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Effects of follicle-stimulating hormone on the proliferation and apoptosis of infantile hemangioma stem cells

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ABSTRACT

Objective: To investigate the effects of different concentrations of follicle-stimulating hormone (FSH) on the proliferation and apoptosis of human hemangioma stem cells, it will provide a basis for studying the mechanism of FSH in treating hemangioma.

Methods: Hemangioma specimens were collected from the Longgang District Maternity & Child Healthcare Hospital of Shenzhen City. Hemangioma stem cells were treated with different concentrations of FSH. Cell viability was detected by CCK8 method and cell apoptosis was analyzed by flow cytometry.

Results: Hemangioma stem cells (HemSCs) were extracted from fresh tissue of infantile hemangioma by the CD133 immunomagnetic bead method. Under the influence of FSH at different concentrations (0, 100, 1000 IU/L), the cell viability of hemangioma stem cells increased significantly in a concentration-dependent manner ($P < 0.05$). At the same time, the apoptosis of hemangioma stem cells decreased with increasing concentrations of follicle-stimulating hormone ($P < 0.05$). Specifically, 1000 IU/L FSH significantly promoted the proliferation of hemangioma stem cells and inhibited their apoptosis.

Conclusion: High concentration of follicle-stimulating hormone can maintain the growth of hemangioma by promoting the proliferation and inhibiting the apoptosis of hemangioma stem cells.

1. Introduction

Infantile hemangioma (IH) is the most common benign tumor found in infants and young children [1–3]. IH can occur anywhere on the body, with 60% of cases appearing on the head and neck, 25% on the torso, and 15% on the limbs. One unique feature of IH is that it occurs more frequently in female infants, with an incidence rate 3–5 times higher than that in male infants [4]. Although most infantile hemangiomas regress without leaving any functional sequelae, a considerable number of cases can cause organ dysfunction or serious complications, such as refractory ulcers, local and systemic infections, vision impairment, and airway obstruction [5,6]. Infantile hemangiomas tend to occur on the head, face, and neck, and can persist. The regression period of an infantile hemangioma can last as long as 5–10 years [4]. Severe infantile hemangiomas can significantly affect facial development in children, causing defects in the lip, soft nose, and even dysplasia. These obvious deformities and dysfunctions often require surgical treatment, which can place a significant psychological and physical burden on both children and parents. However, the pathogenesis of infantile hemangioma is still unclear.

Follicle-stimulating hormone (FSH) is a gonadotropin [7,8]. In a study on the role of follicle-stimulating hormone in vascular malformations, it was reported that the development of infantile hemangiomas was found to be associated with the level of follicle-stimulating hormone [9,10]. This finding suggests a key role for follicle-stimulating hormone in the development of infantile hemangiomas. Clinically, the incidence

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of infantile hemangioma in females is 3–5 times higher than in males, and the level of follicle-stimulating hormone in females during infancy is 5–7 times higher than in males [11,12]. Furthermore, the level of follicle-stimulating hormone in low birth weight newborns is 38% higher than in normal newborns, which is also associated with a higher incidence of hemangiomas in low birth weight newborns [13]. There are many similarities between the placenta and infantile hemangioma, and pathological studies have found high expression of follicle-stimulating hormone receptors on the placenta [14,15]. Further, studies have found that the level of follicle-stimulating hormone receptor in infantile hemangioma is significantly higher than in other vascular malformations. Furthermore, the expression of follicle-stimulating hormone receptor in infantile hemangioma tissue during the proliferative stage is higher than during the regressive stage, suggesting that follicle-stimulating hormone plays a crucial role in the pathogenesis of infantile hemangioma. In addition, it has been suggested that infantile hemangioma pericytes, which are rich in follicle-stimulating hormone receptors, may play a critical role in the pathogenesis of hemangiomas [9,16]. Therefore, studying the pathogenesis of hemangioma can help us understand the cause of the disease and identify high-risk factors.

In summary, our aim is to elucidate the role of follicle-stimulating hormone in hemangioma stem cells. In this study, we analyzed the relationship between follicle-stimulating hormone and the proliferation and apoptosis of hemangioma stem cells. These findings may serve as a possible target for the prevention and treatment of hemangioma in the future, and provide better theoretical support for the management of infantile hemangioma.

