Original Article

Effect of Apigenin on Gap Junctional Intercellular Communication in Human Tenon's Capsule Fibroblasts

Shanshan Liu[1], Jibing Wang[2,*], Huihui Zou[3], Xudong Huang[2]

1 Department of Ophthalmology, Weifang Medical University, Weifang 261042, China

2 Weifang Eye Hospital, National Key Clinical Constructive Project Unit of Ophthalmology, Weifang 261042, China

3 Department of Ophthalmology, Dezhou People's Hospital, Dezhou 253000, China

Abstract

Purpose: To investigate the effect of apigenin on gap junctional intercellular communication (GJIC) in human Tenon's capsule fibroblasts (HTFs) and its underlying mechanism.

Methods: After a 48 h treatment of cultured HTFs with apigenin (80 μmol/L), the GJIC was detected by a scrape-loading/dye transfer technique with Lucifer yellow dye and rhodamine (Rh) dextran. The coupling index represents a quantification of GJIC where a high coupling index is associated with a greater number of cells demonstrating cell-cell communication through gap junction channels. The changes in connexin 43 (Cx43) distribution and the expression of Cx43 at the protein and mRNA levels were statistically compared between the two groups by means of immunocytochemistry, western blotting, and real-time polymerase chain reaction (PCR).

Results: The functioning of GJIC in the HTFs was significantly enhanced after 48 hours by apigenin treatment when compared with the control cells. In the apigenin group, the intercellular dye transfer grade was above 9, while this value was only grade 3–4 in the control group. The coupling index was significantly increased up to 9.205±0.3621 in the apigenin group, compared with 5.1775±0.3177 in the control group ($F=279.581$, $P=0.000$). The expression of Cx43 at the protein and mRNA levels was significantly up-regulated in the apigenin group compared with the control group.

Conclusion: Apigenin can significantly enhance the function of GJIC in HTFs by up-regulating the expression of Cx43 at both the protein and mRNA levels, suggesting that the enhancement of GJIC in HTFs by apigenin probably acts as an important mechanism underlying the inhibitory effect of apigenin on HTF proliferation. (***Eye Science 2013; 28:62–67***)

Keywords: apigenin; gap junctional intercellular communication; Connexin 43; human; Tenon's capsule fibroblast

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* **Corresponding author:** Jibing Wang, Weifang 261042, China, E-mail: wangjb918@163.com

Glaucoma has become the second most prevalent cause of blindness in the world, surpassed only by cataract[1]. Glaucoma treatments are designed to lower intraocular pressure (IOP) and include medication, laser treatments, and surgery, with glaucoma filtering surgery confirmed as the most effective therapy. Scarring of the filtering blebs is the primary cause of surgical failure but the use of mitomycin C (MMC) and 5-fluorouracil (5-FU) improves the success rate. Nevertheless, a variety of adverse events such as filtering bleb leakage, hypotony maculopa thy, and endophthalmitis are also inevitably induced[2]. Consequently, novel strategies for resisting scarring are urgently required.

Gap junctional intercellular communication (GJIC) is a vital process for the transmission of intercellular substances and signals and participates in regulating cell proliferation, differentiation, and apoptosis[3,4]. Human Tenon's capsule fibroblasts (HTFs) cultured in vitro have a relatively strong GJIC[5,6]. Therefore, intervention in cell proliferation and even prevention of vivo scarring is potentially possible via regulating GJIC. This aim of this study was to examine the effect of apigenin on GJIC in HTFs and its underlying mechanism.

Materials and methods

Materials

The study protocol was submitted to the relevant

Ethics Committee for approval. Informed consent was obtained from all participants' relatives before this study. The Tenon's capsule was obtained from 10 patients with strabismus, 5 males and 5 females, aged from 6 to 10 years. None had systemic or ocular inflammation or prior trauma history.

