RESEARCH ARTICLE

Metformin Reduced Collagen Deposition and Contractility, but Increased Collagen Degradation in *in vitro* Posterior Capsule Opacification Model

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Abstract

BACKGROUND: Posterior capsule opacification (PCO) often occurs after cataract surgery. Metformin has been known to have an ability to inhibit fibrosis. This study aimed to investigate the effects of metformin on cell contractility, collagen deposition and degradation in human lens epithelial cells (HLEC) of cataract patients.

METHODS: HLEC were isolated from the anterior lens capsule of patients undergoing cataract surgery. The HLEC culture was carried out using explant culture technique. The *in vitro* PCO model was created by scratching technique on HLEC cultures. The treatment groups were given 0.1, 0.5 and 1 mM metformin, respectively, while the control group were given 10% fetal bovine serum (FBS). On the 7th day after scratching technique, the collagen deposition, collagen degradation and cell contractility were evaluated.

RESULTS: Collagen deposition in HLEC was significantly reduced after given 0.1 mM, 0.5 mM and 1 mM metformin (17.92±6.16 µg/mL, 12.92±4.31 µg/mL, 11.25±5.30 µg/mL, respectively), compared to the control group (31.46±7.52 µg/mL, p=0.002). Collagen degradation significantly was increased in the 0.1 mM, 0.5 mM and 1 mM metformin groups (4.77±9.27 µg/mL, 6.59±1.16 µg/mL, 6.35±1.90 µg/mL, respectively) compared to the control group (2.21±2.78 µg/mL, p=0.002). While, collagen contractility in HLEC was significantly reduced in 0.1mM, 0.5mM and 1 mM metformin groups (16.39±3.89%, 13.89±2.59%, 11.93±2.44%, respectively), compared to the control group (44.25±4.95%, p=0.000).

CONCLUSION: Metformin reduced collagen deposition and contractility, but increased collagen degradation in HLEC of cataract patients through mechanism of extracellular matrix remodeling.

KEYWORDS: metformin, human lens epithelial cell, fibrosis

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Introduction

Posterior capsule opacification (PCO) is fibrosis conditions triggered by inflammatory responses that occur due to tissue trauma in the lens capsules tissue. PCO can be caused by cataract surgery combined with inflammatory reactions caused by foreign objects such as intraocular lens.(1,2) Lens epithelial cells (LECs) remaining in the anterior lens capsule during cataract surgery will undergo proliferation, migration and trans-difference to the posterior capsules that will trigger fibrosis and eventually become PCO.(3)

The common treatment of PCO is Nd:YAG laser capsulotomy but may cause a series of complications such as inflammation of the iris or vitreous body.(4) The use of therapeutic agents as inhibits the PCO process is now under study. The drugs that can be inhibits the LECs inflammation

process are non-steroid antiinflammatory drugs (example: tobramycin dexamethasone eyedrop), antineoplastic agents (example: fluorouracil (5-FU) and methotrexate) and immunosuppresive agents (example: sirolimus, cyclosporine A (CsA), and tacrolimus).(5)

Metformin is rarely used on ophthalmology cases. In the previous study, metformin is known to have good performance in influencing the epithelial to mesenchymal transition (EMT) process. The EMT process has a fairly identical pathway with tissue fibrosis in the formation of PCO.(6) Metformin is a class of biguanide drugs that are widely used in the treatment of diabetes. It has been known to have the ability to inhibit the process of fibrosis. (7,8) However, the mechanism of antifibrotic effect of metformin in the eye are still unclear. Remodelling of extracellurar matrix (ECM) composition, structure, stiffness and abundance provide to several pathological conditions, such as fibrosis.(9) The aim of this study was to investigate the effects of metformin on cell contractility, collagen deposition and degradation in human lens epithelial cells (HLEC) of cataract patients.

Methods

Study Design and Subject Collection

This was an experimental *in vitro* study using human LEC (HLEC), which was conducted at Integrated Surgical Center, Universitas Airlangga Hospital and Stem Cell Research and Development Center, Universitas Airlangga from January to April 2019. The protocol was accepted by Ethic Committee of Health Research Dr. Soetomo General Hospital (No. 1170/KEPK/V/2019). The inclusion criteria were subject aged 50 years or above and were diagnosed with senile cataract grade N2 to N5 BCN 10. Subject who had history of diabetes mellitus, endocrine-metabolic disorder, long-term steroid use, anterior lens fibrosis, or uveitis were excluded. Before the study, all subjects signed the written informed consent.

