Effects of Indocyanine Green on Apoptosis, Proliferation, Migration, Invasion and Transdifferentiation of Human Lens Epithelial Cells Line SRA01/04

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Research Article

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Abstract

Background

To investigate the effect of indocyanine green (ICG) on apoptosis, proliferation, migration, invasion and transdifferentiation in cultured human lens epithelial cells line SRA01/04 (HLECs).

Methods

HLECs were exposed to ICG for 3 minutes at various concentrations of 0.5%, 1.5% and 2.5%. The apoptosis process was detected by flow cytometry, and apoptosis related proteins Bax, Bcl-2, caspase-3 and caspase-9 were detected by Western blot. Cell proliferation process was detected via the cell counting kit-8 (CCK-8) assay and 5-ethynyl-2'-deoxyuridine (EdU) incorporation assay. Wound healing assay was employed to determine cell migration. Transwell assays were performed to assess cell migration and invasion process. Transdifferentiation related proteins α-SMA, N-cadherin, Fibronectin and Vimentin were accessed by Western blot.

Results

ICG significantly increased HLECs apoptosis and decreased cell proliferation, migration, invasion and transdifferentiation after 3 minutes of exposure. Expression of Bax, caspase-3, and caspase-9 pro-apoptosis proteins were up-regulated after ICG exposure, while Bcl-2 anti-apoptosis protein was down-regulated. Transdifferentiation related proteins α-SMA, N-cadherin, Fibronectin and Vimentin expression were down-regulated.

Conclusions

ICG can promote HLECs apoptosis and inhibition HLECs proliferation, migration, invasion and transdifferentiation in a concentration-dependent manner.

Background

Posterior capsule opacification (PCO) is very common during follow-up after cataract surgery. During the 5 years follow-up period, the incidence of PCO has been reported to be as high as 22.6% after cataract surgery for senile patients [1]. Especially in eyes of pediatric, the incidence of PCO increases to almost 100% [2]. The pathogenesis of PCO is because of the proliferation, migration, invasion and transdifferentiation into myofibroblasts, matrix deposition, and contraction occurring in residual lens epithelial cells (LECs) after cataract surgery [3]. Drug inhibition of these key cellular events in PCO pathogenesis appears to be a valuable therapeutic option. In clinical work, the most effective method for treatment of PCO is neodymium:YAG (Nd:YAG) laser posterior capsulotomy. While this treatment may

injure the intraocular lens (IOL), occured cystoid macular edema, result in increased intraocular pressure and the risk of retinal detachment [4, 5]. In recent years, various drugs by inhibiting, clearing, or killing residual LECs to reduce the occurrence of PCO has been studied extensively. Drugs that inhibiting the proliferation of LECs include celecoxib [6], fluorouracil (5-FU) [7] and sirolimus [8]; Hindering the migration include methotrexate (MTX) [9], erlotinib [10] and miRNA-34a [11]; Impeding the epithelial-mesenchymal transition (EMT) include sorbinil [12], resveratrol [13] and THZ1 [14]; Clearing or killing LECs include edetic acid (EDTA) [15], distilled water (DW) [16] and hypertonic saline [17]. Although initial results are promising, these drugs were mainly used in cell and animal models experiments and were not yet available for clinical use. Therefore, an effective and safe PCO prevention drug is needed. ICG is widely used in clinical ophthalmology for fundus fluorescein angiography, internal limiting membrane staining and anterior capsule of the lens staining [18–20]. ICG has been shown to be safe when used at low concentrations in clinically [21]. However, more and more cell experiments have shown the toxic effects of ICG at high concentrations [22–25]. The goal of this research was to evaluate the effect of ICG using cells line SRA01/04 culture model. And then it is expected to kill the residual HLECs through capsule bag injection of ICG to achieve the purpose of preventing PCO formation.