Dulbecco's modified Eagle's medium (DMEM, Hyclone), fetal calf serum (Hyclone), apigenin (Sigma), DMSO (Sigma), vimentin monoclonal antibody (Beijing ZS-Bio Co., Ltd), FITC-conjugated secondary antibody (Beijing ZS-Bio Co., Ltd), Lucifer yellow dye (Sigma), rhodamine dextran (Sigma), mouse anti-human CX43 monoclonal antibody (Invitrogen), reverse transcription and PCR kits (Takara Biotechnology (Dalian) Co., Ltd.) were used in the study.

Main instruments

Instruments included a CO_2 incubator (Thermo Forma, U.S.), optical inverted microscope (Olympus TH4-200, Japan), PCR device (Corbett, Australia), RT-PCR device (Corbett, Australia), and confocal microscope (Leica TCS SPE, Germany).

Methods

Culture and identification of human Tenon's capsule fibroblasts

A previously described tissue culture method was adopted[7]. During strabotomy, the bulbar conjunctiva was incised, along with the corneoscleral limbus, and an approximately 5 mm×5 mm×5 mm portion of the Tenon's capsule was collected and cut into 1mm ×1 mm×1 mm of tissue pieces, attached in a culture dish, and supplemented with a slight amount of DMEM containing 15% FCS, 100 U/mL penicillin and 100U/mL streptomycin; the cells were cultured in a 5% CO_2 incubator at 37℃ for 3 h. When the tissue blocks had tightly attached to wall, the culture medium was added and continuously cultured. The 3rd to 5th passage cells were used for subsequent experiments. Fluorescence immunocytochemistry was employed to identify vimentin.

Measurement of GJIC

The measurement of GJIC was conducted according to the method described by Deng et al[8]. HTFs were inoculated into a 6-well plate at a density of 1× 10^5 cells/mL. Once the cells had reached confluence, apigenin (80 μmol/L) was added (the control group received an equal volume of the DMSO ve hicle). After 48 h of culture, the culture solution was discarded, the cells were rinsed with PBS three times, and 1mL of Lucifer yellow dye (0.5 g/L; molecular weight: 450kDa) and 1ml of rhodamine dextran dye (0.5 g/L; molecular weight: 70000 kDa) were added. The Lucifer yellow (LY) demonstrated transfer between injured and intact cells via GJIC while transfer of rhodamine dextran was limited to injured cells and was not transferred to periphery cells via GJIC. A disposable anterior chamber acupuncture knife was used to scrape several lines on the plate bottom. After 5 min, the dye solution was removed, and the cells were rinsed with PBS three times. The free fluorescent compounds and dislocated cells were discarded, and the remaining cells were fixed in paraformaldehyde for 20 min and observed with a fluorescence microscope. The coupling index (CI), calculated as the ratio of LY-labeled cells/RD-labeled cells, was recorded (a higher CI was associated with a higher number of GJIC-associated fibroblasts). The experimental procedure was repeated three times.

Detection of changes in Cx43expression by immunocytochemistry

Actively growing HTFs were inoculated into a 6-well plate. Upon reaching 75% cellular confluence, apigenin (80 μmol/L) was added to the experimental cells and DMSO was added to the control cells. After 48 h, the culture solution was discarded, and the cells were rinsed with PBS three times, fixed in 4% paraformaldehyde for 60 min, rinsed three times for 3 min with PBS, and then incubated in a blocking solution containing 10% normal goat serum for 30 min at 37℃. The blocking solution was then discarded and the cells were incubated in diluted mouse anti-human Cx43 antibody at 4℃ and cultured overnight. After three rinses with PBS for 3 min, diluted FITC-labeled rabbit anti-mouse IgG secondary antibody was added and incubated for 2h-incubation at 37℃, and then rinsed three times with PBS. The cells were stained with Hoeschst 33258 for 20 min, followed by three rinses with PBS for 3 min, to reveal nuclear morphology. The coverslips were sealed with 50% buffer glycerol before observ-

ing and taking pictures with a laser confocal microscope.

