dark/14 h bright. All procedures regarding the animal studies were approved by Tamkang University (The Use of Laboratory Animal Committee, TKU) with an approval number (AZ-BS-111002), and the ethical issues were followed the Helsinki Declaration.

Guo Min decoction and liquorice extract preparation

The GMD consists of 20% Fang Feng; 20% Wu Mei; 20% Wu Wei Zi; 20% Yin Chai Hu; and 20% Gan Cao. In brief, around 100 mg of GMD powder was solved in distilled water (100 mL) and incubated at 45°C for 1 h. Then, they were filtrated with Whatman paper and adjusted their concentration to 100 mg/dL (known as 1000 ppm), stored in a −20°C refrigerator as a stock. Each test solution was serially diluted with distilled water to the desired concentrations in the following experiments. The same protocol was also applied to prepare the liquorice (Gan Cao) extracts.

High-performance liquid chromatography conditions

The high-performance liquid chromatography (HPLC) system is composed of three different instruments, including a binary pump (Waters 1525), an autosampler injector (Waters 2707), and a UV/Visible detector (Waters 2489). The analysis of compound dissociation was conducted utilizing an XBridge C18 column (size: 4.6 mm × 250 mm, 5.0 μm) coupled with UV detection at 254 nm. Two pumps controlled the gradient to maintain a total flow rate (1 mL/min). The mobile phase consisted of Phase A (0.05% phosphoric acid solution) and phase B (acetonitrile). The extraction process extended for 30 min, during which the initial 20-min interval involved a gradient extraction approach, transitioning from a composition of 75% phase A and 25% phase B to a composition of 50% phase A and 50% phase B. Following this, the subsequent 10-min period was characterized by an isocratic extraction mode, maintaining a composition of 50% phase A and 50% phase B.

Sample preparation for high-performance liquid chromatography

Extraction and preparation of samples were conducted following established procedures. For the extraction of liquorice and GMD, 2 g of each sample was accurately weighed and added to 25 mL of 50% ethanol. Ultrasonic treatment was performed with a power of 200 W and a frequency of 40 kHz for 40 min. The resulting mixtures were then subjected to filtration using No. 1 filter paper, and the filtrates were collected in 50 mL centrifuge tubes. Residues were subjected to a second extraction using the same procedure, and the resulting filtrates were pooled with the initial ones. An additional volume of 50% ethanol was added to the combined filtrates to reach a final volume of 50 mL. Then, the mixtures were subjected to filtration through a 0.22 mm filter. To prepare glycyrrhizin samples, 10 mg of glycyrrhizin was added to 50% ethanol and subjected to filtration through a 0.22 mm filter.

Drug exposure and survival rate recording

One hundred AB strain embryos were collected and considered as a group for the subsequent drug exposure and survival rates calculation. For GMD treatment, 100-1000 ppm of GMD were prepared, the exposure protocol Method I [12-72 hpf, Figure 1] was applied, and their survival rates were calculated. The same protocol was used for treating liquorice (50 and 100 ppm) and glycyrrhizin (5, 10, 25, and 40 ppm; PHR1516, CAS. 1405-86-3, Sigma, USA). All experiments were triple-repeated.

Caudal vein plexus growth patterns recording

A transgenic fish line, *Tg(fli1:egfp)* with green fluorescent blood vessels was used to record the GMD effects on caudal vein plexus (CVP) patterning. Twenty transgenic fish embryos were collected and treated with 500 ppm of GMD by using Method II [12-36 hpf, Figure 1]. By 36 hpf, the CVP patterning was recorded by counting the number of intercapillary spaces following the protocols described in the previously published paper [8].

Alkaline phosphatase staining and subintestinal vein recording

In this study, we used AP staining experiments to make the vascular endothelial cells visible and subjected to the following subintestinal vein (SIV) recording. The protocols for AP staining were described previously [7]. In brief, embryos with or without the drug (GMD, liquorice, or glycyrrhizin) treatment through Method III [48–72 hpf, Figure 1] were collected at the endpoint (72 hpf). The embryos were fixed in 4% paraformaldehyde, washed with PBST (PBS with 10% tween 20), and then NBT/BCIP was added. After 20 min, the NBT/BCIP was removed and the embryos were washed with PBST at least three times (each for 10 min). Finally, the outgrowth (branch points) of the SIV was calculated and subjected to subsequent statistical analysis [9].