Materials And Methods

HLECs Culture and ICG treatment

Human lens epithelial cells line SRA01/04 was purchased from the TongPai Bio Tech Co. Ltd (Shanghai, China). The HLECs cultured in DMEM/F12 medium supplemented with 10% FBS (Thermo Fisher Scientific, USA) in a 5% CO_2 humidified atmosphere incubator at 37°C, every 3 days to replace the medium. Cells were grown to 80-90% confluence before harvesting for experiments. The control group, 5%GS group and ICG groups HLECs were exposed to balanced saline solution(BSS), 5% aqueous glucosevarious and various ICG concentrations (0.5%, 1.5% and 2.5%) for 3 minutes, respectively. Then, each group was washed out with PBS 3 times and then incubated in fresh medium for 24 hours for further experiments.

Dye preparation

ICG (YiChuang Pharmaceutical Co. Ltd, Dandong, China) was prepared by dissolving the sterile powder in 5% aqueous glucose to obtain the concentrations of 0.5%, 1.5% and 2.5%.

Cell apoptosis assay

Cell apoptosis rate was tested using the flow cytometry. The Annexin V-FITC/PI apoptosis detection kit (Biolegend, California, USA) was used to detect the cells apoptotic after HLECs were exposed to ICG. In brief, cells were digested by trypsin without ethylene diamine tetraacetic acid (EDTA) and collected after treated as described above. Cells concentration was adjusted to 1×10^5/ml, washed out with PBS 2 times, adding 500 μl of binding buffer, with 5 μl Annexin V-FITC and 5 μl Propidium Iodide and incubated of 20-25^oC and avoid light for 15 minutes. The samples were tested by Flow Cytometer (Becton,

Dickinson and Company, CA, USA) at the excitation wavelengths of 488 nm within 1 hour, and the results were analyzed using CellQuest.

Western blot

The expression level of apoptosis and transdifferentiation related proteins were detected by Western blot. The treated HLECs were added with appropriate amount of RIPA lysate (Best Bio Tech Co. Ltd, Nanjing, China) for 15 minutes on ice and total cellular protein contents were determined by BCA protein assay kit (Beyotime Bio Tech Co. Ltd, Shanghai, China). Proteins were loaded onto 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis minigel (SDS-PAGE) and separated at 120 V. The separated proteins were transferred to polyvinylidene difluoride (PVDF) blotting membrane (Millipore, Bedford, MA, USA) at 200 mA. The PVDF membrane was immersed in the sealing solution containing 5% non-fat milk, slowly shaken on a shaker, and sealed of 20-25^{O}C for 2 h. Subsequently, probed with antibody specific for α-SMA (Proteintech Group, Chicago, IL, USA), Bax, Bcl-2, caspase-3, caspase-9, N-cadherin, Fibronectin, Vimentin and β-actin (Abcam, Cambridge, UK) incubate at 4°C overnight. Membranes were washed and incubated with goat anti-rabbit horseradish peroxidase-conjugated (Best Bio Tech Co. Ltd, Nanjing, China) or goat anti-mouse horseradish peroxidase-conjugated (Best Bio Tech Co. Ltd, Nanjing, China) secondary antibody for 1 hour of 20-25^{O}C. Protein bands were visualized by addition of ECL chemiluminescence detection solution and band greyscale was measured using image analysis software Image J.

Cell proliferation assay

CCK-8 assay was used to detect HLECs proliferation. Taked 96-well plates and added 100 uL cell suspension to each well. At the time of 24 hours, 48 hours and 72 hours, 10 μl of CCK-8 mixed with 100 μl of medium containing 0.5% FBS was added to each well and incubated for 2 hours at 37◦C. The 96-well plates was placed in a microplate reader and read at 450 nm (OD450).

