Gambogic amide inhibits angiogenesis by suppressing VEGF/VEGFR2 in endothelial cells in a TrkA-independent manner

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ABSTRACT

Context: Gambogic amide (GA-amide) is a non-peptide molecule that has high affinity for tropomyosin receptor kinase A (TrkA) and possesses robust neurotrophic activity, but its effect on angiogenesis is unclear.

Objective: The study investigates the antiangiogenic effect of GA-amide on endothelial cells (ECs).

Materials and methods: The viability of endothelial cells (ECs) treated with 0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 µM GA-amide for 48 h was detected by MTS assay. Wound healing and angiogenesis assays were performed on cells treated with 0.2 µM GA-amide. Chicken eggs at day 7 post-fertilization were divided into the dimethyl sulfoxide (DMSO), bevacizumab (40 µg), and GA-amide (18.8 and 62.8 ng) groups to assess the antiangiogenic effect for 3 days. mRNA and protein expression in cells treated with 0.1, 0.2, 0.4, 0.8, and 1.2 µM GA-amide for 6 h was detected by qRT-PCR and Western blots, respectively.

Results: GA-amide inhibited HUVEC (IC 50 = 0.1269 µM) and NhEC (IC 50 = 0.1740 µM) proliferation, induced cell apoptosis, and inhibited the migration and angiogenesis at a relatively safe dose (0.2 µM) in vitro. GA-amide reduced the number of capillaries from 56 ± 14.67 (DMSO) to 20.3 ± 5.12 (62.8 ng) in chick chorioallantoic membrane (CAM) assay. However, inactivation of TrkA couldn’t reverse the antiangiogenic effect of GA-amide. Moreover, GA-amide suppressed the expression of VEGF and VEGFR2, and decreased activation of the AKT/mTOR and PLCγ/Erk1/2 pathways.

Conclusions: Considering the antiangiogenic effect of GA-amide, it might be developed as a useful agent for use in clinical combination therapies.

Introduction

Garcinia hanburyi Hook f. (Gittiferae) is a tropical plant found in southeastern Asia, and bioactive compounds extracted from its resin named gamboge have biological activities, such as anti-tumor, antibacterial, and anti-inflammatory activities (Jia et al. 2015; Hatami et al. 2020). Gambogic amide (GA-amide) is a derivative of gambogenic acid (GA), the main active compound of gamboge. Previous studies reported that GA-amide is a non-peptide molecule with selective high affinity for the tropomyosin receptor kinase A (TrkA) that upregulates the expression and tyrosine phosphorylation of TrkA (Obianyo and Ye 2013; Shen and Yu 2015). GA-amide can prevent glutamate-induced apoptosis and induce neurite outgrowth in PC12 cells (Jang et al. 2007) and is tolerated in vivo. Additionally, GA-amide can promote osteoblastic differentiation, improve fracture healing in mice, and ameliorate leukemia progression in K562 cell-inoculated nude mice (Chan et al. 2009).

TrkA is a tyrosine kinase receptor with a primary function in neurodevelopment (Singer et al. 1999). Nerve growth factor (NGF) can bind to TrkA, which activates cAMP response element-binding protein (CREB) to regulate the regeneration, survival, and proliferation of neurons (Lu et al. 2010; Hirose et al. 2016). Recent studies have shown that TrkA is associated with angiogenesis in a variety of tumours, such as neuroblastoma, ovarian cancer, and breast cancer (Eggert et al. 2000; Lagadec et al. 2009; Vera et al. 2014). Therefore, TrkA may play important roles in angiogenesis in the brain, ovary, and other organs.

PI3K/AKT, Ras/MAPK, and PLCγ are three important signaling pathways downstream of TrkA. Many researchers have reported that pathways stimulated by TrkA activation can mediate cell proliferation, invasion, angiogenesis, and death (Kruttgen et al. 2006; Molloy et al. 2011; Wang et al. 2016). Upon activation by NGF, the Erk1/2 and AKT pathways can promote human choroidal endothelial cell migration and proliferation (Steinle and Granger 2003), and TrkA activation can promote cell proliferation and angiogenesis through the oncosgenes c-MYC and VEGF in epithelial ovarian cancer (Garrido et al. 2020). However, the overexpression of TrkA in SY5Y NB cells inhibited
angiogenesis and tumour growth by downregulating angiogenic factors (Eggert et al. 2000, 2002).