2. Materials and methods

2.1. Cells and reagent

The hemangioma-derived stem cells (HemSC) were obtained from hemangioma. It was sorted by CD133 immunomagnetic beads method [17]. The immunophenotype of isolated and cultured passage HemSC was examined by flow cytometry. Flow cytometry results showed that the expression rates of CD133 and CD90 in HemSC were 99.8% and 96.7%, respectively. Indicating that the cells isolated in this study are Hem-SCs, which can be used for follow-up related tests. FSH was purchased from MedChemExpress.

2.2. Cell viability assay

Cell viability was analyzed by CCK-8 assay. Cell Counting Kit-8 (MedChemExpress, New Jersey, USA) was used for CCK-8 assays. Cells were plated in 96-well plates at a density of 2000 cells in 100 μL medium per well, starved with medium without FBS for 24 h, followed by treatment with indicated concentrations of FSH in medium containing 0.5% FBS for 24 h, 48 h, 72 h, 96 h. Then 10 μL of the CCK-8 solution was added to each well of the plate and incubated at 37 °C for 2 h. The absorbance at 450 nm was measured to calculate the number of vital cells in each well.

2.3. Colony formation assay

Cells were cultured and seeded into 6-well plates (800 cells/well). The plates were cultured for 14 days with treated with FSH in a 5% CO2 incubator at 37 °C. Colonies with >50 cells were counted. Cells were fixed with methyl alcohol for 25 min. Cell colony was stained with crystal violet for 25 min. The number of cell colonies was counted using a light microscope.

2.4. Flow cytometric analysis

Cells were resuspended at a concentration of 5×10^3/mL in PBS and then cells were stained with anti-CD133/CD29/CD44/CD90 antibody. The cells were detected by BD AccuriTMC6 Flow Cytometer (BD Biosciences, USA). The data were analyzed by Flowjo software and obtained from 3 independent experiments.

2.5. Cell cycle assay and apoptosis analysis

For cell cycle assay, after treated with FSH, cells were harvested with trypsin and fixed in 70% ice-cold ethanol at 4 °C for overnight. After washing with phosphate buffered saline, the cell pellet was resuspended in 20 μg/mL PI staining buffer and incubated at 37 °C for 60 min in the dark.

Apoptosis was detected using Annexin V/PI double staining. Approximately cells were seeded into six-well plates and treated with FSH the next day. After cultured for 48 h, the cells were incubated in 5 μl Annexin V and 20 μl PI at 37 °C in the dark in the dark for 60 min. Apoptosis and cell cycle status were analyzed by flow cytometry (BD FACSCalibur, BD Biosciences, San Jose, CA, USA).

2.6. Caspase-3 activity assay

Caspase-3 activity was assessed in accordance with the manufacturer's protocol using a Caspase-3 assay kit. In brief, cells were lysed with 50 μl of lysis buffer and incubated for 10 min. Subsequently, protein concentrations in the resulting supernatant were determined using the BCA protein assay. A total of 100 μg of protein, dissolved in 50 μl of lysis buffer, was then transferred to a 96-well plate. To initiate the assay, reaction buffer, DL-dithiothreitol, and the caspase-3 catalytic substrate DEVD-pNA were added to the protein samples. This was followed by an incubation period of 2 h at 37 °C to allow for the enzymatic reaction. The optical density (OD) at 405 nm was measured using a microplate reader.

2.7. Statistical analysis

All experiments were repeated three times, and SPSS 20.0 software was used for statistical analysis. Measurement data are expressed as mean ± standard error. Statistical methods used included *t*-test, one-way ANOVA. $P < 0.05$ indicated a statistically significant difference.

3. Results

3.1. Cultivation and identification of hemangioma stem cells

It is currently recognized that infantile hemangioma tissues are rich in poorly differentiated CD133-positive cells, which are hemangioma stem cells (HemSCs). We collected the fresh tissues from infants aged 2–4 months who were clinically diagnosed.

HemSCs were extracted from fresh tissue of infantile hemangioma by CD133 immunomagnetic beads method. After the cells were observed, the cells were spindle shaped (Fig. 1A). The markers (CD29, CD44, and CD90) were reported as HemSCs surface markers [18,19]. Thus, the expressions of HemSCs surface markers CD29, CD44, and CD90 were further detected by flow cytometry (Fig. 1B–D). Taken together, HemSCs were obtained and cultured.