EdU staining assay

To verify the effect of ICG on HLECs growth, cell proliferation was measured by the Edu Cell Proliferation kit (Beyotime Bio Tech Co. Ltd, Shanghai, China) according to the instructions. After two hours, replacd the original medium with fresh medium containing 50 μM EdU. Then, the cells were fixed with acetone for 10 minutes, washed with PBS for 5 minutes. After that, the cells were incubated in Apollo reaction buffer containing FITC-fluorescein in the dark for 30 minutes. Subsequently, the cells were counterstained with DAPI and mounted. Images were captured using a confocal fluorescence microscope (Carl Zeiss, Overkochen, Germany). Cells were counted using image analysis software Image J.

Wound healing assay

The HLECs were seeded into 6-well plates and cultured until 60-80% confluency. Then, the medium was replaced with 1% penicillin/streptomycin without serum medium and cultured for another 18 hours. The cells were scratched in the center of each hole along a straight line. After that, the cells were washed with PBS 2 times. The cells were treated as described above. The wounded areas images were photographed at 0 hour and 48 hours. The wounded areas were measured using image analysis software Image J.

Transwell assay of migration and invasion

Transwell assay was used to detect cell migration and invasion abilities. A 24-well transwell chamber (Thermo Fisher Scientific, USA) with 8 μm pore polycarbonate membrane was used for migration assay. A 24-well transwell chamber with Matrigelprecoated membrane (BD Biosciences, Franklin Lakes, NJ, USA) was used for invasion assay. In both assays, the upper chamber per well were seeded 4×10^4 cells and cultured in DMEM/F12 without serum, while in the lower chamber contained 600 ul of 12% FBS was used as chemoattractants. The cells were treated as described above. After 24 hours, the membranes were washed with PBS 3 times, fixed in 4% paraformaldehyde for 10 minutes and then stained with crystal violet for 15 minutes. The number of migrated and invaded cells were photographed by microscopy (Olympus, Tokyo, Japan) and counted by Image J soft-ware.

Statistical analysis

Independent experiments were repeated three times and all results were presented as mean ± standard deviation (SD). One-way analysis of variance(ANOVA)combined with Least Significant Difference (LSD) statistical analysis for multiple comparisons. A P value < 0.05 was considered as statistically significant.

Results

ICG increases the apoptosis of SRA01/04 cells in vitro

To investigate whether ICG can induce apoptosis of HLECs, we used gradient concentrations of ICG on HLECs for 3 minutes. Compared to the blank control group, HLECs cells showed no significant apoptosis after cultured with 5%GS for 24 h. All the concentrations of ICG induced a significant amount of apoptosis in HLECs when compared to the blank control group after 24 hours of culture (P < 0.05, Fig. 1a and b). Flow cytometry results showed that the apoptosis of HLECs by ICG in a concentration-dependent manner. Western blot showed that all the concentrations of ICG significantly up-regulated the expression of caspase-3, caspase-9 and Bax, while Bcl-2 was down-regulated by 1.5% and 2.5% concentrations of ICG compared to the blank control group (Fig. 1c and d). No statistically significant changes of 2.5% ICG group compared with 1.5% ICG group on cell apoptosis related proteins (P > 0.05).

ICG inhibits the proliferation, migration and invasion of SRA01/04 cells in vitro

To further investigate the effects of ICG on cell proliferation, HLECs were exposed to different concentrations of ICG for 3 minutes. The EdU staining assay revealed that all the concentrations of ICG inhibited HLECs proliferation at 3 minutes (P < 0.05, Fig. 2a and b). The CCK-8 assay also showed that all concentrations of ICG inhibited the proliferation of HLECs after 3 minutes treatment (P < 0.05, Fig. 2c). There was no statistical significant of 2.5% ICG group compared with 1.5% ICG group on cell proliferation (P > 0.05). The scratch method was used to observe of cell migration. After HLECs were treated with ICG for 3 minutes and cultured for 48 hours, the results showed that ICG significantly inhibited cell migration

($P < 0.05$, Fig. 3a and b). No statistically significant changes of 2.5% ICG group compared with 1.5% ICG group on cell migration ($P > 0.05$). We further detected the migration and invasion characteristics of HLECs via a transwell assay. The number of migrated and invasioned cells decreased by all concentration of ICG compared to the blank control group ($P < 0.05$, Fig. 4a and b). No statistically significant changes of 2.5% ICG group compared with 1.5% ICG group on cell migration and invasion ($P > 0.05$).