Detection of Cx43 mRNA expression by RT-PCR

Following a 48 h treatment with apigenin (80μ mol/L), the total RNA of HTFs was extracted and reverse transcribed into cDNA. The primers included: Cx43 forward primer 5'-ATA GAC GGA TCT GAG TGC CTG AA-3'; reverse primer 5'-GCT CCA GTC ACC CAT GTT G-3'; GAPDH forward primer 5'-GCA CCG TCA AGG CTG AGA AC-3'; reverse primer 5'-TGG TGA AGA CGC CAG TGG A-3'. The reaction volume was 20 μl. The reaction protocol was as follows: 95℃ pre-denaturalization for 5 s, 40 cycles of 95℃ for 30 s and 58℃ for 20 s. Three parallel experiments were repeated three times for experimental and control cells. The relative expression level of each gene was calculated based on 2-△△Ct.

Detection of expression of Cx43 protein by western blotting

After a 48 h apigenin treatment (80 μmol/L), the HTFs were harvested and lysed to extract total protein according to the method of Sambrook et al[9]. The protein concentration was measured, and the proteins were denatured at 95℃ for 5 min and subjected to polyacrylamide gel electrophoresis. The gel was blocked with 5% defatted milk powder for 1 h, mouse anti-human Cx43 monoclonal antibody (1:250) was added and incubated overnight at 4℃. After washing with PBS, horseradish peroxidase-conjugated goat anti-mouse IgG (1:2500) was added and the gel was incubated at room temperature for 1 min and exposed to X-ray film. β-actin was used as an internal reference and to the gel was scanned. The protein expression was expressed in relation to the gray values on the scanned image.

Statistical analysis

SPSS 11.0 statistical software was used for data analysis. $P<0.05$ was considered as statistically significant. Single-factorial ANOVA was adopted. $P<0.05$ was considered as statistically significant.

Results

Culture and identification of HTFs

At the 8th day of human Tenon's capsule tissue incubation, free cells were seen surrounding the tissue block. The cells were transparent, had a typical long spindle shape, were rich cytoplasm, and had round and oval shaped nuclei. These fibroblasts proliferated relatively fast and reached confluence after approximately 3 weeks. The cultured cells were confirmed to be HTFs according to sampling site, cellular morphology, and immunofluorescence. The cells were arranged in a monolayer irradiative or swirling pattern. The HTFs cultured in vitro were positive for vimentin, and immunohistochemistry revealed green fluorescence in the cytoplasm while the nuclei remained nonfluorescent (Figure 1).

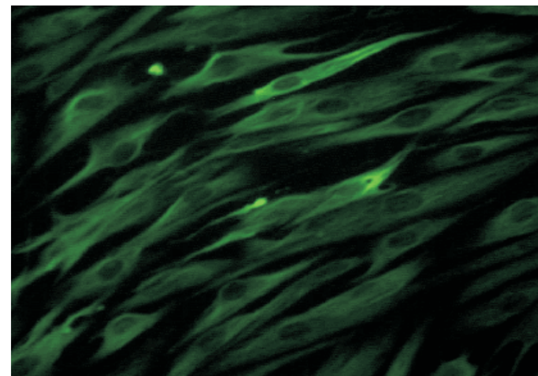

Figure 1 The cultured human Tenon capsule fibroblasts were identified by both in cell shape observation and immunocytochemistry for vimentin (green fluorescence). (Flurorescence microscope×400)

Effect of apigenin on GJIC among HTFs

The scrape-loading/dye transfer technique revealed that the coupling index can be an indicator of GJIC. In the control and apigenin-treated groups, the coupling indexes were 5.1775±0.3177 and 9.205±0.3621, respectively, and the difference was statistically significant ($F=279.581$, $P=0.000$) (Figure 2). Apigenin significantly upregulated the GJIC among the HTFs.

Effect of apigenin on Cx43 expression in HTFs

Compared with the control group, the HTFs treated with apigenin showed significantly upregulated expression of Cx43 and more intense green fluorescence distributed along the cell outline, hinting that Cx43 was distributed on the cell membrane (Figure 3).

