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Submission date: 28-Jul-2021 12:59PM (UTC+0800)
Submission ID: 1624948409
File name: ial_Cells_Through_Transforming_Growth_Factor-Beta_Inhibition.pdf (246.93K)
Word count: 2934
Character count: 15976

DOI : 10.35124/bca.2019.19.S2.4911

Biochem. Cell. Arch. Vol. 19, Supplement 2, pp. 4911-4915, 2019 | www.connectjournals.com/bca | *ISSN 0972-5075*

METFORMIN EXERTS ANTIFIBROSIS EFFECT ON HUMAN LENS EPITHELIAL CELLS THROUGH TRANSFORMING GROWTH FACTOR-β INHIBITION

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(Received 11 August 2019, Revised 14 October 2019, Accepted 25 October 2019)

ABSTRACT : To investigate the antifibrosis effect of metformin human lens epithelial cells (HLECs) *in vitro*. HLECs were isolated from single donor of anterior capsule and treated with culture media supplemented with various doses of metformin as 0.1mM, 0.5 mM and 1mM. While the media was treated with 2% FBS as control group, with the cells stained using TGF-β FITC conjugated antibody and α-SMA used to examine the antifibrosis effect. Data were analyzed using oneway ANOVA and posthoc test with significant level of $p<0.05$. TGF-β antibody expression levels decreased after the administration of metformin 0.1mM (6.96×10^6 pixels $\pm$ 1.03×10^6 pixels), metformin 0.5mM (5.35×10^6 pixels $\pm$ 1.23×10^6 pixels) and metformin 1mM (1.93×10^6 pixels $\pm$ 0.94×10^6 pixels). The Tukey HSD post hoc test showed a significant decrease in expression levels in the 0.5mM metformin group ($p = 0.027$) and 1mM metformin ($p = 0.000$) compared to 2% FBS control. The levels of α-SMA antibody expression decreased after the administration of metformin 0.1mM (4.49×10^6 pixels $\pm$ 0.74×10^6 pixels), 0.5mM (3.34×10^6 pixels $\pm$ 0.47×10^6 pixels) and 1mM (2.26×10^6 pixels $\pm$ 0.55×10^6 pixels) metformin. The Games-Howell post hoc test showed a significant decrease in expression levels between the control groups compared and the three treatment groups consisting of 0.1mM ($p = 0.035$), 0.5mM ($p = 0.008$) and 1 mM ($p = 0.002$) metformin with 2% FBS. Metformin tends to have antifibrotic effect on HLECs through TGF-β inhibition.

Key words : Metformin, fibrosis, lens epithelial cells,TGF-β, α-SMA.

INTRODUCTION

Posterior capsular opacity (PCO) is a common complication experienced after cataract surgery, which isdetrimental to visual acuity. It is characterized by the presence of fibrosis in the posterior capsule of plaque-form on the intraocular lens. Morphologically, PCO is characterized by the transformation of epithelial lens cells from the normal form of cuboid into spindle-shaped myofibroblasts opting for cell elongation, loss of organelles and nucleus chromatin condensation. The incidence of PCO is quite high by 40% after the cataract extraction intraocular lens implantation especially in patients with risk factors of uveitis and diabetes. These events gave rise to the response of inflammatory cytokines, especially TGF-β, to trigger fibrosis. This process was characterized by the formation of Epithelial Mesenchymal Transition (EMT) and expression of á-smooth muscle actin (α-SMA), which produces contractile strength in injured tissue, which continuously increased the deposition of the extracellular matrix, causing opacity in the posterior capsule of the lens (Nibourg *et al*, 2015; Saika *et al*, 2014; Shirai *et al*, 2014).

The various strategies currently along with the rapid development of medical science, utilized to halt PCO have not provided optimal results. Anti-hyperglycemic drug has been studied to possess antifibrotic and antimitotic effects with theability to inhibit TGF-β expression signals, which is an important key in the EMT formation process of fibrosis. This study aimed to prove the antifibrosis effect of metformin on HLECs culture as an vitro model of PCO (Awasthi *et al*, 2009; Wormstone *et al*, 2016; Zheng *et al*, 2016).