ICG inhibits the transdifferentiation of SRA01/04 cells in vitro

To explore the effects of ICG on cell EMT, we observed the changes on the expression of EMT related proteins. Western blot analysis showed that all the concentrations of ICG significantly down-regulated the expression level of α-SMA, N-cadherin, Fibronectin, while the expression level of Vimentin was down-regulated only by 1.5% and 2.5% concentrations of ICG (Fig. 5a and b). No statistically significant changes of 2.5% ICG group compared with 1.5% ICG group on cell transdifferentiation related proteins ($P > 0.05$).

Discussion

PCO is an important cause of the gradual decline of visual acuity after cataract surgery. In terms of pathogenesis, because these residual cells are the cause of visual axis opacification, so remove of these cells during the surgery could reduce or eliminate the incidence of proliferation of LECs and the occurrence of PCO.

Zhang et al [26] study showed that IOLs were modified with ICG and then triggered by laser could obviously inhibit LECs proliferation and migration meanwhile other tissues in the eye were not affected in-vitro. In-vivo study found that ICG-IOL not only prevent PCO occurrence but also was safe for other tissues in the eye. Melendez et al [25] found that after LECs were cultured in 0.1 mg/mL ICG for 30 minutes, the cells were irradiated by 806-nm laser for 1 minute could significantly decrease cells activity. When the concentration of ICG reached 5 mg/mL, only less than 5% LECs survived. Therefore, we studied the effects of ICG on the apoptosis, proliferation, migration, invasion and transdifferentiation of HLECs and revealed the potential value of ICG in solving PCO.

Flow cytometry showed that ICG could promote HLECs apoptosis in a concentration-dependent manner. At present, the apoptosis signaling pathway induced by ICG is not clear. Therefore, this study assessed the relationship between ICG exposure and apoptosis in HLECs by research Bax, Bcl-2, caspase-3 and caspase-9, which are associate with controlling apoptosis events. The anti-apoptotic protein Bcl-2 inhibits apoptosis by decrease the level of cytochrome c release, while the pro-apoptotic protein Bax induces apoptosis by increase the level of cytochrome c release [27]. The release of cytochrome c promotes the activation of caspase-9 [28], which then activates downstream caspases and causes cell apoptosis. The study revealed that ICG treatment leads to down-regulated of Bcl-2, while up-regulated of Bax, caspase-3 and caspase-9 expression, which may eventually lead to caspase-dependent cell death.

In this study, we evaluated the effect of ICG on HLECs proliferation, migration and invasion in vitro. The CCK-8, EdU assay and wound healing assay studies showed that HLECs were immediately affected after 3 minutes ICG treatment. Besides, the effect was concentration-dependent, in other words, higher concentrations of ICG leaded to more HLECs injure, and the inhibition effect was strongest when the ICG concentration was 1.5%. The occurrence of PCO requires the migration of equatorial LECs to the center of posterior capsule. The study results showed that the migration and invasion capabilities of HLECs were significantly decreased after ICG treatment. This can slow down the migration speed of HLECs to the posterior capsule. The EMT of LECs plays an important role in PCO formation. In the process of EMT, overexpression of α-SMA, N-cadherin, Fibronectin and Vimentin are considered key factors. Western blot showed that the expression of mesenchymal protein were decreased after ICG treatment. In conclusion, ICG may play a negative role in EMT by inhibiting mesenchymal protein expression.