Real-time polymerase chain reaction

Four primer sets (*flt1, cdh5, nrp1a,* and *β-actin*) were synthesized to evaluate the effects of GMD on gene expressions

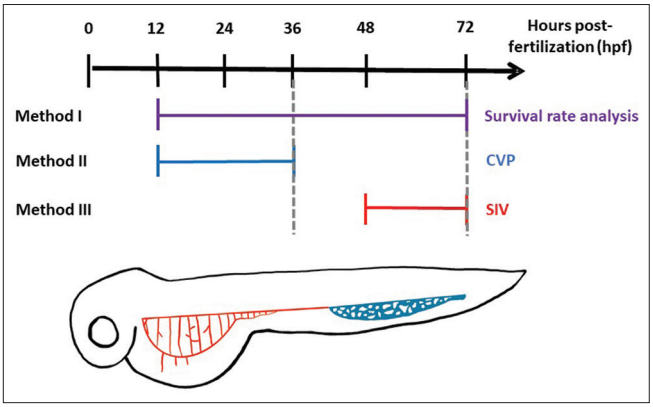

Figure 1: Illustration representation of three exposure methods. Method I (12–72 hpf) is applied for survival rate analysis; Method II (12–36 hpf) for caudal vein plexus recording, and Method III (48–72 hpf) for subintestinal veins observation. CVP: Caudal vein plexus, SIV: Subintestinal vein

by real-time polymerase chain reaction (PCR experiments. The gene accession numbers and primer sequences are listed in Table 1. The total RNA preparation, reverse transcription, and real-time PCR experimental procedures used standard protocols as previously described [10,11].

Statistical analysis

We used three software, Matlab software (R2023a), one-way ANOVA, and the Tukey-Kramer honestly significant difference (HSD) test, to examine the statistical significance of the mean number of branch points among different groups. Microsoft Excel software was used to examine the statistical significance of CVP, the data were presented as means ± standard deviation, $P < 0.05$ would be considered statistically significant.

Results

Using high-performance liquid chromatography to confirm the quality of Guo Min decoction

To confirm the quality of GMD, we used the HPLC chromatograms to analyze the GMD aqueous extract, liquorice (Gan Cao), and glycyrrhizin [Figure 2]. The detailed elution conditions can be found in the Material and Methods section. Results showed that all three chromatograms displayed a characteristic elution peak at ~18 min, suggesting that GMD used in this experiment contains the same component as liquorice, and glycyrrhizin. These results also confirmed the quality of GMD is effective for the following experiments.

Survival rates analysis of zebrafish embryos after exposure to Guo Min decoction, liquorice, and glycyrrhizin

To find appropriate concentrations of GMD, liquorice, and glycyrrhizin in a zebrafish model, we used exposure protocol Method I to calculate their survival rates [Figure 1]. As shown in Figure 3a, 83.7% ± 13.5%–100% ± 0% ($n = 100$; n: numbers of embryos, triple repeated) of embryos survived after 0–500 ppm of GMD exposure, but the survival rates decreased to 19.8% ± 7.2% and 3.0% ± 3.0% after exposure to 600 ppm and 700 of GMD. No survival embryos were observed if the exposure concentrations increased to 800–1000 ppm. The same exposure protocol was also applied in calculating the survival rates after liquorice or glycyrrhizin exposure experiments. As shown in Figure 3b, the survival rates were 93.5% ± 3.7%, 100% ± 0%, and 96.9% ± 2.5% ($n = 100$, triple repeated) of the embryos after exposure to 0 ppm (Mock), 50 ppm, and 100 ppm of liquorice. For glycyrrhizin exposure, the survival rate was 97.8% ± 3.2% in the Mock group. Still, the survival rates were 96.0% ± 0% and 100% ± 0% ($n = 100$, triple repeated) in the 5–40 ppm of glycyrrhizin exposure groups [Figure 3c]. These results indicated that 500 ppm of GMD could be the best concentration in a zebrafish model. As to the exposure concentration, 50 and 100 ppm of liquorice; and 5–40 ppm of glycyrrhizin could be applied for the following experiments.