Effect of apigenin on Cx43 mRNA expression in HTFs

The relative expression of Cx43 mRNA was 1 in

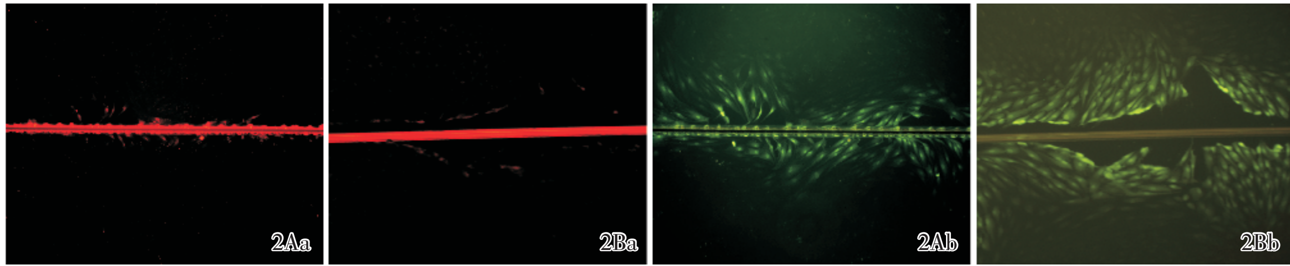

Figure 2 Effect of apigenin on GJIC of HTFs.(fluorescence microscope×100).

2A: The control group;

2Aa. HTFs were stained into red by rhodamine (Rh)- dextran(RD) fluorescence dye, restrained in damaged cells along scraping line because failed to transfer through GJIC due to large molecules.

2Ab. The lucifer yellow dye can transfer to 3–4 grades cells away from scraping line.

2B: 48 hours after using 80μmol/L of apigenin.

2Ba. The red fluorescence RD dye restrained to the damaged cells by scraping almost the same as 2Aa, GJIC not affected by apigenin;

2Bb: The transporting degree of lucifer yellow dye was > 8–9 grades away from scraping line through GJIC of HTFs, the ability of GJIC was enhanced significantly by apigenin.

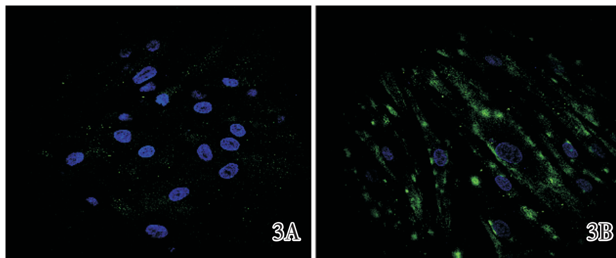

Figure 3 3A.Negative control group. The nucleus of HTFs were stained into blue by Hoechst33258, Cx43 was stained into green by immunocytochemistry. Green fluorescence was distributed on the cell membrane. 3B.Forty eight h-treatment by 80 μmol/L of apigenin. Green fluorescence was significantly condensed than the control group, suggesting up- regulated expression of Cx43 of HTFs enhanced by apigenin.

the control group and 1.991±0.155 in the apigenin-treated group, with a significant difference between the two groups (F=122.969, P=0.000), as shown in Table 1 and Figure 4.

Detection of changes in expression of Cx43 protein by Western blotting

The results of western blotting were consistent with those of fluorescence immunocytochemistry. Compared with the control group, the expression of Cx43 protein was significantly up-regulated by apigenin (F=133.264, P=0.000), as illustrated in Figure 5 and Table 1.

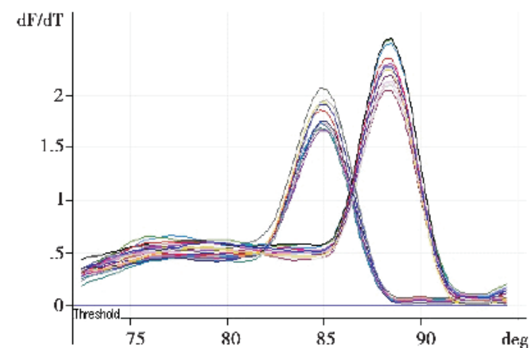

Figure 4 Melting curve of Cx43/mRNA detected by Real-time PCR. Specific single peak was seen for both internal reference (GAPDH) and Cx43. The lower peak was for GAPDG and the higher one for Cx43.