In summary, ICG could not only promote the apoptosis of HLECs, but also inhibit the proliferation, migration, invasion and transdifferentiation of HLECs in a concentration-dependent manner. These preliminary results revealed that ICG might be a promising drug for the prevention of PCO in vivo application. However, further clinical studies are needed to assess the efficacy and tolerability of ICG when applied in the capsule bag.

Conclusions

ICG promotes HLECs apoptosis by up-regulated Bax, caspase-3, and caspase-9 proteins expression and down-regulated Bcl-2 protein expression. ICG inhibits HLECs proliferation, migration, invasion and transdifferentiation in a concentration-dependent manner, and the inhibition effect was strongest when the ICG concentration was 1.5%.

Abbreviations

ICG: Indocyanine green; HLECs: Human lens epithelial cells line SRA01/04; CCK-8: Cell counting kit-8; EdU: 5-ethynyl-2'-deoxyuridine; PCO: Posterior capsule opacification; LECs: Lens epithelial cells; IOL: Intraocular lens; 5-FU: Fluorouracil; MTX: Methotrexate; EDTA: Edetic acid; EMT: Epithelial-mesenchymal transition; DW: Distilled water; PVDF: Polyvinylidene difluoride;

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All authors agree with the publication of this study.

Availability of data and material

The datasets used or analysed during the current study available from the corresponding author on reasonable request.

Competing interests

All authors promise that they have no conflict of interest in this article.

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Authors' contributions

Zhenggao Xie: designing of the experiment; Yajun Liu and Yingdi Zhao: operating of the experiment and manuscript writing; Wenwen Zhang, Si Zhang, Zifang He, Feifei Chen: analysis and revision of the manuscript. All authors have read and approved the manuscript.