Guo Min decoction affects caudal vein plexus patterning

During the zebrafish CVP development, the formation of intercapillary spaces will be observed. More intercapillary spaces mean that the CVP is well-growth and patterning. In this regard, the intercapillary spaces are often used as an indicator to evaluate the proper remodeling of the CVP [8]. Our data showed that intercapillary spaces were more likely to be observed in the GMD-treated embryos than in Mock control embryos [indicated by white arrows in Figure 4a and b]. The numbers of intercapillary spaces in the Mock control and GMD-treated groups are 11.1 ± 1.8 ($n = 20$; n: numbers of embryos) and 16.2 ± 1.9 ($n = 20$), respectively [Figure 4c]. These observations indicate that GMD treatment increased the remodeling of CVP.

Effects of Guo Min decoction, liquorice, and glycyrrhizin on subintestinal vein out-growth

Next, we examine the effects of GMD, liquorice, and glycyrrhizin on SIV out-growth by AP-staining. Results showed that after exposure to 500 ppm of GMD, the embryos displayed an evident change in increasing SIV out-growth, and their branches were counted as shown in Figure 5a and b (indicated by white arrow). Figure 5c showed the average numbers of branch points (for the Mock control: 0.170 (standard error [SE] = 0.063, $n = 47$); GMD-treated groups: 0.667 (SE = 0.124, $n = 48$)). The two-sample t-tests indicated a significant difference ($P < 0.001$) between the Mock control and GMD-treated groups.

For liquorice exposure, we found that both 50 ppm and 100 ppm of liquorice-treated embryos led to increased SIV out-growth [indicated by white arrows in Figure 6b and c] compared to that of Mock control group [Figure 6a]. The mean number for three treatment groups (Mock control, 50 ppm, and 100 ppm) are 0.811 (SE = 0.093, $n = 106$), 1.422 (SE = 0.128, $n = 83$), and 1.308 (SE = 0.138, $n = 78$), respectively. Figure 6d indicated that the mean number of the Mock control group was significantly lower than that of the 50 ppm and the 100 ppm group ($P < 0.001$). However, the 50 ppm and the 100 ppm liquorice-treated groups did not show significance ($P > 0.05$, assayed by the pairwise comparison). These observations indicated that GMD and liquorice possessed pro-angiogenic effects in the zebrafish embryos.

Glycyrrhizin is one of the components of the liquorice. Consequently, we examined whether glycyrrhizin possesses pro-angiogenic effects or not. Results showed that glycyrrhizin seemed to have no evident effects on zebrafish

Table 1: Primers used for real-time polymerase chain reaction to detect the angiogenesis-related gene expressions

Gene	Accession number	Forward primer	Reverse primer
β-actin	AF057040.1	CGAGCAGGAGATGGGAACC	CAACGGAAACGCTCATTGC
flt1	BC163921.1	AACTCACAGACCAGTGAACAAGA	TTAGCCTTCTGTGGGTATGTCCA
cdh5	BC163467.1	GGTGCCTCCGACAAGGATGA	AACACTCTTTTGCTCTGGCGT
nrp1a	BC163888.1	CTCCAACAAACCCTACCAGGT	TCGGTGATGTCCACCATGATTTC

cdh5: Cadherin 5, *nrp1a*: Neuropilin 1a, *flt1*: Fms-related receptor tyrosine kinase 1