3.2. FSH promotes cell proliferation of hemangioma stem cells

To study whether follicle-stimulating hormone can affect the cell proliferation ability of hemangioma stem cells, we treated the cells with different concentrations of follicle-stimulating hormone (0, 100 IU/L and 1000 IU/L). The dosages of 100 and 1000 IU/L for FSH may have been commonly used and well-established in prior researches [20–22]. The cell proliferation abilities were enhanced by FSH in a dose-dependent manner by CCK8 assay (Fig. 2A–B). When the cells were treated for different periods (24, 48, 72 and 96 h), the cell proliferation abilities were enhanced in a time-dependent manner. Meanwhile, we further assessed the role of FSH on proliferation by other assays like

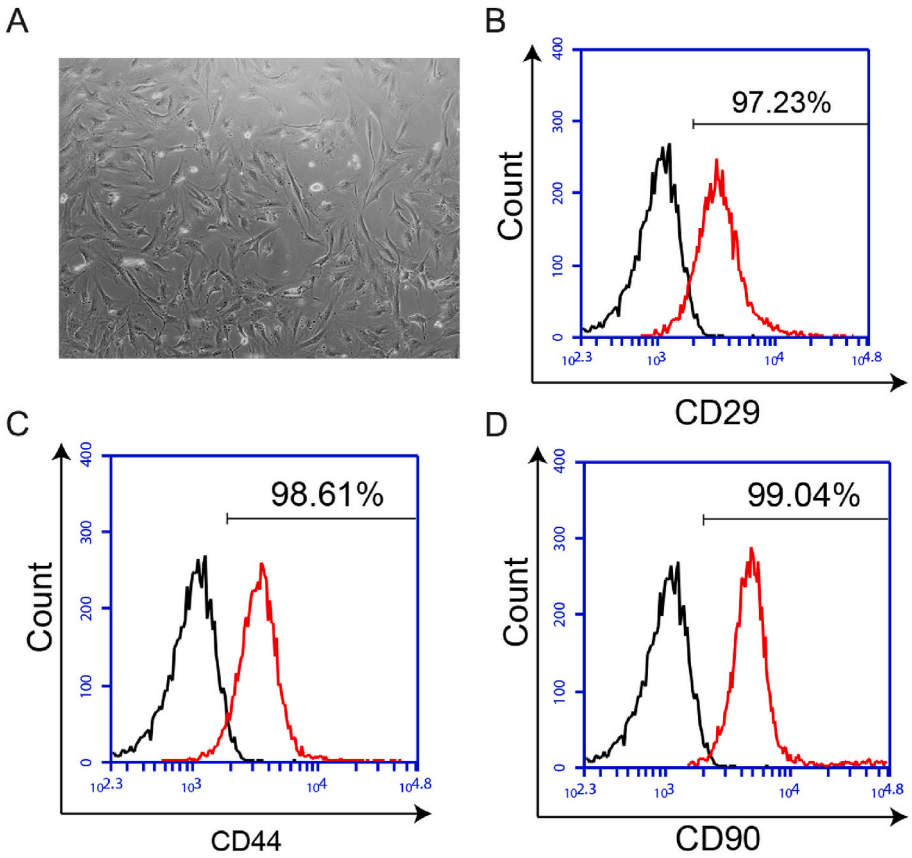

Fig. 1. Extract and verify hemangioma stem cells. (**A**) Hemangioma stem cells were extracted from fresh tissue of infantile hemangioma and observed in microscope. (**B-D**) Flow cytometry analysis of CD29, CD44 and CD90 expression in the hemangioma stem cells. The black stands for IgG group, red stands for positive group indicating cells stained with CD29-PE/CD44-FITC/CD90-FITC antibody. Data are shown as the means of three independent experiments or representative data. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

colony formation assay. The cells were treated with 1000 IU/L follicle-stimulating hormone. It showed that FSH promoted the colony-forming ability (Fig. 2C–D). These data suggested FSH could enhance cell proliferation of hemangioma stem cells and promote tumor progression.