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Figures

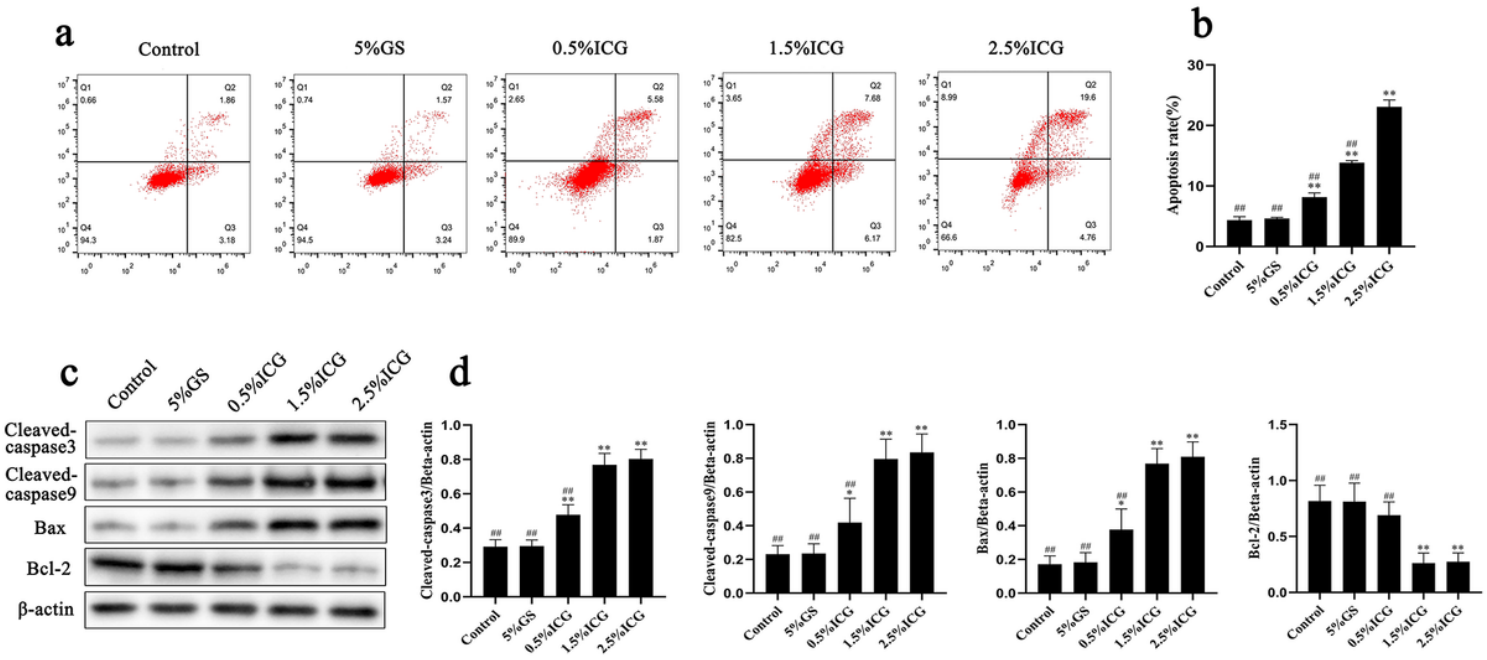

Figure 1

ICG increases the apoptosis of SRA01/04 cells in vitro. a The apoptosis of SRA01/04 cells were measured by flow cytometry after treatment with ICG (0.5%, 1.5%, 2.5%) for 3 minutes. b ICG increases the apoptosis of SRA01/04 cells in a concentration-dependent manner (*P < 0.05 vs. control, * *P < 0.01 vs. control, ##P < 0.01 vs. 2.5% ICG). c SRA01/04 cells were treated with ICG (0.5%, 1.5%, 2.5%) for 3

minutes and examined by Western blot. d Western blot analysis showed that ICG increased the expression of Caspase-3, Caspase-9 and Bax proteins in SRA01/04 cells, decreased the expression of Bcl-2 protein (*P < 0.05 vs. control, * *P < 0.01 vs. control, ##P < 0.01 vs. 2.5% ICG).