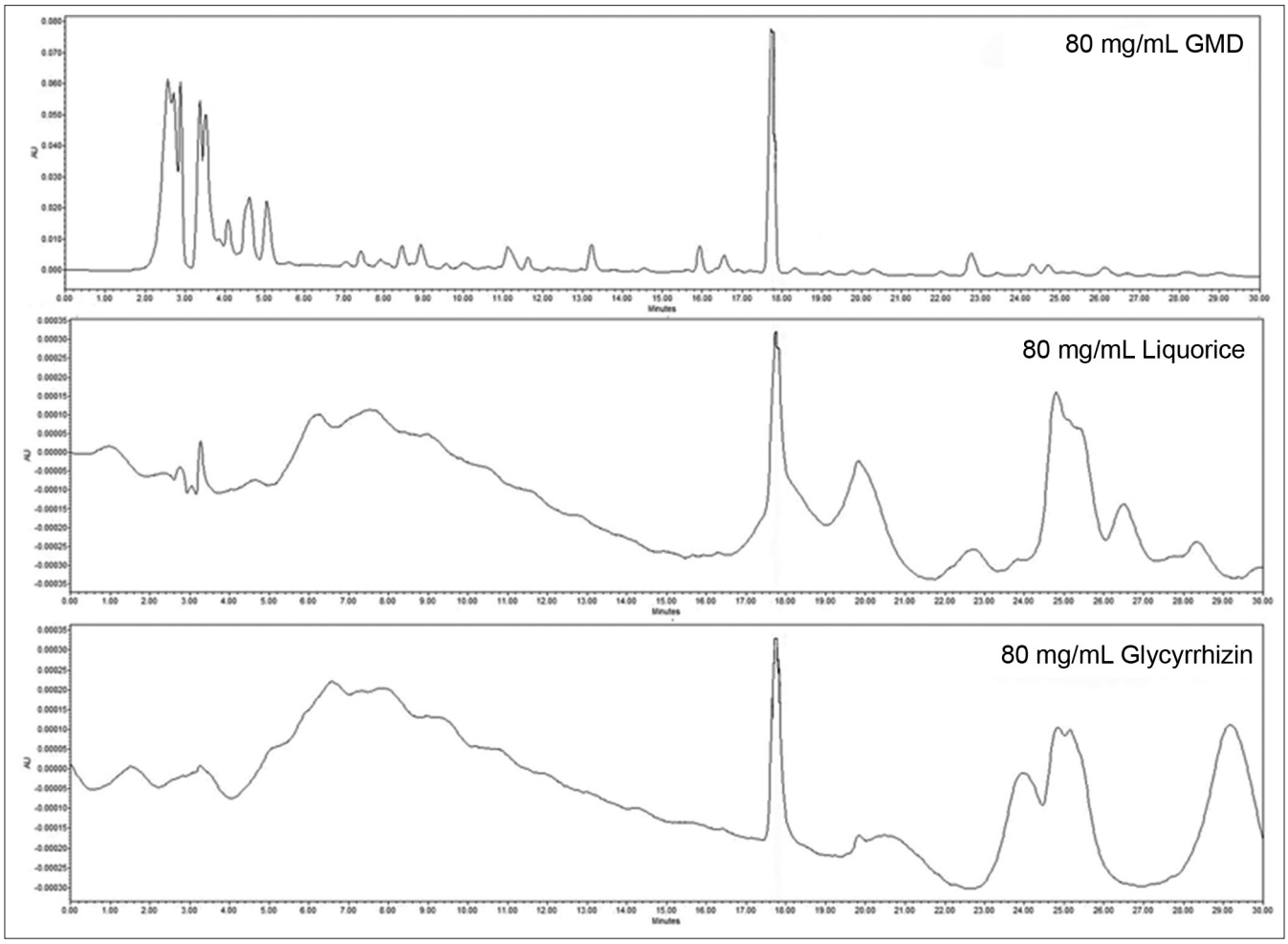

Figure 2: High-performance liquid chromatography (HPLC) chromatograms for Guo Min decoction (GMD), liquorice, and glycyrrhizin. A total volume of 50 mL of GMD and liquorice (crude extracts) were analyzed by HPLC, and their chromatograms were compared to that of glycyrrhizin (which was used as a standard). The X-and Y-axis represented the retention time (minute) and the height of peak, respectively. GMD: Guo Min decoction