Vascular disorders are found in many kinds of diseases, such as type 2 diabetes mellitus (D2M) (Hassanpour et al. 2017), glioma (Chow et al. 2016; Ameratunga et al. 2018), non-small cell lung cancer (Tian et al. 2020) and diabetic retinopathy (Cheung et al. 2010), so it is important to find drugs which have antiangiogenic effect.

The effects of TrkA on angiogenesis are not clear, and limited studies have indicated a possible role for GA-amide in angiogenesis. As the formation of vascular is based on the differentiation, proliferation, and migration of endothelial cells (ECs) (Teleanu et al. 2019), ECs is an important mode used in the study of antiangiogenic therapies. In this study, we used human umbilical vein endothelial cells (HUVECs), a well-known ECs model (Hassanpour et al. 2018; Rezabakhsh et al. 2018; Rezabakhsh et al. 2019), and normal human brain microvascular endothelial cells (NhECs) as in vitro models and the chick chorioallantoic membrane (CAM) assay as an in vivo model to explore the effect of GA-amide on angiogenesis, and to explore the relationship between the effects of GA-amide and TrkA and the pathways potentially affected by GA-amide.

Materials and methods

Cell lines and cell culture

HUVECs were obtained from the National Infrastructure of Cell Line Resource (Beijing, China). NhECs were purchased from Cell Systems (Seattle, WA, USA). ECs were maintained in EBM-2 medium (Lonza, Switzerland) containing 2% foetal bovine serum (FBS), 0.1% ascorbic acid, 0.1% GA-1000, 0.1% hEGF, 0.1% heparin, 100 U/mL penicillin, and 100 mg/mL streptomycin (Lonza, Switzerland).

Viability assay

MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, is a tetrazolium compound widely used in cell viability assay (Yu et al. 2016; Chen et al. 2020). The cells were seeded in 96-well plates at a density of 1 × 10^4 cells per well and incubated overnight in EBM-2 medium. Cells were treated with DMSO and GA-amide (0.1, 0.15, 0.2, 0.3, 0.4, and 0.5 μM) for 48 h. Cell viability was assessed by MTS assays (Cell Titer96; PROMEGA. Madison, WI, USA) according to previously described methods (Flores-Arriaga et al. 2017).

Wound-healing assay

Cell motility was measured by the wound-healing assay as reported previously (Zhou et al. 2017). Typically, HUVECs (4 × 10^5 cells/well) or NhECs (3 × 10^5 cells/well) were seeded in 6-well plates and grown for 24 h to reach 90–100% confluence. Then, a linear wound was scratched across the middle of the well surface using a 200 μL pipette tip. Next, culture media were replaced with fresh culture media containing 0.2 μM GA-amide, and the cells were incubated for 12 h. DMSO was used as a vehicle control. At 0, 6, and 12 h, the wound was photographed with a Nikon inverted microscope at 100× magnification, and relative cell migration was quantified using ImageJ image analysis software (Bethesda, MD, USA).

In vitro angiogenesis assay

HUVECs (2 × 10^5 cells/well) or NhECs (1.5 × 10^5 cells/well) were seeded to 6-well plates overnight and treated with DMSO or 0.2 μM GA-amide for 6 h. Then, the cells were trypsinized, counted, and resuspended in basal medium. HUVECs (1 × 10^5 cells/well; 100 μL) or NhECs (1 × 10^5 cells/well; 100 μL) were seeded in 96-well plates precoated with 60 μL Matrigel at 37°C for 2 h. After 6 h of incubation at 37°C and 5% CO_2, tube formation was photographed with a Nikon inverted microscope, and tubular structures were analyzed and quantified by ImageJ image analysis software. Data were collected by measuring the total mass segment length (pixel) of capillary tubes in five randomly selected standardized fields (the central field of view and four fields up, down, left, and right) of each well under 100× magnification.

