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Full Length Research Paper

Genistein, the phytoestrogen induces *heart-and-soul* (*has)* phenotypes in zebrafish embryo

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Zebrafish (*Danio rerio*) embryos treated with a small plant molecule, genistein generated a mimic of zebrafish VEGF-A loss of function model. These embryos develop with an enlarged pericardium and major blood vessel deficiencies and their morphological assessment after 48 h post fertilization indicates a nearly complete absence of both axial and intersegmental vasculature with no or reduced numbers of circulating red blood cells resulting in distinctive phenotype of "heart and soul". Thus, zebrafish provides an excellent vertebrate model system to study developmental process and identify novel genes involved during development.

Key words: Genistein, VEGF, has, vertebrate development, zebrafish, angiogenesis.

INTRODUCTION

New blood vessel formation is important during embryogenesis, wound healing as well as pathological situation such as tumour growth (Carmeliet and Jain, 2000) during which vascular endothelial growth factor (VEGF) gene family is implicated. The major regulations of angiogenesis involve signaling of the VEGF and their receptors (VEGFRs) promoting endothelial cell differentiation, survival and migration (Ferrara and Alitalo, 1999; Carmeliet, 2000). The biological hurdle to the genetic investigation of VEGF-A has resulted in a series of experiments using conditional knock-out strategies. Pioneering work in mice with vascular endothelial growth factor-A demonstrates the extreme lose responsiveness of the mouse embryo to VEGF-A signaling during development (Ferrara et al., 1996).

Zebrafish provides an alternative vertebrate model to study angiogenesis as it possess a complex circulatory system similar to mammals with reasonable counterparts (Kimmel et al., 1995). The formation of blood vessels and evaluation of blood flow is extremely easy to score in the zebrafish embryo, making it an ideal model for the study of angiogenesis (Peterson et al., 2000). Further its small embryo size, transparency, large clutch size and permeability to small molecules, has enabled to use this model as a promising method to screen for antiangiogenic growth factor receptor drugs (Eisen, 1996; Fishman, 1999).

Some of these attributes formed the basis for highly successful genetic screens that provided many insights into zebrafish development and thereby angiogenesis. Circulation begins around 30 hpf and it is present in the major angiogenic vessels (ISV, CV and SIV). Chemicals can be added directly to the fish water or injected into embryos. Embryos can survive and develop for at least 1 week without a circulatory system; defective vessel formation does not cause immediate embryonic lethality (Seng et al., 2004).

Genistein, a naturally occurring isoflavonoid in soybeans is known to exhibit strong antiangiogenic activity and it has wide-ranging effects on a number of cellular processes. Also shown to be an anti-oxidant, possess weak estrogenic and antiestrogenic properties, and it is found to inhibit the activity of ribosomal S 6 kinase, tyrosine kinase and topoisomerase as well as angiogenesis *in vitro* (Shao et al., 1998; Buchler et al., 2003).

In this study, we have assessed the effects of genistein the most potent kinase inhibitor of angiogenesis on development of zebrafish embryo and show that it can be

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Abbreviations: VEGF-A, Vascular endothelial growth factor-A; **hpf,** hours post fertilization; **ISV,** intersegmental vessel; **CV,** cardinal vein; **CA,** caudal artery; **DA,** dorsal artery; **has,** heart-and-soul; **SIV,** subintestinal vessel; **VEGFR,** vascular endothelial

Table 1. Effects of 1.0 µM Genistein on various stages of embryonic development. Embryos were treated at 50% epiboly stage.

Hours of post ferti (hpf)	Severe defect[a](%)	Moderate defect[b](%)	Mild defect[c](%)	Percentage of death(%)	Number of embryos
24	50	32	08	10	120
48	74	10	05	07	120
72	80	17	05	05	120

[a]Intersegmental vessels at <30% the normal number; [b]Intersegmental vessels at 30 to 50% the normal number; [c]Intersegmental vessels at >50% the total number.

Table 2. Analysis of blood vessel formation in live zebrafish embryos treated with Genistein.

Genistein concentration (µM)	Dorsal artery present (%)	Number of embryo
5.0	0	90
2.0	22	98
1.5	70	110
1.00	90	115
0.50	92	105
0.10	95	100

Embryos were treated at 50% epiboly stage. Observation was recorded at 48 hpf.

used to determine the timing of critical developmental events. We phenocopied the loss of VEGF ligand function brought about by injecting morpholino in zebrafish embryos, where blood vessel formation was blocked during development (Nasevicius et al., 2000; Chan et al., 2002).