MATERIALS AND METHODS

The main reagents include Type I collagenase (Roche USA), alpha modified eagle medium (α-MEM, Gibco-Life Technologies, USA), nonessential amino acid (NEAA, Sigma, USA), Trypsin (Gibco - Life Technologies, USA), fetal bovine serum (FBS, Biowest, USA), basic fibroblast growth factor (bFGF, Gibco- Life Technologies, USA), and anticoagulant citrate phosphate dextrose acid (CPDA), $CaCl_2$ (sigma). Primary FITC conjugated antibodies for immunochemistry of TGF-β and α-SMA vimentin were purchased from BIOSS (USA) and Santa Cruz (USA), respectively.

Study design

This is an in vitro study in HLECs of senile cataract's eye, conducted in Stem Cell research and development center, Universitas Airlangga. All experiments were conformed to local ethics review in Dr. Soetomo Hospital. Furthermore, *in vitro* scratch technique was performed for each group to promote wound on HLECs, which was then divided into 4 groups which consists of the control group, treated with 2% FBS in culture media, as well as 0,1 mM, 0,5 Mm and 1 mM metformin. TGF-β and α-SMA antibody expression levels were measured on the 7th day after treatment.

Isolation of HLECs

Human lens epithelial cells were isolated from a patient (n = 1) suffering from cataract aged 54 years old with no ocular abnormality. Tissue pieces were placed in 60 mm culture petri containing 2 mL of 0.2mg/mL collagenase type I and incubated for 30 minutes at 37°C. Collagenase was removed and 4 ml of culture media (alpha-modified eagle medium; αMEM + 10% fetal bovine serum; FBS + gentamicin reagent fluid + and 5ng/mL bFGF) was added to the petri, stored in an incubator at 37°C, while 5% CO_2 was stored for 48 hours. Culture media were changed every 3 days prior to 90% confluency, which were sub-cultured by warm trypsination techniques. This method was based on the protocol developed by Ibaraki *et al* (2002).

Characterization of Lens Epithelial Cells

Approximately 5×10^3 cells were seeded in each multi-well plate 96 prior to 70% confluency andfixated using 10% formaldehyde for 15 minutes at room temperature. Cells were washed with PBS tween 0.2% for three times and stained with vimentin and P63 FITC conjugated antibody. Furthermore, it was incubated at 37°C for 45 minute followed by overnight incubation at 4^0C and DAPI counterstained. The stained cells were further visualized under inverted immunofluorescence microscope (Olympus).

Posterior Capsular Opacification Model

The *in vitro* PCO model was made by scratching techniques in accordance with the protocol developed by Stamm *et al* (2015). The epithelial cells of the lens are cultured in 96 microplate plates with a density of 5×10^3 cells to reach 90% confluence. These were further scratched using a white micro pipette tip perpendicular vertically to the petri meridian. Cell cultures were washed with PBS after scratching and ready to be treated.

TGF-â and á-SMA staining

After the 7th day treatment, cells werefixated using formaldehyde 10% for 15 minutes at room temperature andwashed with 0.2% PBS tween three times. They were further stained with TGF-â and á-SMAFITC conjugated antibody, incubated at 37°C for 45 minute which was followed by an overnight incubation at 4^0C. The stained cells were then visualized under inverted immunofluorescence microscope (Olympus), with the level of expression analyzed using the ImageJ software and presented as pixel of positive cells.

Statistical analysis

The mean difference of TGF-â and á-SMA expression levels among groups was conducted with one way ANOVA using SPSS version 19.0 software, with p=0.05 considered statistically significant.

RESULTS

Human Lens Epithelial Cells isolation

This study successfully isolated HLECs in a relatively short time (an average of 7 days) which was required to reach first monolayer at 90% confluency. Supplementation with bFGF and NEAA were able to promote cells proliferation while maintaining its phenotype and viability (Fig. 1).

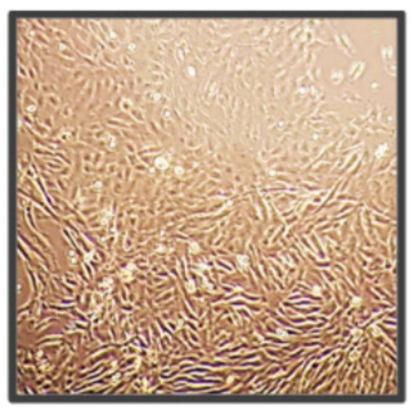

Fig. 1 : HLECs morphology on passage 3.

Immunofluorescent staining of vimentin and p63, a specific marker of fibroblast and epithelial, revealed that near 100% of the cells after passage-3 were p63-positive (Fig. 2).