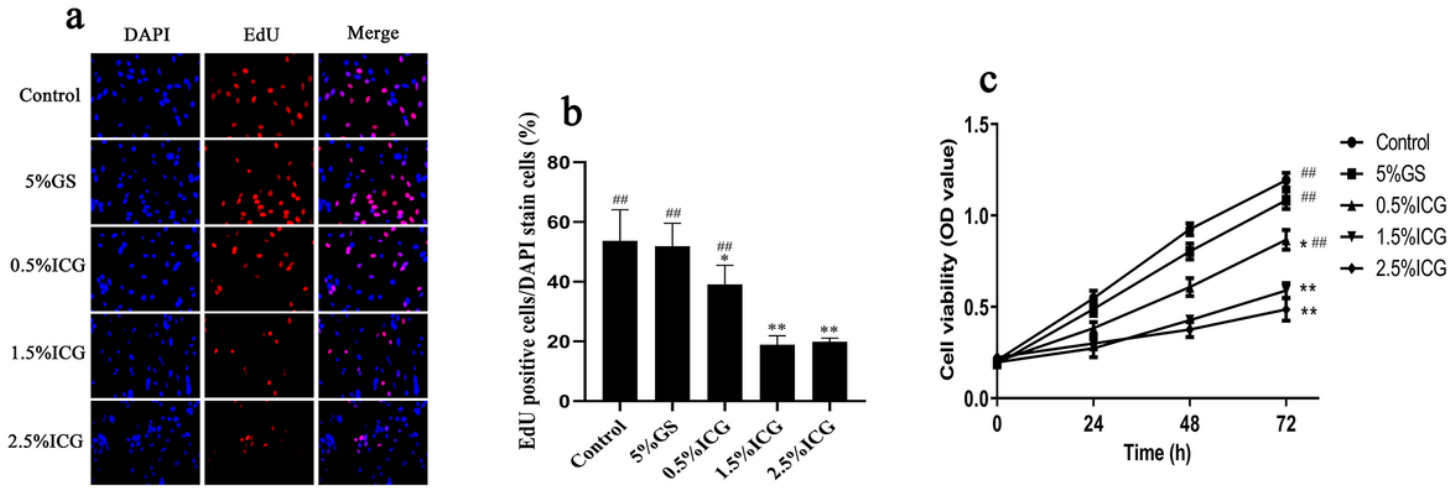

Figure 2

ICG inhibits the proliferation of SRA01/04 cells in vitro. a The proliferation of SRA01/04 cells were measured by EdU staining assay after treatment with ICG (0.5%, 1.5%, 2.5%) for 3 minutes. b The percentage of cell proliferation in ICG group decreased with the increase of ICG concentration (*P < 0.05 vs. control, * *P < 0.01 vs. control, ##P < 0.01 vs. 1.5% ICG). c The CCK-8 assay showed that ICG inhibited the proliferation of SRA 01/04 cells (*P < 0.05 vs. control, * *P < 0.01 vs. control, ##P < 0.01 vs. 1.5% ICG).

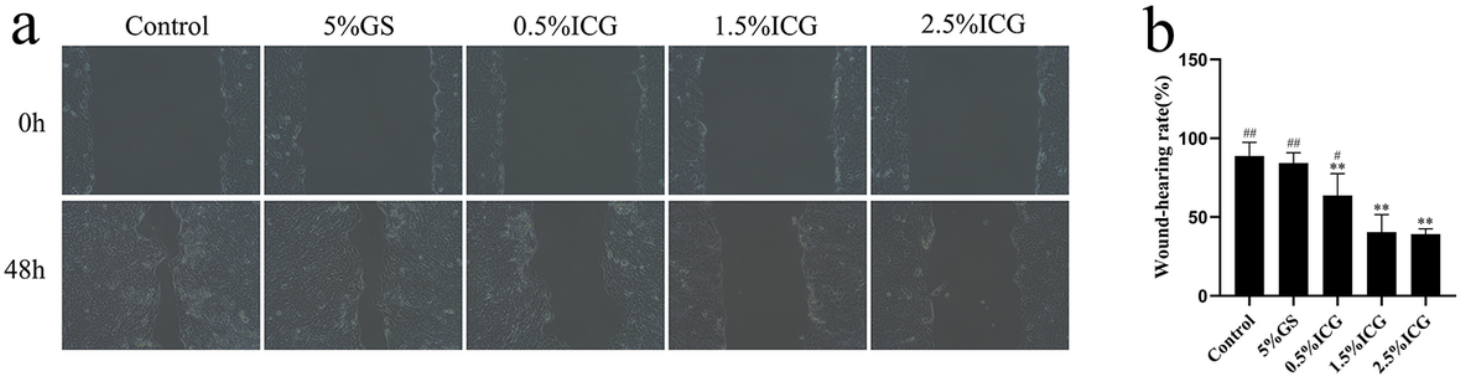

Figure 3

ICG inhibits the migration of SRA01/04 cells in vitro. a Wound healing at 0 and 48 hours. b Wound healing assays showed that ICG significantly inhibited the migration of SRA01/04 cells (* *P < 0.01 vs. control, #P < 0.05 vs. 1.5% ICG, ##P < 0.01 vs. 1.5% ICG).