3.3. FSH inhibits the apoptosis of hemangioma stem cells

At the same time, to study whether follicle-stimulating hormone can affect the apoptosis of hemangioma stem cells, cells were analyzed by flow cytometry following Annexin V-FITC and propidium iodide (PI) dual labeling. Apoptosis rate was reduced from 7.07% to 3.43% when the cells were treated with 100 IU/L follicle-stimulating hormone (Fig. 3A–B). In addition, the apoptosis rate was just 1.39% when cells were treated with 1000 IU/L follicle-stimulating hormone. Meanwhile, as evidenced by caspase-3 activity, cell apoptosis of hemangioma stem cells was inhibited after FSH treatment (Fig. 3C). Therefore, the cell apoptotic abilities were decreased in a dose-dependent manne. Taken together, our results indicate FSH could inhibited cell apoptosis.

3.4. FSH promoted cell cycle progression in hemangioma stem cells

The cell cycle plays a crucial role in the development and progression of cancer. We further assessed the role of FSH in cell cycle progression of hemangioma stem cells. The cells were treated with 1000 IU/L follicle-stimulating hormone. It showed that the percentages of the cells in the S phase among the cells treated with control group and FSH group were 16.02 ± 0.74% and 43.53 ± 1.13%, respectively (Fig. 4). These data indicated that FSH could speed up the G1/S phase transition.

4. Discussion

Follicle-stimulating hormone (FSH), a gonadotropin, plays a crucial role in hemangioma. The present study revealed that high concentrations of FSH regulate proliferation and apoptosis in hemangioma. We have demonstrated that FSH promotes tumor proliferation and inhibits cell apoptosis in hemangioma.

Accumulating evidence indicates that FSH plays an important role in tumors. FSH and its receptor have been shown to be involved in various cancers [15,21,23–25]. FSH has multiple biological functions, including promoting cell proliferation, metastasis, and angiogenesis [15,26–28]. As is known, the combination of FSH and its receptor can up-regulate HIF-1α, increase the expression of downstream VEGF, and thus promote angiogenesis in ovarian granulosa cells [29]. FSH can regulate angiogenesis in follicle development, which may also be consistent with the angiogenesis caused by endothelial cell proliferation in infantile hemangioma. It has been reported that the expression of follicle-stimulating hormone receptor was found in the specimens of various cancer patients, and it was mostly expressed around the vascular endothelium [15,30]. There are also studies reporting the expression of follicle-stimulating hormone receptors in invasive breast cancer, which is involved in the remodeling of peri-tumoral blood vessels.

The interaction between FSH and its cognate receptor (FSHR) plays a crucial role in cancers [31,32]. Upon binding, FSH triggers the rapid activation of various signaling molecules, resulting in diverse effects within tumour cells. One extensively studied signaling cascade controlled by the FSH–FSHR interaction involves the generation of second messenger molecules, namely cAMP, IP3, and DAG. These second messengers, in turn, activate downstream protein kinases, which