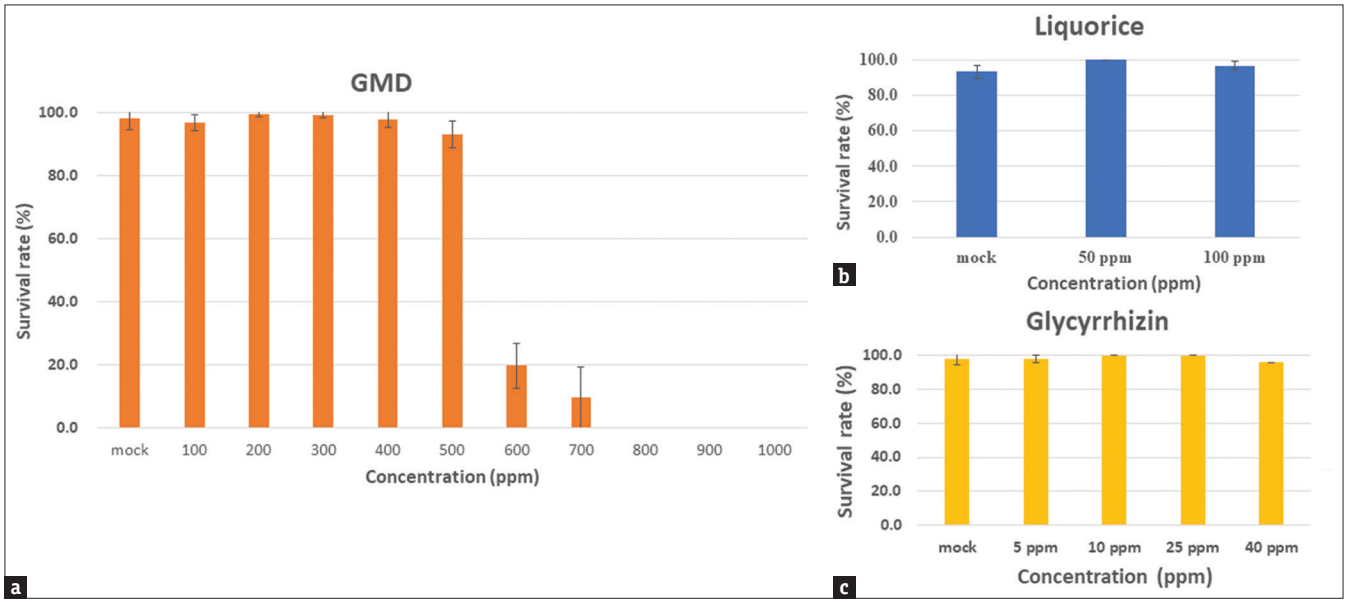

Figure 3: Survival rate analysis of Guo Min decoction (GMD), liquorice, and glycyrrhizin. Zebrafish embryos (AB strain) developed at 12 hpf were treated in the test chemicals ((a) GMD, 100–1000 ppm; (b) liquorice, 50 and 100 ppm; (c) glycyrrhizin, 5, 10, 25, and 40 ppm) through exposure Method I (12–72 hpf). X-axis: concentrations; Y-axis: survival rates. GMD: Guo Min decoction

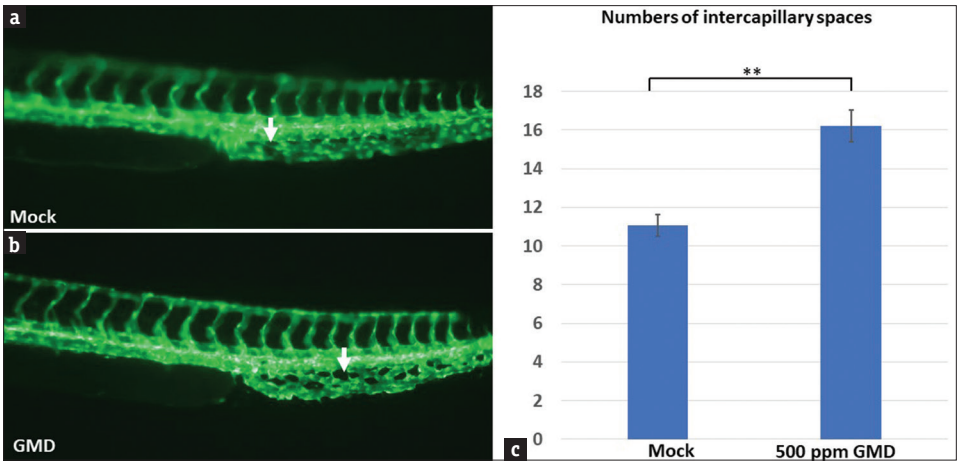

Figure 4: Guo Min decoction (GMD) exposure enhances caudal vein plexus (CVP) growth. (a and b) CVP of 36 hpf Tg(*fli1:egfp*) embryos from Mock control (0 ppm) and 500 ppm GMD-treated group were presented. (c) Statistical analysis from both groups. X-axis: number of intercapillary spaces; Y-axis: experimental group.(**$P < 0.01$). GMD: Guo Min decoction; White arrow: intercapillary space

