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DOWNREGULATION OF THE EXPRESSION OF MMP-3 AND TIMP-1 BY METFORMIN REGARDLESS OF TNF-α LEVEL IN AN *IN VITRO* LENS CAPSULE FIBROSIS MODEL

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ABSTRACT : Metformin, of biguanide groups, was found to possess some anti-inflammatory and anti-fibrotic ability. The drug also showed the capability of inhibiting human lens epithelial cells (HLECs) migration *in vitro*. Therefore, the aim of this study was to investigate the potential of metformin in reducing the expression of TNF-α as the key cytokine of inflammatory process as well as MMP-3 and TIMP-1 in the early proliferative phase of epithelial-mesenchymal transition (EMT) in lens capsule fibrosis. This study uses HLECs, as initiator of EMT in the development of posterior capsular fibrosis after surgery. These were isolated from the anterior capsule ofacataractous eye. The HLECs were cultured and divided into 4 groups, *i.e.*, FBS 10% control group, as well as the metformin 0.1 mM, 0.5 mM and 1.0 mM treatment groups. The expression of TNF-α was assessed 6 hours after intervention using ELISA, while MMP-3 and TIMP-1 immunofluorescence were evaluated 7 days following metformin administration. The data were analyzed using through One-way ANOVA followed by post hoc test at 95% confidence interval ($p<0.05$). Low concentration of TNF-á was detected in all groups, with the lowest in FBS 10% group (5.9896±0.0357 pg/mL). Similarly, metformin decreases the expression of MMP-3 and TIMP-1 with mean intensity of $1.8069 \times 10^5 \pm 1.2715 \times 10^5$ pixels and $2.6662 \times 10^5 \pm 1.6261 \times 10^5$ pixels, which were respectively found to be significantly lower than control group ($p<0.05$). This result showed metformin role in early proliferative phase of lens EMT directly without TNF-á attenuation. Metformin has an anti-fibrotic role by downregulating proteolytic enzyme such as MMP-3 and TIMP-1 regardless the expression of TNF-á.

Key words: Metformin, lens epithelial cells, MMP-3, TIMP-1, fibrosis.

INTRODUCTION

Posterior capsular opacification (PCO) is the most common postoperative complication of cataract surgery with varying incidence in adult patients ranging from roughly 38.5% to 67% (Jorge *et al*, 2014; Jick *et al*, 2016). The condition arises from residual hyperproliferative human lens epithelial cells (HLEC) after extracapsular cataract surgery. Also, this HLEC phenotype modification is mediated through epithelial-mesenchymal transition (EMT) towardsfibrocytic morphology accompanied with aberrant synthesis of the basal membranes. Additionally, mechanical injury-activated cells attenuate gene transcription associated with their survival maintenance within 24 hours,thereby initiating the expression of the genes responsible in regulating fibrosis (Jiang *et al*, 2018). Such mechanical stress leads to the expression of aldose reductase which induces the phosphorylation of akt/mTOR signaling axis,as well as theupregulation of numerous inflammatory cytokines such as TNF-á, IL-6 and TGF-â. The main effector of fibrosis, TGF-â, orchestrates EMT via Smad, p38 MAPK, JNK and ERK 1/2 which are accessible by TNF-á to regulate the same process. In addition, the initiation of EMT by TNF-á is aided by IL-6 and NF-êB resulting in the upregulation of MMP and TIMP. The imbalance in MMP3 and TIMP1 playsa role in fibrotic cataract (Tan *et al*, 2006). Also, the overexpression of both enzymes results in E-cadherin downregulation which subsequently affects cellular proliferation, differentiation, and migration (West-Mays and Korol, 2014; D'Angelo *et al*, 2014). Furthermore, overexpression of MMP-3 alter FGF-2 trait to further increase HLEC migration.

The strategy for PCO prevention and treatment remains a challenge, with no idyllic result to date. Metformin of biguanide group, is found to possess anti-inflammatory and anti-fibrotic activity. The drug downregulates NF-κB by activating AMPK through mTORC1, inhibiting inflammatory cytokines including IL-1â, IL-6, TNF-á and TGF-â, repressing catabolic gene encoder for MMP-3, downregulating TIMP-1 and

increasing promoter gene for E-cadherin expression in various cells (Hwang and Jeong, 2010; Park *et al*, 2010). Also, metformin is shown to reduce á-SMA, fibronectin, as well as LEC proliferation and migration in the development of EMT on HLEC *in vitro* (Lasiste *et al*, 2018). Therefore, the purpose of current study is to investigate both anti-inflammatory and anti-fibrotic effect of metformin on inhibiting the expression of TNF-á, MMP-3 and TIMP-1 in an *in vitro* lens capsule fibrosis model.