Sample Collection

Lens anterior capsule tissue was obtained from early phase of cataract surgery. General anesthesia was performed during cataract surgery. Capsulorhexis performed with Continuous Curvilinear Capsulorhexis (CCC) technique using utrata capsulorhexis forceps to collect 3x3 mm lens anterior capsule tissue. The tissue then transported to the Stem Cell Research and Development Center, Universitas Airlangga less than 24 hour after sample collected.

Lens Anterior Capsule Tissue Culture

The HLEC culture was carried out using explant culture technique. Lens anterior capsule tissue was isolated according the protocol established (10) with some modification. The anterior lens capsule tissue was cultured in 60 mm dishes using media containing alpha modified eagle medium (αMEM) (Gibco-Life Technologies, Waltham, MA, USA), beta fibroblast growth factors (βFGF) (Gibco-Life Technologies), non-essential amino acid (NEAA) (Sigma-Aldrich, St. Louis, MO, USA), 10% fetal bovine serum (FBS) (Biowest, Riverside, MO, USA), 5 ng/mL FGF (Gibco-Life Technologies), and 1% gentamicin (Sigma-Aldrich) until reaching 80-90 confluence; then cells were distributed into microplate size 96 wells. HLEC characterization were identified with immunofluorescence staining and vimentin and p63 antibody. Cells were placed in a fibrin coated dish and characterized using 4′,6-diamidino-2-phenylindole (DAPI) staining to identify cells with a nucleus, and FITC staining with vimentin and p63in order to identify mesenchymal and epithelial cells. The positive expression of vimentin and p63 antibody indicated a lens epithelial cell culture.

in vitro PCO Model Establishment

The *in vitro* PCO model was made with scratching technique developed that were previously developed.(11) HLEC were seeded into microplate 96 wells, at a density of $5x10^3$ cells/well (6 wells for each group). The cells then scratched using yellow micropipette tips vertically in perpendicular position on the plate meridian and washed out with culture medium to remove dead cells.

in vitro PCO Model Treatment

The cells were then divided into 4 groups; each group located in 8-well microplate. Every cell in each group given 10 µg/mL of lipopolysaccharide for 60 minutes to induce the inflammation process and epithelial cells fibrosis. Control group consisted of cell culture with confluence greater than 90% with growth medium DMEM, 1% NEAA, 1% gentamicin and 1% FBS. While treatment groups were cell culture with confluence greater than 90% with the same growth medium and were given metformin with concentration of 0.1 mM. 0.5 mM and 1 mM.

Sample Measurement

Analysis of deposition, degradation and contractility collagen were evaluated on the 7th day after scratching and treatment by measuring collagen deposition, collagen degradation and cell contractility. Observations were done

by multiple examiners consist of 3 resident of opthalmologist using inverted microscope OLYMPUS CKX53 (Olympus Corporation,Tokyo, Japan).

The amount of collagen deposited and degraded in each group was measured by Sirius Red-based colorimetric assay (Sigma-Aldrich). The amount of collagen deposited and degraded was then calculated according to the standard collagen curve determined from the amount of type I collagen rat tail at a wave length of 550 nm in µg/mL.

The collagen contractility test was carried out based on a previously developed protocol (12) using type 1 collagen rat tail (Santa Cruz Biotechnology, Dallas, TX, USA). The diameter changes of recorded in several time point by using digital camera at fixed distance above the gels. ImageJ software was used to assess the diameter of the collagen gel, which illustrated the collagen contractility due to cellular response of the lens epithelial cells.

Statistical Analysis

The levels of deposited and degraded collagen levels were determined based on a standard curve using type I collagen rat tail. Differences in the level of collagen contractility between groups were analyzed by Multivariate ANOVA and continued with post hoc Tukey with $p \leq 0.05$ was considered statistically significant. Whereas the amount of collagen deposited and degraded between groups were not normally distributed, statistical analysis used was Kruskal Wallis non-parametric test and continued with post hoc Mann-Whitney test with $p \leq 0.05$ considered statistically significant.

Results

HLEC Isolation and Charaterization

HELC was successfully isolated, reached its first confluent monolayer of 80-90% in 14 days (Figure 1). NEAA and βFGF supplementation promoted proliferation while maintaining viability of the cells. The identified mesenchymal and epithelial cells stained by p63 and vimentin were shown in Figure 2 and Figure 3.