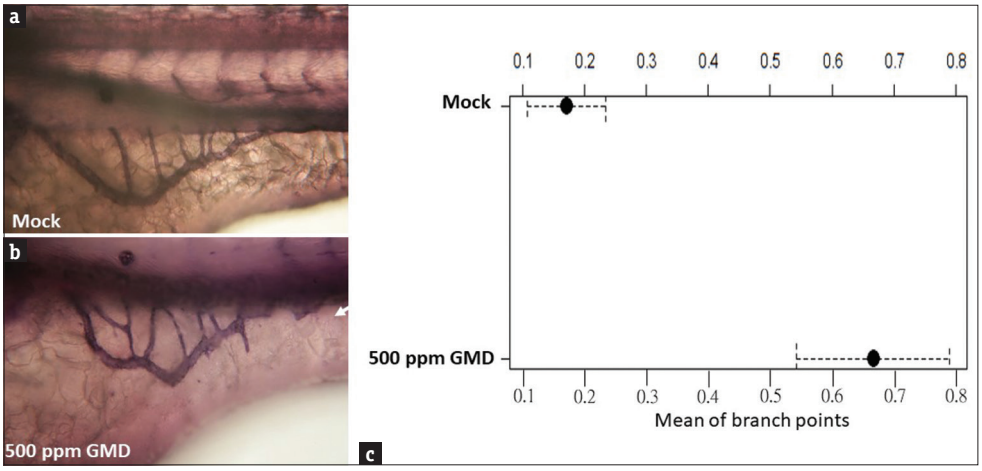

Figure 5: The pro-angiogenic effects of Guo Min decoction (GMD) on the zebrafish. (a and b) Photos of alkaline phosphatase-stained subintestinal vein branch points (marked by white arrow) of embryos from Mock, or 500 ppm GMD group. (c) Data were subjected to the statistical analysis (Tukey-Kramer honestly significant difference test) listed below. Two group means (Mock and 500 ppm GMD) are significantly different because their intervals do not overlap. GMD: Guo Min decoction; White arrow: SIV out-growth

angiogenesis [Figure 7a-e]. Figure 7f indicated that the mean number of branch points of the Mock control and glycyrrhizin-treated groups (5, 10, 25, and 40 ppm) did not show significance ($P > 0.05$, Tukey-Kramer HSD test). That is, glycyrrhizin had no significant pro-angiogenic effects in the zebrafish embryos.

Guo Min decoction treatment changes the blood vessel growth-related gene expressions

To further investigate which genes were affected after GMD treatment, real-time PCR experiments were used to explore the GMD-induced gene expression changes. Three angiogenesis-associated genes (*cdh5, flt1*, and *nrp1a*) were detected. Among them, Cdh5 is an endothelial cell marker; Nrp1a is a co-receptor of VEGF receptor (VEGFR), that functions both in angiogenesis and axon growth; and Flt1 has VEGFR activity [1,5,12]. As a result, GMD-treated groups increased the *cdh5* and *flt1* to 1.21 and 1.99 folds [Figure 8]. Moreover, the expression level of *nrp1a* in the GMD-treated group was decreased to 0.86 by fold changes. These observations may explain the GMD-induced blood vessel out-growth.

Discussion

For toxicological studies, dose is an important issue that needs to be discussed. In humans, the daily dose of GMD is around 50 g. In other words, 50 g/60 kg (adult body weight) is around 833 ppm. In this study, we used 100–1000 ppm of GMD to carry out zebrafish survival rates analysis, and the concentration "500 ppm" was selected for the subsequent study. Compared to the human dose (833 ppm), we thought "500 ppm" of GMD exposure was appropriate.