TGF-â antibody expression

TGF-â antibody expression levels decreased after

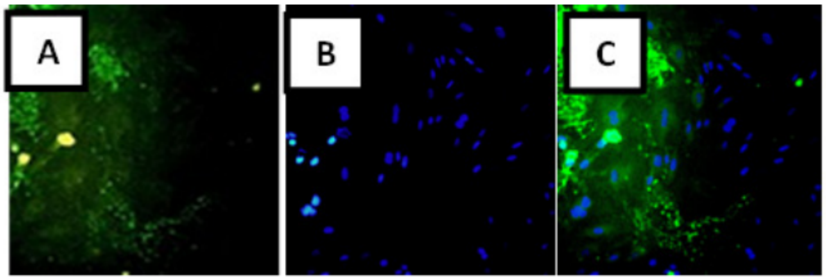

Fig. 2 : HLEC with immunofluorescent staining of p63. (a) green fluorescence: p63 positive, 200x. (b) DAPI, 200x. (c) Merge, 200x.

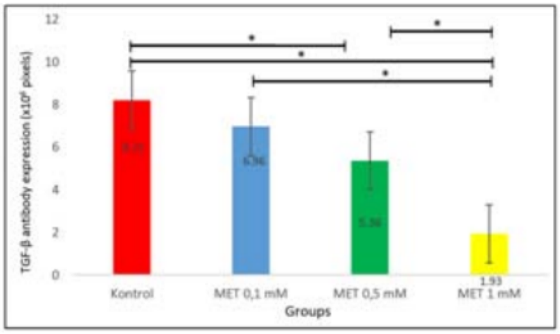

Fig. 3 : TGF-β antibody expression (Turkey test, *statistically significant).

administering 0.1mM (6.96×10^6 pixels $\pm$ 1.03×10^6 pixels), 0.5mM (5.35×10^6 pixels $\pm$ 1.23×10^6 pixels) and 1mM (1.93×10^6 pixels $\pm$ 0.94×10^6pixels)metformin. The Tukey HSD post hoc test showed a significant decrease in the 0.5mM metformin ($p = 0.027$) and 1mM metformin groups ($p = 0.000$) compared to the 2% FBS control (Fig. 3).

TGF-â antibody expression levels using immunofluorescence gradually decreased after administering metformin compared to to the 2% FBS control. The lowest stain was shown in 1mM metformin group (Fig. 4).

á-SMA antibody expression

The levels of á-SMA antibody expression decreased after the administration of 0.1mM (4.49×10^6 pixels $\pm$ 0.74×10^6 pixels), 0.5mM (3.34×10^6 pixels $\pm$ 0.47×10^6 pixels) and 1mM (2.26×10^6 pixels $\pm$ 0.55×10^6 pixels) metformin. The Games-Howell post hoc test showed a significant decrease in expression levels between the control groups compared with the three treatment groups with 0.1mM ($p = 0.035$), 0.5mM ($p = 0.008$) and 1 mM ($p = 0.002$) metformin consisting of 2% FBS control (Fig. 5).

á-SMA antibody expression levels using immunofluorescence gradually decreased after administration of metformin compared to the 2% FBS control. The lowest staining showed in 1mM metformin group is shown in Fig. 6.