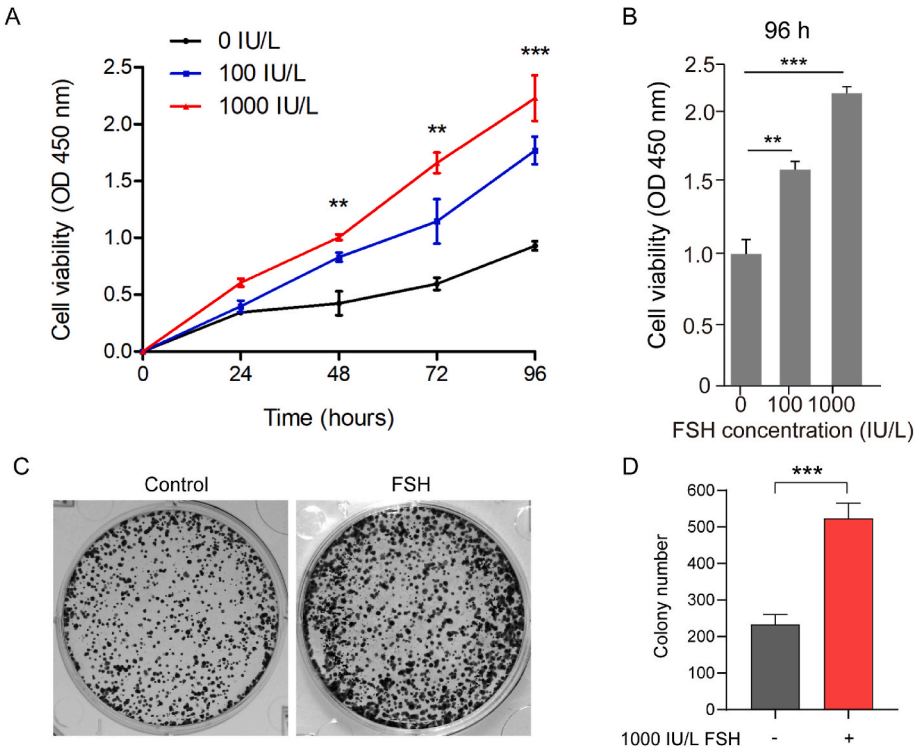

Fig. 2. FSH promotes cell proliferation. (**A**) Different concentrations of follicle-stimulating hormone were treated hemangioma stem cells for 24 h, 48 h, 72 h and 96 h. Cell viability was measured by CCK8 assay. (**B**) At the end of time (96 h), it's quantitative analysis of FSH-induced cell proliferation. (**C-D**) The colony-forming ability of hemangioma stem cells treated with 1000 IU/L follicle-stimulating hormone for 48 h. Colonies stained with crystal violet. Graphic quantification of the colonies. Data are shown as the means of three independent experiments or representative data. Data are expressed as mean ± standard deviation. $^{**}P < 0.01$, $^{***}P < 0.001$, by Anova One Way. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

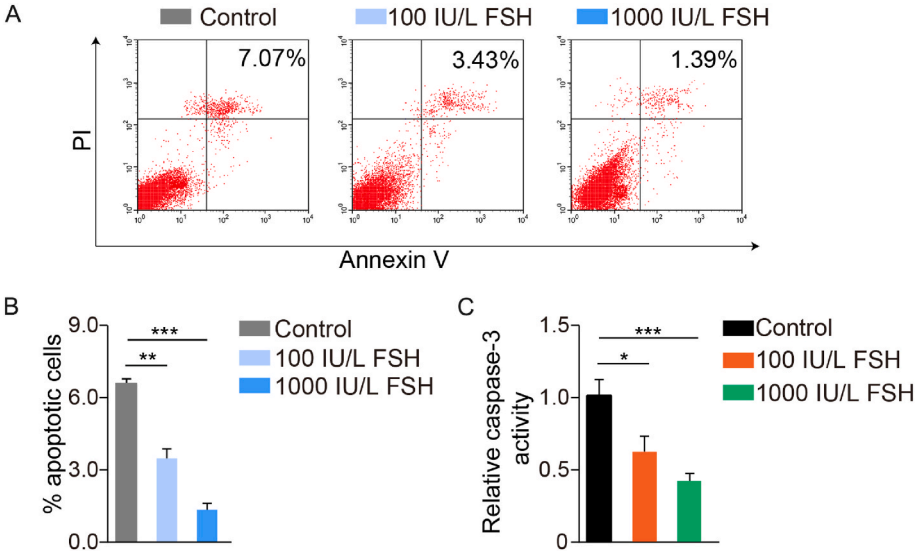

Fig. 3. FSH inhibits cell apoptosis. (**A**) Different concentrations of follicle-stimulating hormone were treated hemangioma stem cells for 48 h. Cell apoptosis was measured by flow cytometry. (**B**) Quantitative analysis of FSH-reduced cell apoptosis is shown. (**C**) The caspase-3 activity of hemangioma stem cells was measured for 48 h. Data are shown as the means of three independent experiments or representative data. Data are expressed as mean ± standard deviation. $^{*}P < 0.05$, $^{*}P < 0.01$, $^{***}P < 0.001$.

subsequently trigger the activation of different transcription factors [32]. In summary, the FSH–FSHR interaction leads to the activation of multiple signaling pathways and transcription factors.