In Traditional Chinese Medicine formulations, liquorice stood out as the predominant herbal remedy, renowned for its ability to balance and synergize with other herbs. In other words, liquorice is usually used as a "guide drug" [13]. In GMD, liquorice not only acted as a guide drug but also possessed immune-modulating activity. In addition, our data showed that liquorice extract had pro-angiogenic effects in zebrafish. Similar results were also reported from previous studies. For example, liquorice extract was reported to be capable of increasing angiogenesis during the wound-healing process in rats [14]. One of the active compounds in the

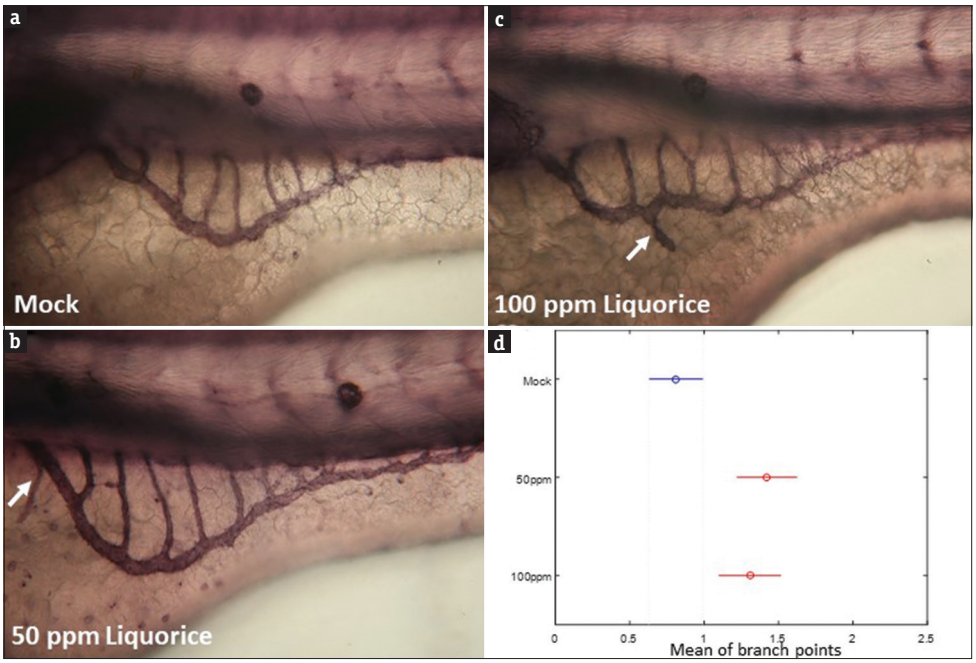

Figure 6: The pro-angiogenic effects of liquorice on the zebrafish. (a-c) Photos of alkaline phosphatase-stained subintestinal vein branch points (marked by white arrow) of embryos from Mock or liquorice (50 and 100 ppm) group. (d) Data were subjected to the statistical analysis listed below. The means from liquorice-treated groups (50 and 100 ppm) and the Mock group are significantly different because their intervals do not overlap. White arrow: SIV out-growth