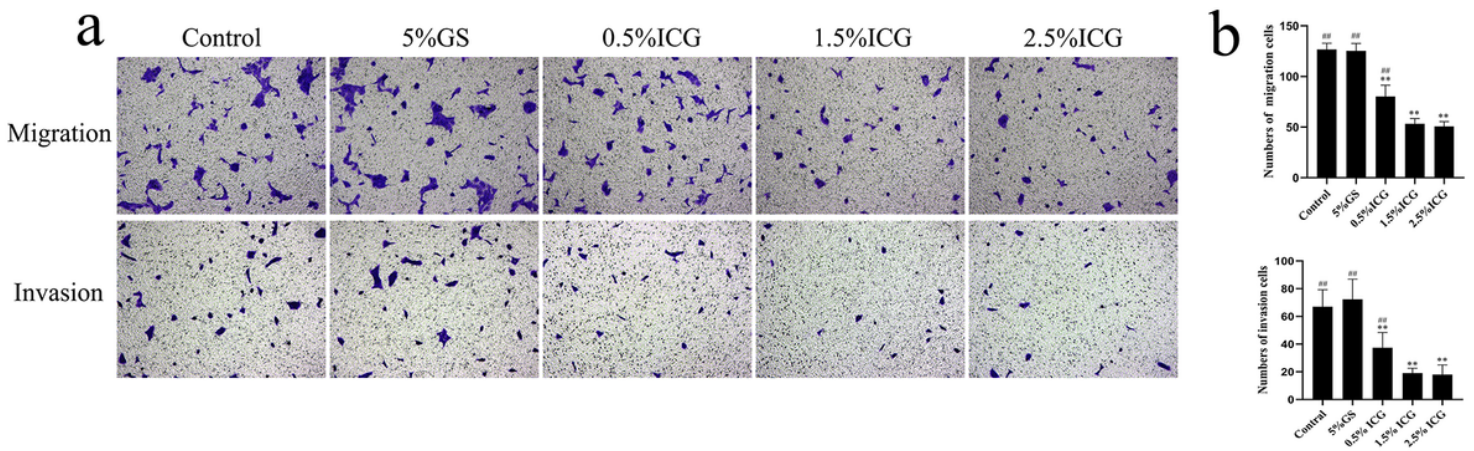

Figure 4

ICG inhibits the migration and invasion of SRA01/04 cells in vitro. a Transwell migration and invasion assays for 24 h. b Transwell migration and invasion assays showed that ICG decreased numbers of migrated and invasioned cells (* *P < 0.01 vs. control, ##P < 0.01 vs. 1.5% ICG).

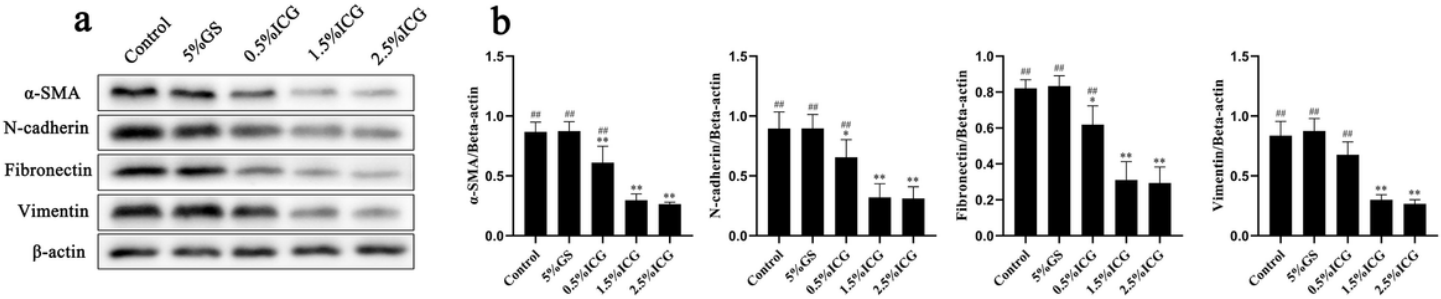

Figure 5

ICG inhibits the transdifferentiation of SRA01/04 cells in vitro. a SRA01/04 cells were treated with ICG (0.5%, 1.5%, 2.5%) for 3 minutes and examined by Western blot. b Western blot analysis showed that ICG decreased the expression of α-SMA, N-cadherin, Fibronectin and Vimentin proteins in SRA01/04 cells (*P < 0.05, * *P < 0.01 vs. control, ##P < 0.01 vs. 1.5% ICG).