It has been confirmed that high levels of follicle-stimulating hormone can promote the proliferation of hemangioma stem cells to maintain the growth of hemangioma, while low levels of follicle-stimulating hormone can inhibit the proliferation of hemangioma stem cells. The hormone can promote the proliferation of hemangioma stem cells. And follicle-stimulating hormone could speed up the G1/S phase transition and even inhibit cell apoptosis, thereby contributing to hemangioma progression. Some studies suggested follicle-stimulating hormone (FSH) stimulates protein kinases including protein kinase B (PKB/Akt), p38

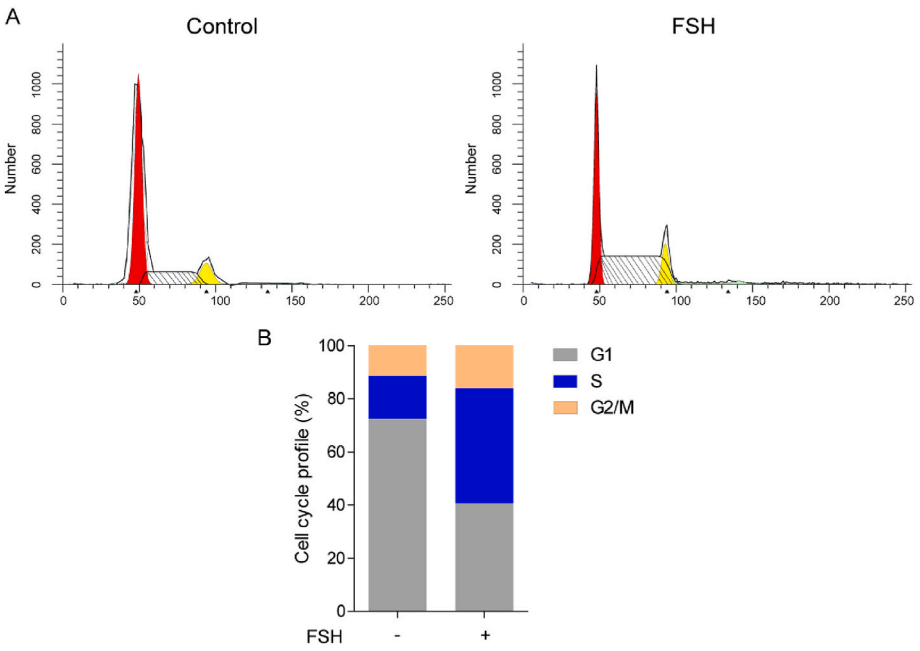

Fig. 4. FSH promotes cell cycle progression. (**A-B**) Hemangioma stem cells were treated with 1000 IU/L follicle-stimulating hormone for 48 h. Cell cycle was measured by flow cytometry. Acceleration of the G1/S phase transition by FSH. Data are shown as the means of three independent experiments or representative data.

mitogen-activated protein kinase (p38MAPK) and serum and glucocorticoid-lnduced kinase (Sgk) [33]. Meanwhile, upregulation of VEGF/VEGFR-2 expression activates PI3K/AKT signaling and promotes the elevation of the antiapoptotic protein Bcl-2, thereby inhibiting apoptosis of HemSCs [2]. In the future, we'll dig deeper into the underlying mechanisms and protein kinases would be involved in the process of FSH-reduced the apoptosis rate of hemangioma stem cells. In addition, the markers like p53 and p53-upregulated modulator of apoptosis (PUMA) were closely associated with apoptosis. FSH inhibits the expression of PUMA by ROS/PI3K/Akt in mouse granulosa cells [34]. It may be the potential mechanism of FSH inhibiting apoptosis. A limitation of this paper is the lack of a specific mechanism. Based on this, we will further explore the molecular mechanism of follicle-stimulating hormone on hemangioma stem cells and hope to identify the specific pathway and key genes involved. Thus, it will serve as a possible target for the prevention and treatment of hemangioma in the future, and may help to understand the role of hemangioma stem cells during the prenatal and perinatal periods. In summary, interventions aimed at the prevention and treatment of infantile hemangioma may benefit from this research, which provides valuable theoretical support.

Author contributions

Z.H. conceived and designed the experiments. Z.H., J.K. performed the in vitro experiments. Y.G. performed and guided the statistical analysis. G.Z., and Z.Z. provided or collected the study materials or patients. Z.H. and L.J. wrote and reviewed the manuscript. All the authors read and approved the final manuscript.

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Declaration of competing interest

The authors declare no competing interests

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