Apoptosis assay

HUVECs (2 × 10^5 cells/well) or NhECs (1.5 × 10^5 cells/well) were seeded in 6-well plates overnight and treated with DMSO and GA-amide (0.1, 0.2, 0.4, 0.8 and 1.2 μM) for 6 h. Apoptosis assays were performed using a FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, CA, USA) as previously described (Hu Y et al. 2019). Cells were collected after treatment with DMSO or GA-amide and resuspended in 1× binding buffer. Then, 5 μL FITC Annexin V and 5 μL PI were added to the cell suspension, and the mixture was incubated for 15 min at room temperature in the dark. Cell apoptosis was determined by flow cytometry within 1 h.

Reverse transcription real-time quantitative PCR (qRT–PCR)

HUVECs (2 × 10^5 cells/well) or NhECs (1.5 × 10^5 cells/well) were seeded in 6-well plates overnight and treated with DMSO or 0.8 μM GA-amide for 6 h. Total RNA was isolated using TRIzol reagent (Life Technologies, CA, USA) according to the manufacturer’s protocol, and qRT–PCR was performed using SYBR Premix Ex Taq Master Mix and a 2-Step kit (TaKaRa, Dalian, China) as previously described (Hu et al. 2017). All primers were synthesized by TSINGKE (Beijing, China), and the following primers were used to amplify target mRNA and the internal control: vWF forward (5′-AGGCTTTGAACTCGGAAGCAT-3′) and reverse (5′-GGCCATCCCAGTCCATCTG-3′); CD31 forward (5′-AACAGTGTTGACATGAAGAGCC-3′) and reverse (5′-TGTTAAAACAGACGCTATCCCTG-3′); CD31 forward (5′-AACAGTGTTGACATGAAGAGCC-3′) and reverse (5′-TTGTTAAAACAGACGCTATCCCTG-3′); CD105 forward (5′-TGACCTTGGCCTACAATTCCA-3′) and reverse (5′-GGCCATCCACTCAAGGATCT-3′); and GAPDH forward (5′-GTCATCATGACAATTGG-3′) and reverse (5′-GGCCATCACGCCACAG-3′).

Western blot assay

HUVECs (2 × 10^5 cells/well) or NhECs (1.5 × 10^5 cells/well) were seeded in 6-well plates overnight and treated with DMSO or GA-amide (0.1, 0.2, 0.4, 0.8, and 1.2 μM) for 6 h. Western blot assays were performed as previously described (Tian et al. 2020). Cells were lysed with Tris-NaCl-Triton-EDTA (TNTNE), and 8 μg protein was separated by SDS-polyacrylamide gel electrophoresis (SDS–PAGE) according to molecular weight. Then, the separated proteins were transferred to NC membranes, and antibodies were used to detect the expression of the corresponding proteins. The following antibodies were used: anti-CD31 (Ab28364,
Abcam), anti-CD105 (Ab169545, Abcam), anti-VEGFR2 (2479S, Cell Signalling Technology [CST], MA, USA), anti-vWF (65707S, CST), anti-VEGF (Ab53465, Abcam), anti-mTOR (2983T, CST), anti-p-mTOR (2974T, CST), anti-AKT (2938S, CST), anti-p-AKT (9018S, CST), anti-Erk1/2 (4695T, CST), anti-p-Erk1/2 (4370S, CST), anti-PLCγ (3872T, CST), anti-p-PLCγ (3871T, CST), anti-PARP (9542L, CST), anti-caspase-3 (9665S, CST), anti-p-TrkA (9141S, CST), anti-TrkA (Sc-118, Santa Cruz Biotechnology) and anti-β-actin (A5441, Sigma–Aldrich, St. Louis, MO, USA).

**siRNA-mediated gene knockdown assay**

Cells were seeded into 6-well plates and transfected with siRNA oligonucleotides for 72 h using INTERFERin® (PolyPlus, NY, USA) according to the manufacturer’s protocol. The siRNA sequence was as follows: siTrkA sense: 5′-CGAGAACCCACAAUACUUCAGUGAU-3′.