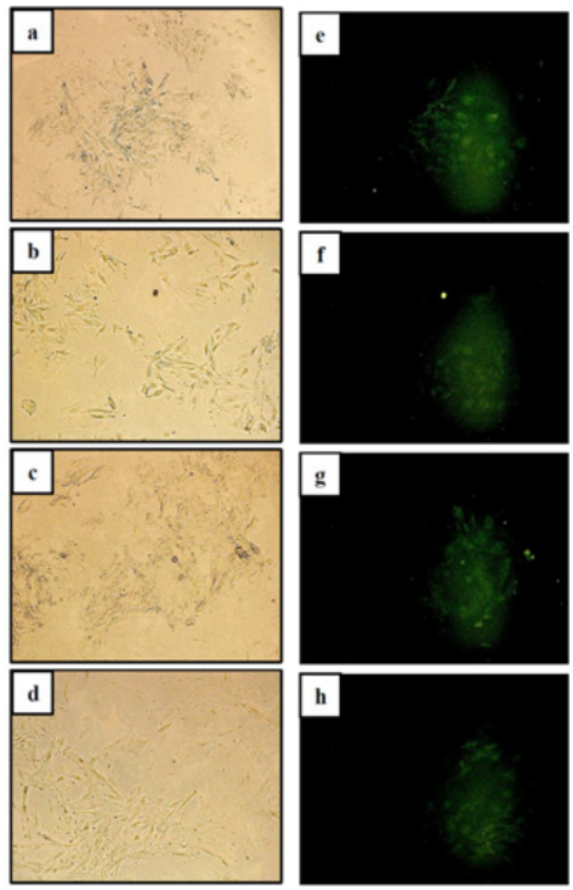

Fig. 4 : TGF-β antibody expression. Contrast phase photo after immunofluorescence staining with TGF-β FTC antibody on (a) control group, (b) metformin 0,1 mM, (c) metformin 0,5 mM, (d) metformin 1 mM. (inverted microscope, zoom in 100x). Immunofluorescence staining with TGF-β FITC antibody on (e) control group, (f) metformin 0,1 mM, (g) metformin 0,5 mM, (h) metformin 1 mM (fluorescence microscope, 200x).

DISCUSSION

The present experimental work was designed to evaluate the condition that metformin has the ability to ameliorate the fibrosis process in lens epithelial cells.

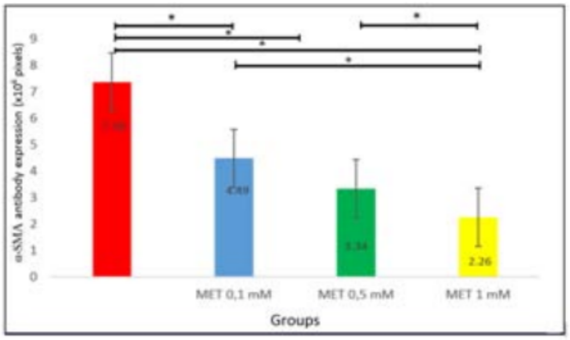

Fig. 5 : á-SMA antibody expression levels(Games-Howell, *P<0.05).

Fibrosis mechanism related with PCO manifests as excessive production of extracellular matrix (ECM) secretion and remodeling process. A significant evidence revealed that the epithelial-to-mesenchymal transition (EMT) is an important component of this process. The mechanism of EMT is conducted by major inflammatory cytokine TGF-â. Meng *et al* (2013) demonstrated that TGF â induces EMT promoting excess synthesis and deposition of ECM proteins such as fibronectin and type I collagen in the Human Lens Epithelial Cells (HLECs), and mTOR is activated during TGF â induce EMT. TGF-â also stimulates generation of ROS and recruitment of inflammatory cells by activating the Smad 2/3 and mitogen-activated protein kinase (MAPK) signals (Ahn *et al*, 2011; Chen *et al*, 2014; Meng *et al*, 2013).

Furthermore, it has an antifibrotic effect through AMPK signal activity along with the rapid development of medical science, and metformin which is known as anti-hyperglycemic drugs. Gamad *et al* (2018) conducted a study on the activity of AMPK by metformin, which inhibits TGF-â expression in Bleomycin Idiopathic Pulmonary Fibrosis (BIPF) models. Lasiste *et al* (2018) also revealed that metformin activity lower EMT process on lens epithelial cells with PCO induction media (PCOM). It causes a decrease in migration and increase in cell apoptosis. In addition, AMPK activity suppresses TNF-á through the inflammatory pathway (Gamad *et al*, 2018; Lasiste *et al*, 2018; Wang *et al*, 2016).

This study indicates that metformin was able to decrease TGF-â and á-SMA expression levels. TGF-â antibody expression levels significantly decreased after administration of 0.5mM metformin compared to the control group. This study was consistent with Zheng *et al* (2016), which demonstrated that the alleviation of metformin peritendinous tissue fibrosis lesions was after the tendon injury. It has been showed that metformin inhibited abnormally activated proliferation *in vitro* and *vivo*. Activation of TGF-â1 signaling has been shown to