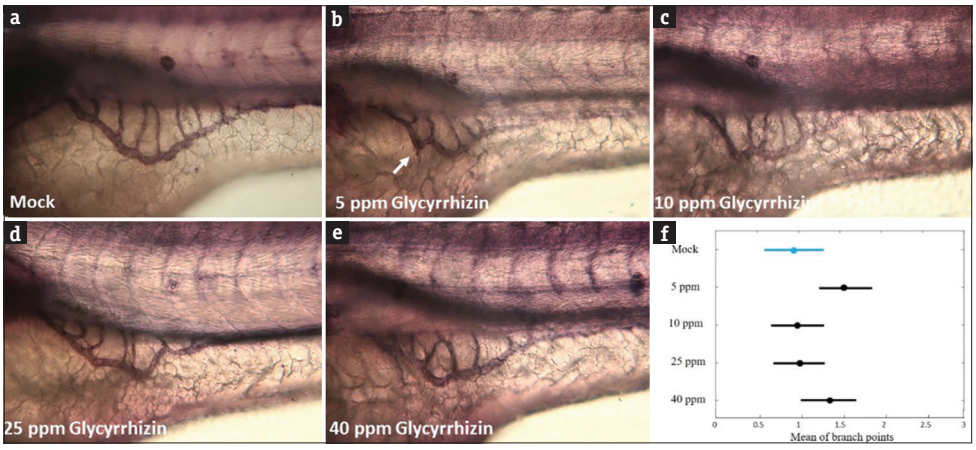

Figure 7: Glycyrrhizin exposure had no evident pro-angiogenic effects. (a-e) Photos of alkaline phosphatase-stained subintestinal vein branch points (marked by white arrow) of embryos from Mock or glycyrrhizin (5, 10, 25, and 40 ppm)-treated groups. (f) Data were subjected to the Tukey-Kramer honestly significant difference analysis listed below. In this figure, the intervals from each glycyrrhizin exposure group overlapped, suggesting that no significant differences were observed. White arrow: SIV out-growth

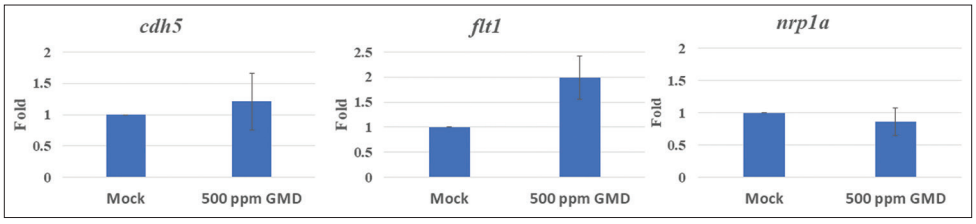

Figure 8: Guo Min decoction (GMD) exposure changes the expressions of *cdh5*, *nrp1a,* and *flt1*. We used the comparative CT method (CT: cycles of real-time polymerase chain reaction) to analyze the relative folds between mock and GMD-treated groups. The expression folds for target genes were calculated relative to the internal control group (*β-actin*) using $2^{-\Delta\Delta CT}$ method)

liquorice extract, isoliquiritin, had pro-angiogenic activity both in human umbilical vein endothelial cells and in zebrafish embryos [15]; and it has also been found to promote angiogenesis during zebrafish wound healing [16]. Another component in the liquorice extract, calycosin, was reported to have pro-angiogenesis in zebrafish embryos [17-19]. Glycyrrhizin is also one of the pure chemicals in the liquorice extract, but in this study, we found that it had no significant

pro-angiogenic effects in zebrafish embryos. Thus, we suggested that the pro-angiogenic activities of GMD might be partially from some chemicals in the liquorice extracts (properly from isoliquiritin and/or calycosin).

The relationship between angiogenesis and immune response is very complicated and needs to be further investigated. It was reported that some pro-angiogenic factors (e.g.: VEGF and placental growth factor) were able to cause immunosuppression [20,21]. For example, VEGF can induce the accumulation of immature dendritic cells and inhibit T lymphocytes migrate toward the tumor [22]. GMD is often used to be an immunoregulatory medicine, in this regard, activation of angiogenesis might contribute to the GMD's immunoregulation activity. Our observations demonstrated that *flt1* (possessed VEGFR activity) gene expressions increased to 1.99 folds, but *nrp1a* gene expressions decreased to 0.86 folds in the GMD-treated embryos [Figure 8]. To our knowledge, Nrp1a played important roles both in angiogenesis and axon growth [23], the downregulation of *nrp1a* expressions might be due to the unexamined effects of GMD during the zebrafish neuron development.

Conclusions

This study demonstrated that GMD possesses pro-angiogenic activity in a zebrafish model, providing a link between immune response regulation and angiogenesis. This is the first report to prove that GMD possessed pro-angiogenic activity in a zebrafish model.

Data availability statement

All data generated or analyzed during this study are included in this published.

Financial support and sponsorship

The study is supported by the Buddhist Tzu Chi Medical Foundation (Taiwan). The grant number is TCMF-CM1-111-03.

Conflicts of interest

There are no conflicts of interest.

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