**Chick chorioallantoic membrane (CAM) assay**

The CAM assay was performed as described in a previous study (Varinské et al. 2018). Chicken eggs at seven days post-fertilization (Boehringer Ingelheim, Beijing) were cleaned with 70% ethanol, and a window of ∼1.5–2.0 cm² was gently opened on the blunt end of the egg without damaging the embryo. A sterilized silicone ring (inner diameter, 6 mm) was positioned on the CAM surface avoiding major blood vessels. Then, DMSO, 18.8 ng GA-amide, 62.8 ng GA-amide, or 40 μg Bevacizumab (positive control) was placed within the ring. The eggs were incubated in a forced-draft incubator at 38 ± 0.2°C, with ~60–65% humidity. After 72 h of treatment, the large vessels, small vessels, and capillaries of the CAM were counted. The angiogenesis index was calculated as the difference between the number of vessels after 72 h and the number of vessels before each treatment. Photographs of CAM blood vessels forming inside the rings were obtained using a Nikon stereomicroscope (Tokyo, Japan).

**Statistical analysis**

All experiments were repeated at least three times and representative results are presented. All experimental data were processed by GraphPad Prism 9.0 software. The data are presented as the mean± standard deviation (S.D.). Significant differences between individual groups were determined by one-way ANOVA or two-way RM ANOVA. A t-test was used for statistical analyses between two independent groups. *p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001 indicated statistical significance.

**Results**

**GA-amide inhibited the proliferation and induced the apoptosis of HUVECs and NhECs**

To find a safe dose for use in the angiogenesis assays that does not affect the proliferation of HUVECs and NhECs, we first analyzed the inhibitory effects of GA-amide on EC proliferation by MTS assays. As shown in Figure 1(A), GA-amide inhibited the proliferation of HUVECs and NhECs after treatment for 48 h in

![Figure 1](image-url)
the range of 0.1–0.5 μM, with IC\textsubscript{50} values of 0.1269 and 0.1740 μM against HUVECs and NhECs, respectively. As shown in Figures 1(B–E), GA-amide induced the apoptosis of HUVECs and NhECs in a dose-dependent manner in the range of 0.4–1.2 μM after treatment for 6 h. After treatment with 0.4, 0.8, and 1.2 μM GA-amide for 6 h, ECs showed enhanced expression of apoptosis-related proteins, such as cleaved PARP and cleaved caspase-3 (Figures 1(F,G)). From the above experiments, we also observed that treatment with 0.2 μM GA-amide for 6 h did not induce EC death, so the treatment conditions for the angiogenesis assay were set as 0.2 μM GA-amide for 6 h.

**GA-amide suppressed HUVECs and NhECs migration in vitro**

EC migration is essential for angiogenesis; thus, we assessed the antimigration effects of GA-amide on HUVECs and NhECs using a relatively safe dose of 0.2 μM in vitro. As shown in Figures 2(A,B), ECs actively migrated into the wounded area in the DMSO, and 0.2 μM GA-amide treatment slowed EC migration. The statistical analysis revealed no significant difference in relative cell migration between HUVECs treated with GA-amide or DMSO for 6 h, but 0.2 μM GA-amide significantly inhibited the migration of HUVECs after 12 h of treatment (Figure 2(C)). Moreover, 0.2 μM GA-amide significantly inhibited the migration of NhECs after 6 and 12 h (Figure 2(D)).

**GA-amide inhibited angiogenesis both in vitro and in vivo and suppressed the expression of EC markers at both the mRNA and protein level**

Since angiogenesis is characterized by the formation of tubular structures by capillary ECs, we conducted capillary tube formation assays to assess the effects of GA-amide on tube formation. As shown in Figures 3(A,C), when grown freely on Matrigel, HUVECs and NhECs became aligned into cords on the Matrigel, and highly organized capillary tubes formed. Treatment of HUVECs and NhECs with 0.2 μM GA-amide effectively suppressed tube formation compared with the control (Figures 3(A–D)). We also evaluated the in vivo antiangiogenic effects of GA-amide by using the CAM assay. We counted the number of large vessels, small vessels, and capillaries in CAM models treated with DMSO, bevacizumab, and GA-amide. As shown in Figures 3(E,F), GA-amide had the same antiangiogenic effects as bevacizumab (positive control), and 62.8 ng GA-amide suppressed the formation of capillaries but not of large or small vessels. To further confirm the antiangiogenic effects of GA-amide, we detected the expression of EC markers, such as vWF, CD31, and CD105; the results showed that GA-amide downregulated the expression of vWF, CD31, and CD105 at both the mRNA and protein levels (Figures 3(G–J)).