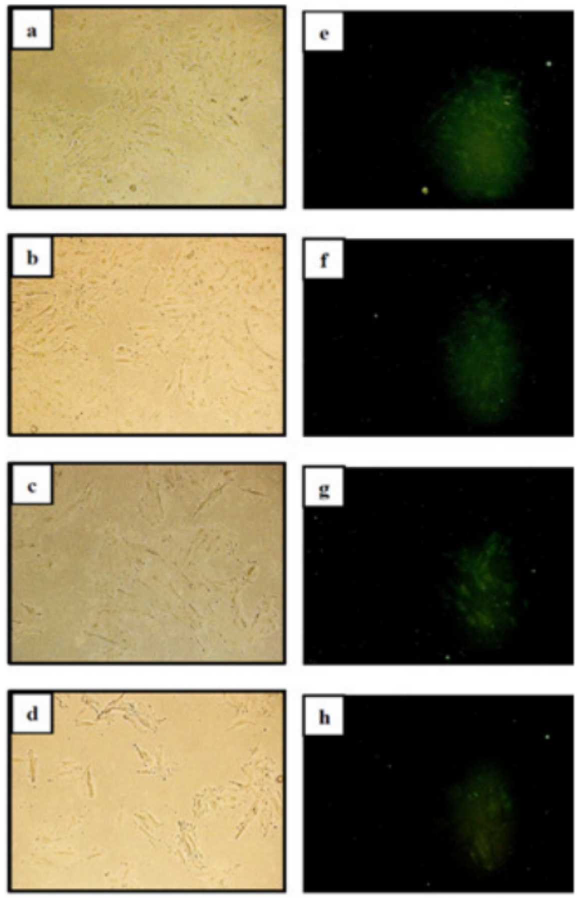

Fig. 6 : α-SMA antibody expression. Contrast phase photo after immunofluorescence staining with α-SMA FITC antibody on (a) control group, (b) metformin 0,1 mM, (c) metformin 0,5 mM, (d) metformin 1 mM. (inverted microscope, zoom in 100x). Immunofluorescence staining with α-SMA FITC antibody on (e) control group, (f) metformin 0,1 mM, (g) metformin 0,5 mM, (h) metformin 1 mM (fluorescence microscope, zoom in 200x).

promote fibrosis in multiple tissues. Zheng *et al* (2016) stated that *in vitro* also demonstrated that metformin inhibits TGF-â1 induced peritendinous tissue fibrosis by activating AMPK signaling. In this study, TGF-â1-treated NIH/3T3 fibroblasts exposed to metformin, which decreased cell viability and metformin treatment in promoted fibroblast apoptosis and inhibited proliferation (Gamad *et al*, 2018; Zheng *et al*, 2016).

In this study, the expression of á-SMA after treatment was decreased which indicated EMT inhibition. Zheng *et al* (2016) revealed that 5mM metformin tends to decrease expression of á-SMA and inhibit those induced by TGF-â1. Lasiste *et al* (2018) showed á-SMA expression as a marker of EMT which decreased after treatment with metformin 0,1mM on HLECs with PCOM media. TGF-â modulates the EMT process, with the trans-differentiation of epithelial cells into mesenchymes, which is characterized by an increased expression of á-smooth muscle actin (á-SMA), gene expression, and contractile MMP-2 protein levels. In this process, epithelial cells tend to turn into myofibroblasts with high contractility in fibrosis. This mechanism increases collagen production and accumulation to make the extracellular matrix fiber more rigid. EMT was characterized by trans-differentiating of epithelial phenotype to mesenchymal cell with a contractil action as known as myofibroblast. The decreasing levels of á-SMA expression in this study convinced the antifibrotic mechanism effect of metformin to HLECs (Dawes *et al*, 2009; Lasiste *et al*, 2018; Saika *et al*, 2014; Shirai *et al*, 2014; Zheng *et al*, 2016).

This study suggested that the administration of metformin alleviates EMT by decreasing its TGF-â expression. It played a pivotal role in the proccess of EMT and fibrosis, which characterized by an increase in á-SMA, with a continuous administration of metformin which significantly reduced the expression of TGF-â as well as á-SMA expression. Presently, pharmacological PCO prophylaxis has not been achieved. Although, several approaches have been developed to prevent PCO using chemicals, none of these have been clinically applied. The results of the present study demonstrated that metformin may be potential agents for preventing and treatment of PCO. However, further inves-tigations are required to investigate other signaling pathways capable of regulating EMT in HLECs (Sinha *et al*, 2013; Wormstone *et al*, 2016).

CONCLUSION

Metformintends to have antifibrosis effect on HLECs by inhibitingTGF-β and α-SMA expression, which indicates the EMT process.

ACKNOWLEDGEMENT

Thank to Airlangga Health Science Institute Research.

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PAGE 1

PAGE 2

PAGE 3

PAGE 4

PAGE 5