MATERIALS AND METHODS

Embryo collection

Zebrafish mutants (Albino, Brass) were obtained from local suppliers and maintained at 28° C on a 14 h light/10 hour dark cycle in 40 liters glass tanks, each having 4 females and 8 males. Embryos were collected by natural spawning with 2:1 male to female ratio (Westerfield, 1995) and staged according to Kimmel et al (Kimmel et al., 1995). Embryo stages were given as hours post fertilization (hpf).

Drug treatment

Genistein (Calbiochem cat.#345834) was dissolved in DMSO at stock concentrations of 2 mM and then diluted with dose concentration of 0.1, 0.5, 1.0, 1.5, 2.0, 5.0 µM. All these were added into embryo media in a 6 well culture plate in which the synchronized embryos at 50% epiboly stages were arranged by pipette, 20 embryos per well containing 2 ml of embryo medium for each well. Control embryos were treated with the equivalent amount of DMSO solution.

Visual screen

After drug treatment, the embryos were maintained in individual wells of culture plates at 28°C until 72 hpf. After 24, 48 and 72 h of drug addition to the wells, embryos were visually inspected for viability, gross morphological defects, heart rate and circulation. Circulation was assayed by visually comparing the movement of blood cells in treated and control embryos to assess the relative flow rate. The phenotypes exhibited by every embryo in a well were recorded at 48 hpf (Tables 1, 2 and 3).

Photography

After 24, 48 and 72 h of drug treatment, embryos with or without chorion were then examined on stereo-microscope (Euromax) and images were collected and stored using a digital camera and image acquisition software attached to a computer.

RESULTS

Genistein treated embryos developed with moderate to severe phenotypic changes during 24, 48 and 72 hpf (Table 1) and exhibited an enlarged pericardium(Figure 1D), with no circulating red blood cells. A slight reduction in neural tube and over all body size, little or no functioning vasculature (Figure 1G) in a subset of embryos was observed after drug treatment. There was an accumulation of red blood cells and were found in the anterior hypochord (Figure 1H). Strikingly, the eye development is also affected (Figure 1E) in drug treated embryos at 48 hpf. When compared to the controls (Figure 1B).

At 1.0 µM, we observed a 30% vessel inhibition in our system (Table 2) which may be compared to its use at plasma concentrations above 1 µM in mouse tumor studies.