Effect of Metformin on Collagen Deposition, Degradation and Contractility

Intergroup differences showed a significant difference ($p<0.05$). Mann-Whitney post hoc test showed significant intergroup differences between the control group and all groups given metformin. Mann-Whitney post hoc test also showed significant differences between groups given metformin. The average collagen cells deposited in each group were shown in Table 1.

Effect of metformin on collagen degraded showed significant difference. The average collagen cells degraded in each group were shown in Table 2. Test on degraded collagen showed significant ($p=0.002$) differences between the control group and 0.1mM metformin group.

Differences in the effect of metformin on collagen contractility between groups showed significant differences ($p=0.000$). The highest mean collagen contractility was shown in the control group, while the lowest was in the 1 mM metformin group. The differences in collagen contractility were shown in Table 3.

Discussion

Tissue culture from human eye donors provided the closest model to clinical studies on humans and provided information about the proliferation and trans-differentiation of contact epithelial cells.(6) In this study, isolation of the anterior capsule epithelium as a PCO model was used to prove the effect of metformin in PCO. Human epithelial isolation material obtained from cataract surgery on male

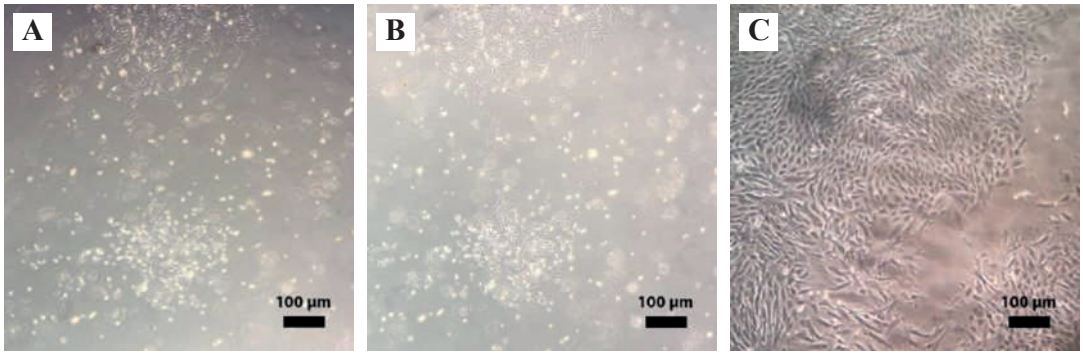

Figure 1. Primary culture of anterior lens epithelial cells by semi-enzymatic explant method. A: On the 7th day post culture, cell sprouting was seen; B: 80% cell confluence rate on the 14th post-culture day; C: cell culture with a confidence level of 90-100%.