Table 1 Expression of mRNA and protein of Cx43 between two groups

Groups	Relative expression of CX43 mRNA	Expression of Cx43 protein(gray scale)
Control group	1	74.812±4.537
80μm/L- apigenin	1.991±0.155	114.531±5.729
F	122.969	133.264
P	0.000	0.000

Discussion

GJIC is a vital channel for information transmission among intercellular gap junctions and allows the passage of small molecules < 1 to 2 kDa, such as

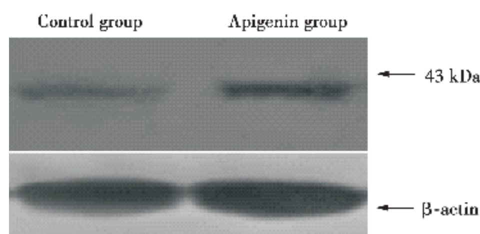

Figure 5 The protein expression of Cx43 was detected by Western blotting
Compared with the control, protein expression of Cx43 of HTFs in the 80 mmg/L-apigenin group was significantly up-regulated after 48 h.

metal ions, second messengers, and small molecular metabolites. The movement is direct and accomplished without passage through the extracellular space[10]. Each gap junction channel is composed of two connexons (or hemichannels), which connect the intercellular spaces of adjacent cells. Each connexon consists of 6 dumbbell-shaped connexins.

Currently, a total of 21 connexins have been identified; Cx43 is the most studied and most widely distributed of these gap junction proteins[11]. At present, gap junctions are believed responsible for intercellular communication, regulation of cellular metabolism, and maintenance of a stable environment. In addition, they play a significant role in accelerating and regulating embryonic development, cell proliferation, and differentiation. Disruption of gap junction communication is probably a common phenomenon during cancer progression. In particular, the proliferative and diffusive growth of carcinoma cells is associated with this type of disruption. Certain upregulators of GJIC, such as apigenin and lycopene, are able to suppress the growth of cancer cells, presumably by maintaining GJIC[12].

A synergistic role for GJIC in wound healing has been rarely reported; therefore, the potential effect of GJIC on subconjunctival tissue healing remains elusive. Previous research revealed that gap junctions exist in wound-derived fibroblasts and that GJIC optimizes the collagen lattice contraction induced by fibroblasts. GJIC is significantly reduced in human skin scars, especially in fibroblast scars, and lithium chloride (LiCl), an up-regulator of GJIC, can facilitate advanced maturity of granulation tissues[13]. In the eye, injection of heptanol endosulfan (an uncoupler of GJIC) into an embedded sponge increased the density of fibroblasts within the capsule after 7 days and decreased the number of myofibroblasts and the number of cells passing through the sponge. Observations made under polarized light indicated that the uncoupler decreased organization and sedimentation of collagen and subsequently interrupted the synergistic phenotype changes typically undergone by fibroblasts during the repair period. During the transition of granulation tissues into scars, GJIC played a vital role in fibroblast migration to apoptosis.

Previous studies[14] indicated that apigenin can upregulate the GJIC among hepatic epithelia in rats and inhibit the proliferation of tumor cells. GJIC has an important mechanism known as "bystander effect," which enhances the killing effect of medicines[5]. The present study indicated that apigenin at a concentration of 80 μmol/L can significantly enhance GJIC among HTFs, allow for a grade 9-GJIC, as determined by permeation of low molecular weight Lucifer yellow dye, but had no effect on movement of high molecular weight rhodamine dextran. Compared with the control group, the coupling index was significantly improved (F =279.581, P =0.000). Immunocytochemistry, RT-PCR, and western blot confirmed that apigenin significantly upregulated the expression of Cx43, while the DMSO vehicle had no adverse effects. Combined with our previous findings, the results suggested that apigenin and lithium chloride can suppress the proliferation of HTFs via a mechanism involving up-regulation of Cx43 expression.

In this study, HTFs that showed GJIC were selected as target cells to study a novel treatment for regulating the scarring caused by glaucoma filtering surgery. However, the potential inhibitory effects of apigenin in vivo and related complications remain to be elucidated.

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