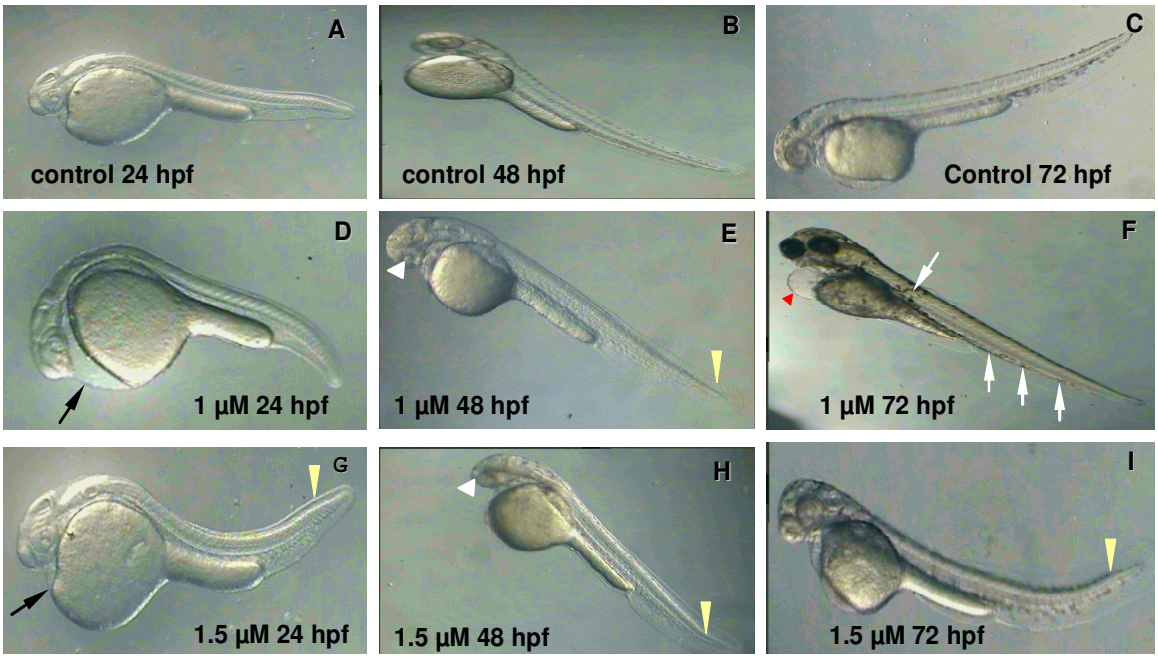

Figure 1. Morphological features of Genistein treated embryos (A, B, C 24, 48 and 72 hpf. (D, G) Loss of Vasculature and enlarged pericardium at 24 hpf (black arrow). (E, H) Indistinct eye development (white arrow head). (F) Pericardial edema (red arrow head), blood accumulation in anterior hypochord (white arrow). (G, H, I) Curved body axis at 24, 48 and 72 hpf (yellow arrow head).

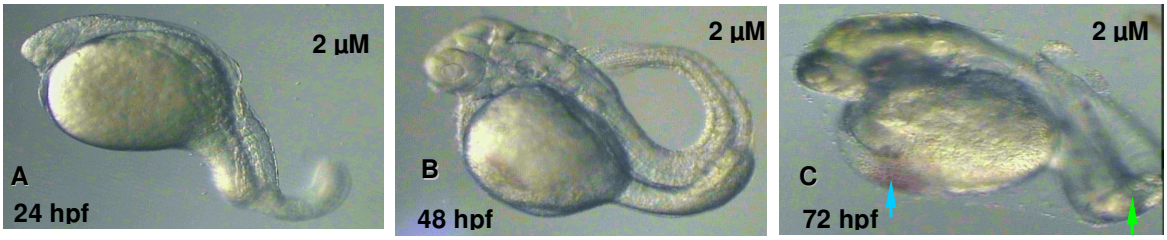

Figure 2. *has* phenotype: Tyrosine kinase inhibitor Genistein can phenocopy known *in vivo* effects. Embryos treated with 2 µM genistein at 50% epiboly (A,B) generated a curved body axis similar to the genetic mutant *heart-and-soul (has*) at 24 and 48 hpf. C; embryos treated with 2 µM genistein at 50% epiboly displayed a hemorrhage (blue arrow) and loss of posterior structure (green arrow).

The phenotypes observed from our study were similar to the phenotypes observed by chemical genetic approach to study angiogenic signaling in zebrafish by Chan et al (2002). At 1.5 µM concentration of genistein, the only vasculature detectable in these embryos was found in the heart and yolk (Figure 1I). The vasculature either fails to form at all or contains no functioning connections to the heart in these embryos. We also observed a less severe phenotypic class of embryos with normal heart, yolk and head blood vessels but without axial or intersegmental vasculature (Table 3). The penetrance of these phenotypic classes is very much dose dependent (Table 2) and consistent with the storage dose dependence of VEGF-A function in mouse embryos.

Embryos treated with 2 uM genistein exhibited with typical body curvature, enlarged yolk and hemorrhages (Figure 2C). Treatment of live zebrafish embryos with genistein at 5 µM concentration blocked the formation of dorsal aorta and other blood vessels such as cardinal artery and cardinal vein which were not prominent (Table 2).

The heart in genistein treated zebrafish embryos were shown essentially normal appearance with a slightly enlarged atrium and ventricle(Figure 1D) and these findings are reminiscent of the phenotype observed following knock down of VEGF-A in the zebrafish by antisense morpholino

Table 3. Phenotype analysis of Genistein treated Zebrafish live embryos at 48 h. The embryos were treated at 50% epiboly stage.

Observed phenotype	"Genistein" Concentration (µM)					
	0.1	0.50	1.0	1.5	2.0	5.0
Heart yolk vasculature	4	4	10	29	25	28
No axial or inter segmental vasculature	7	7	20	20	20	22
No/reduced inter segmental vasculature	62	65	10	29	25	28
Normal vasculature	25	20	2	0	0	2
Pericardial edema	13	20	20	30	20	10
Blood accumulation in anterior hypochord	1	0	2	2	5	0
Blood accumulation in tail	2	3	8	5	2	6
Total Embryo count	114	119	107	104	102	100

The experiment was performed with 120 embryos (20 embryo × 6 wells) for each concentration of genistein. The observed phenotype frequencies in each dose were entered into the table after deducting dead embryos and embryos with normal phenotypes.