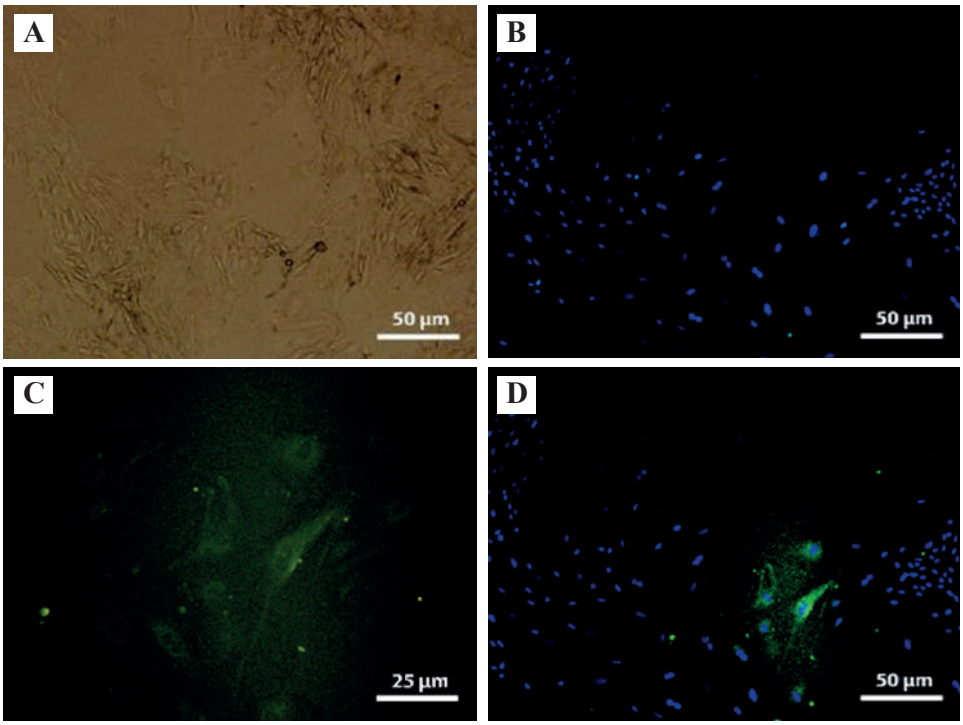

Figure 2. Characterization of lens epithelial cells with p63. A: Photograph of contrast phase of culture of lens epithelial cells with confluence of 80-90%; B: Nuclei with DAPI staining; C: positive p63 FITC antibody; D: Merge of picture B and C.

patients 60 years or above, varies depending on the age of the donor. Cultures were carried out using aseptic semi-enzymatic techniques in laminar flow.(10) In this study, although anterior epithelial cell insulating material was obtained from senile cataract donors, culture and subculture were successfully performed with a 90-100% cell confluence rate on the 14th day.

We successfully isolated HLEC to make a PCO model. The positive results of vimentin indicated that the cells tested contain vimentin and can be interpreted as the cells that are bred in this study are lens epithelial cells. (13,14) There were correlation between the intensity of p63 expression in re-epithelialization, in response to injury. (15) *In vitro* wound healing models are used to uncover the

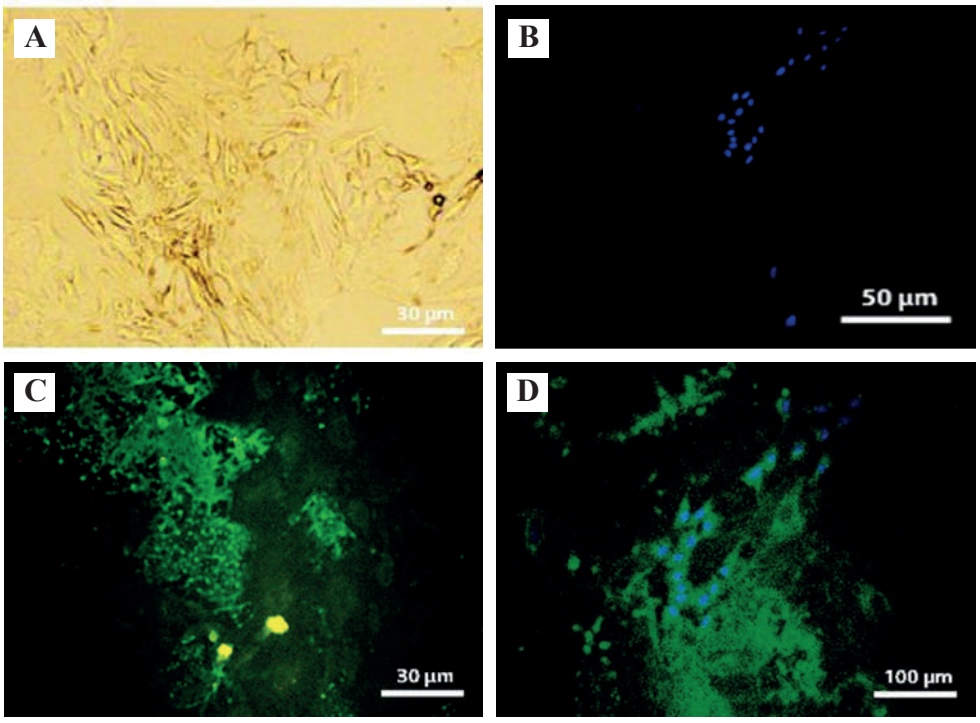

Figure 3. Characterization of lens epithelial cells with vimentin. A: Photograph of contrast phase of culture of lens epithelial cells with 80-90%; B: Nucleus with DAPI staining; C: positive FITC vimentin antibodies: D: Merge of picture B and C.

Table 1. Average levels of deposited collagen.

Group	Mean±SD (µg/mL)	*p*-value
Control	31.45±7.52	0.002
0.1 mM Metformin	17.91±6.16	
0.5 mM Metformin	12.91±4.30	
1 mM Metformin	11.25±5.30	

p-value tested with Kruskal-Wallis.

process of cell migration. The destruction of confluent cell monolayers, thus creating cell-free regions, which allow cells to migrate and repair them.(11) The desctruction of confluent cell monolayers were using scratch technique.