**TrkA knockdown did not reverse the antiangiogenic effects of GA-amide on HUVECs and NhECs**

GA-amide can activate TrkA, so we wanted to determine whether the antiangiogenic effects of GA-amide occur through TrkA. First, we found that 0.2 μM GA-amide enhanced the tyrosine phosphorylation of TrkA in HUVECs and NhECs (Figure 4(A)). Then, we used siRNA to knockdown the expression of TrkA in ECs (Figure 4(B)). As shown in Figures 4(C–F), GA-amide reduced tube formation by TrkA-knockdown ECs. Meanwhile, the tube formation capability was not significantly

![Figure 2](image-url)
different between normal ECs and TrkA-knockdown ECs treated with GA-amide (Figures 4(G–J)).

The inhibition of TrkA could not reverse the antiangiogenic effects of GA-amide on HUVECs and NhECs

To confirm that the antiangiogenic effects of GA-amide were unrelated to TrkA, we treated ECs with a selective TrkA inhibitor, GW441756, and evaluated the antiangiogenic effects of GA-amide. As shown in Figure 5(A), we verified that GW441756 inhibited the phosphorylation of TrkA in ECs, and this treatment did not affect the antiangiogenic activity of GA-amide in ECs (Figures 5(B,C)). Additionally, there was no significant differences in tube formation by ECs upon treatment with GA-amide in the presence or absence of GW441756 (Figures 5(D,E)).

GA-amide suppressed the expression of VEGF/VEGFR2 and downregulated the activation of the AKT/mTOR and PLCγ/Erk1/2 pathways

AKT/mTOR and PLCγ/Erk1/2 pathways are the two main pathways related to angiogenesis (Ahir et al. 2020), so we investigated the influence of GA-amide on the activation of these two pathways. As shown in Figures 6(A,B), GA-amide downregulated the activation of the AKT/mTOR and PLCγ/Erk1/2 pathways in cells treated for 6 h. The expression of VEGF and VEGFR2 can be regulated by the AKT/mTOR and PLCγ/Erk1/2 pathways (Nicolas et al. 2019; Wang et al. 2019; Zhong et al. 2020), and our results showed that GA-amide suppressed the expression of VEGF and VEGFR2 in cells treated for 6 h (Figures 6(C,D)). According to previous studies, GA-amide might act as an NGF analogue to activate TrkA and its downstream pathways to help neuronal cell to survive (Roux and Barker 2002; Szegedi et al. 2008; Greenberg et al. 2009). In our study, we found that GA-amide suppressed the expression of VEGF and VEGFR2 and decreased AKT/mTOR and PLCγ/Erk1/2 pathway activation in ECs; this may reduce VEGF secretion into the extracellular space and the binding of VEGF to VEGFR2, resulting in the observed antiangiogenic effects of GA-amide (Figure 6(E)).

Discussion

GA-amide is a derivative of GA, and most studies on GA-amide, have focussed on its’ function as a selective TrkA agonist (Jang et al. 2007; Obianyo and Ye 2013; Shen and Yu 2015). In our present study, we showed for the first time that GA-amide has an antiangiogenic effect in vitro and in vivo, and this effect does not involve TrkA.
Angiogenesis, the formation of new blood vessels from pre-existing vessels by the proliferation, migration, and differentiation of vascular endothelial cells (ECs), is an important process in building the mature vascular network essential for the delivery of oxygen and nutrients in normal tissues. Researchers have found that angiogenesis is overactivated in tumors and can promote tumor growth, progression, and metastasis (Mazzone et al. 2009; Nagy et al. 2010; Carmeliet and Jain 2011; De Bock et al. 2011; Farzaneh Behelgardi et al. 2018). After Judah Folkman established the antiangiogenic concept in cancer therapy (Folkman 1971), an increasing number of preclinical and clinical studies have focused on this topic. Currently, angiogenesis has been validated as a target in several tumor types through randomized trials, and vascular endothelial growth factor (VEGF) pathway inhibitors have been incorporated into the therapeutic armory (Kerbel and Kamen 2004; Kim et al. 2006).