(Nasevicius et al., 2000).

Heart-and-soul (has)

Treatment of zebrafish embryos with 2 µM dose of genistein generated distinct phenotypes of curvature of the body axis after 48 hpf (Figure 2A and B) which phenocopies that of the genetic zebrafish mutant "heart and soul" (has) (Chan et al., 2003). This drug caused the same phenotypic effects on development including a dose-dependent reduction of vascular function (Table 3) and a similar accumulation of blood in the ventral tail fin (Figure 1F). Treated embryos has also showed a blockade of major blood vessel formation as the dorsal artery and posterior cardinal vein fail to form by 48 h of development (Figure 2A and B).

The embryos treated with 2 uM exhibited severe pericardial edema at 72 hpf and accumulation of blood cells at anterior and posterior hypochord (Figure 1F). Further complications with loss of posterior structure with hemorrhage in yolk were also observed (Figure 2C) failure and embryonic death on the general morphology of embryonic structures by 24 hpf (Figure 2A) argues strongly for the selectivity of genistein for VEGF receptors. The results observed from this study are similar to the results already shown by Chan et al. (2003) and Nasevicius et al. (2000).

DISCUSSION

Previous study shows that genistein exhibits multiple effects on the human malignant cells via the inhibition of tyrosine kinases (Shao et al., 1998; Buchler et al., 2003; Kellof et al., 1996). Tyrosine kinase and receptor tyrosine kinases are critical components of the biological control network that govern cellular growth and differentiation. This further implicates the use of genistein as an antiangiogenic since tyrosine kinase-specific inhibitors may be potentially employed as anti-cancer agents (Akiyama et al., 1987). *In vitro* trials have shown that genistein is capable of inhibiting the activity of tyrosine kinase (Yarden and Ullrich, 1988).

Treatment of embryos with 1.5 µM genistein for 48 h inhibited SIV growth in zebrafish by 30%, and treatment with 2.0 µM was sufficient to completely inhibit vessel growth (Table III). The effective concentrations of other small molecules like SU5416 that inhibit VEGF-dependent proliferation of mammalian endothelial cells *in vitro* were in the micromolar range (0.05 to 1.0 µM) (Haigh et al., 2000), consistent with the effective concentrations in zebrafish.

Continuous exposure of zebrafish embryos to 5 µM of genistein inhibited SIV formation; and also caused a reduction in overall growth. Concentrations higher than 5 µM caused lethality. The results observed from our study is reminiscent of the results already obtained by treating the embryos with SU5416 and TNP470 (Serbedzija et al., 1999) and PTK787 and ZK 222584 (Chan et al., 2002).

Earlier reports have demonstrated that inhibitors of VEGF signaling could inhibit angiogenesis and lead to developmental defects in zebrafish (Nasevicius et al., 2000). VEGF receptors are receptor tyrosine kinases and are critical cellular components that govern endothelial cell proliferation, migration etc. Genistein, a well known inhibitor of receptor tyrosine kinase thus could strongly inhibit major blood vessel formation as observed (Figure 2C). Since the phenotypic changes observed in zebrafish due to genistein treatment mimic VEGF A loss of function model namely the "heart-and-soul" (has) disease, genistein could be considered a potential inhibitor of angiogenesis and might exert its mechanism of inhibition through VEGF signaling pathways.

Genistein potently inhibits angiogenesis and tumor growth (Cao et al., 2008). Neoangiogenesis is probably is

inhibited via inhibition of hypoxic activation of HIF-1, which in turn reduces VEGF gene expression (Sassi-Messai et al., 2009). *In vitro*, hypoxia was found to be a potent stimulus for VEGF gene expression in all pancreatic carcinoma cell lines, and genistein was found to inhibit hypoxic activation HIF-1 in dose-dependent manner and this was accompanied by the dose-dependent down-regulation of VEGF *in vitro* (Cao et al., 2008).

From the results described here, we demonstrate that the zebrafish is a viable model for screening small molecules inhibitor of angiogenesis and its effects on blood vessel formation during embryonic development. Also strongly suggesting that genistein might bind to VEGF receptor, a receptor tyrosine kinase and inhibit its signaling pathways.

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