After cataract surgery, an increase in pro-inflammatory cytokines and growth factors, modulating tissue response to injury. TGF-β has an important role in the development of PCO, because signaling regulation of the epithelial-to-mesenchymal transition (EMT) in the lens epithelial cells and fibrosis capsules lens.(16) EMT was contributed as main mechanism underlying the emergence of PCO.(17) An inhibition of TGF-β1, in liver fibrosis cases, known to has positive effect on decelerates the fibrosis process (18).

The reference to the benefits of metformin on eye disease is quite limited.(16) Metformin in other organs of the body, has been known to have an effect on the pathophysiology of the fibrosis process. Metformin induces AMPK phosphorylation, which has an effect on TGF-β1 inhibition in triggering collagen production type I rat kidney fibroblasts.(19) In the heart, metformin reduces cardiac fibrosis by reducing collagen synthesis, CF proliferation, or increased collagen degradation. Metformin inhibits the occurrence of cardiac fibrosis by blocking the TGF-β1-Smad3 signaling pathway thus protecting the heart against injury to myocardial ischemia reperfusion, and inhibiting cardiac hypertrophy through AMPK activation.(20) The role of metformin in the brain published that metformin blocks the EMT-like process and cancer-like properties in glioblastoma cells.(21) Besides having an effect on the proliferation and survival of cancer cells, metformin has also been shown to specifically inhibit EMT.(22)

The results of the study appeared to be in line with the description of the role of metformin in fibrogenic reactions through the EMT mechanism, which suppresses extracellular matrix accumulation and reduces collagen contraction. In addition, other study states that metformin has role in inhibiting the EMT process in lens epithelial cells, which results in collagen deposition, degradation, and contractility.(23)

We also found that the difference in metformin dosage showed a different effect. These results, compared with other study (23) which showed that metformin was able to significantly suppress the ability of cells to migrate with significant inhibition of migration at 0.5 mM or higher concentrations of metformin. The level of ability to maintain morphology and suppress the ability to migrate lens epithelial cells, was related to the EMT process with the product of extracellular collagen organization. The dosage of metformin seems did not have a significant effect on the amount of collagen contractility. Other study states that increasing metformin concentration increases the ability to inhibit proliferation and EMT in the PCO model *in vitro*.(23)

Metformin has a very good pharmacological profile: cheap, widely available, and safe in its use. In eye cases, from our study, metformin known to have effect to preventing PCO. Further investigate such as the 3D culture method with extensive testing, and research on animal models, is needed to determine the efficacy of other drugs, optimal dosage, route of administration, toxicity, time and duration of administration of metformin.

Increasing the concentration of metformin does not have the effect of increasing the inhibition of fibrosis in the lens anterior capsule cell culture. Bearing in mind that EMT is the main pathophysiology underlying the formation of PCO. The inhibition of EMT, through cell contractility, collagen deposition, and degradation can be translated as PCO prevention. Metformin can be considered on further experiment as an topical agent to prevent PCO. Further, the effectivity of metformin can be compared with other known drugs to prevent PCO as *in vitro* experimental study.

Table 2. Average levels of degraded collagen.

Group	Mean±SD (µg/mL)	*p*-value
Control	2.21±2.78	0.002
0.1 mM Metformin	4.78±9.27	
0.5 mM Metformin	6.59±1.16	
1 mM Metformin	6.35±1.90	

p-value tested with Kruskal-Wallis.

Table 3. Mean collagen contractility.

Group	Mean±SD (%)	*p*-value
Control	44.25±4.95	0.000
0.1 mM Metformin	16.39±3.89	
0.5 mM Metformin	13.89±2.59	
1 mM Metformin	11.93±2.44	

p-value tested with One-way ANOVA.

Conclusion

Metformin reduced collagen deposition and contractility, but increased collagen degradation in human HLEC of cataract patients through the inhibition mechanism of EMT, which can be translated as PCO prevention.

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Authors Contribution

FSB, GS, IW, and MR were involved in concepting and planning the research; FSB, GS, and IW performed the data acquisition/collection; FSB, GS, IW, and WP perfomed the data analysis and results interpretation; FSB, IW, and MR drafted the manuscript; FSB also designed the figures.

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