In the past decade, first-generation antivascular drugs have undergone unprecedented development and have shown very promising therapeutic effects in the treatment of various tumors, such as prolonging progression-free survival and overall survival. However, antivascular drugs have side effects, and tumors can develop intrinsic and acquired resistance, which leads to therapeutic failure (Ellis and Hicklin 2008). Bevacizumab is an antivascular treatment for glioblastoma, but because of its low blood–brain barrier permeability, this agent is administered at a high dose, which can cause serious systemic side effects, including intestinal perforation and pulmonary embolism (Riina et al. 2009). GA-amide possesses robust neurotrophic activity, indicating that it can pass through the blood–brain barrier and enter brain tissue. Our research showed that GA-amide can inhibit EC angiogenesis, indicating that it has potential for the treatment of brain tumors in the future.

Previous studies have demonstrated the important roles of VEGF and VEGFR2 in angiogenesis, and VEGFR2 is the main mediator of VEGF-triggered angiogenesis (Gho et al. 2019). When VEGF binds to VEGFR2, the receptor autophosphorylates its tyrosine residues, increasing its tyrosine kinase activity and the activation of downstream intracellular signalling molecules, including AKT/mTOR and PLCγ1/Erk1/2 (Zhang et al. 2016; Assareh et al. 2019). Our research found that GA-amide could downregulate the expression of VEGF and VEGFR2. Considering the VEGF and VEGFR2 can be regulated by the AKT/mTOR pathway, we speculated that GA-amide could also downregulate the AKT/mTOR and PLCγ1/Erk1/2 pathways.
and PLCγ/Erk1/2 pathways (Nicolas et al. 2019; Wang et al. 2019; Zhong et al. 2020), we hypothesized that GA-amide can suppress the expression of VEGF and VEGFR2 by downregulating the activation of the AKT/mTOR and PLCγ/Erk1/2 pathways, thereby inhibiting angiogenesis by ECs.

Many phytochemicals can inhibit angiogenesis by inhibiting the expression of VEGF/VEGFR2 and the activation of downstream pathways. Gambogic acid can inhibit angiogenesis by suppressing the activation of VEGFR2 and its downstream protein kinases c-Src, FAK, and AKT in prostate cancer (Yi et al. 2008; Wang and Chen 2012). Polydatin can significantly decrease the VEGF-induced phosphorylation of AKT, eNOS, and Erk (Hu WH et al. 2019). Curcumin can inhibit the activation of FAK, AKT, and Erk in HUVECs and inhibit tumour growth in a VEGF-overexpressing tumour model (Fu et al. 2015).

Overall, we found that GA-amide, a phytochemical that has antiangiogenic effects, has potential in angiogenesis-related diseases, such as cancer and diabetic retinopathy. However, our current research mainly focussed on the effect of GA-amide on angiogenesis by normal ECs. To further clarify the role GA-amide in tumours and fundus vascular diseases, we need more in vivo and in vitro research models, such as tumour-derived endothelial cells, tumour cells, and diabetic retinopathy animal models. By exploring the effects of agent in these models, we can further evaluate the therapeutic effects of GA-amide on diseases and promote its translation into clinical applications.

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**Figure 5.** The inhibition of TrkA could not reverse the antiangiogenic effects of GA-amide on HUVECs and NhECs. (A) The inhibitory effects of GW441756 (TrkA inhibitor) on TrkA in HUVECs and NhECs were evaluated by Western blot. (B,C) Representative images showing the antiangiogenic effects of GA-amide with or without GW441756 on HUVECs (B) and NhECs (C). (D,E) Quantitative analysis of (B) and (C). Images were analyzed by ImageJ software, and the numbers of capillary-like structures in five separated fields of one well was calculated. Scale bar, 200 μm. Data are presented as the means ± S.D. of three independent determinations. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with DMSO by two-tailed Student's t-test.
Conclusions

Our research demonstrated the antiangiogenic property of GA-amide in vitro and in vivo via the suppression of VEGF/VEGFR2 and the downstream AKT/mTOR and PLCγ1/Erk1/2 pathways in a TrkA-independent manner. These findings provide pharmacological support for GA-amide as a candidate agent in anticancer therapy.

Disclosure statement

The authors declare that they have no conflicts of interest.

Author contributions

Tongtong Sui designed and performed research, analyzed data, and wrote the paper. Bojun Qiu, Jiaorong Qu, Yuxin Wang, and Kunnian Ran collected data and performed the statistical analysis. Wei Han and Xiaozhong Peng directed the experimental design and data analysis.

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