MATERIALS AND METHODS

Materials

The main reagents used in this study include Dulbecco's modified eagle medium (DMEM) from Gibco-Life Technologies, USA; non-essential amino acid (NEAA) from Sigma, USA; trypsin from Gibco – Life Technologies, USA; fetal bovine serum (FBS) from Biowest, USA; basic fibroblast growth factor (bFGF) from Gibco-Life Technologies, USA and Insulin-transferin-selenium (ITS) from Gibco-Life Technologies, USA. Also, the human TNF-á ELISA kit (E-EL-H0109) from Elabscience, USA. Then, the primary FITC antibodies for immunochemistry to vimentin, p63, MMP-3 and TIMP-1 all purchased from BIOSS, USA.

Methods

Study design

This is an *in vitro* study involving the HLECs of cataract patient, conducted in Stem Cell Research and Development Center, Universitas Airlangga. All experimental procedures were in line with local ethics review board in Dr. Soetomo Hospital. The cultures of HLECs were divided into four groups, *i.e.*, FBS 10%-treated control group in culture media, metformin 0.1 mM, 0.5 mM and 1.0 mM groups. TNF-á concentration was assessed from the supernatant collected from each groupat 6 hours after treatment. Expression levels of MMP-3 and TIMP-1 were measured on 7 days after treatment.

Tissue collection and isolation of HLECs

The lens capsule explant was obtained through routine uneventful cataract surgery conducted on asingle patient, aged 54 years,with senile nuclear immature cataract at Dr. Soetomo Hospital, Surabaya. The cell isolation was in accordance to theprotocol established by Ibaraki (2002) and modified byAndjeliæ *et al* (2015) through dissecting the anterior portion of the lens capsule. Primary HLEC cultures were obtained bycutting the capsule into a size of 0.5×0.5 mm, plated firmly into collagenase A-coated 60-mm petri dish and storedthe dish in a CO_2 incubatorat 37°C and 5% CO_2 for 30 minutes.The tissues were then cultured in 3mL growth media (10 % FBS + 5 ng/mL FGF + 1% gentamicin), until 90% confluency was achieved. The cells were harvested and passaged after 5 to 7 days of culture. In order to confirm epithelial cells with multipotent nature, the cultured HLECs were characterized at passage 3 for the expression of vimentin and p63-FITC antibody, then counterstained with DAPI. The stained cells were visualized under fluorescence microscope (BH2-RFL-T3 model fluorescence attachment, Olympus) and positive expressions were characterized by glowing green cells.

Metformin preparation

The preparation of metformin in this study was in accordance with the protocol established by Dowling *et al* (2010) and Lasiste *et al* (2018). Metformin hydrochloride (Alpha Science Innolabs, Jakarta, Indonesia) was dissolved to a concentration of 10 mM in 20 mL alpha modified eagle medium (á-MEM). The range of concentrations utilized in this study in growth media were 0.1-1 mM metformin.

In vitro lens capsule fibrosis model

HLECs at 3×10^5 cells/well were seeded into 96-well culture dishes and allowed to attach and grow to confluency. Scratch wounding were performed on the cells by yellow pipette tip. Intervened cells were then washed with medium to remove loose or dead cells. Treatment with 10% FBS for control group, and metformin of 0.1-1 mM concentration were administered for 7 days.

TNF-á expression analysis using ELISA

Supernatants were collected 6 hours post-wounding from the control group and 6 hours after adding metformin from the treatment groups for TNF-á assessment.100 ìL supernatant of each group were relocated into a 96-well plate and incubated for 90 minutes at 37°C. The liquids were then removed from each well and 100 µL of Biotinylated detection antibody working solution was added to each well and the plate was incubated for 1 hour at 37°C. The solution was aspirated from the wells and 350 µL of wash buffers were added to soak and wash the plate for at least 3 times. HRP conjugate, substrate reagent, and stop solution application to the wells were done as instructed in the datasheet from the kit. Optical density values were read at 450 nm.

Immunofluorescence staining of MMP-3 and TIMP-1

Each group was tested for immunofluorescence to measure the expression levels of MMP-3 and TIMP-1 after 7 days of treatment. Firstly, the culture media were

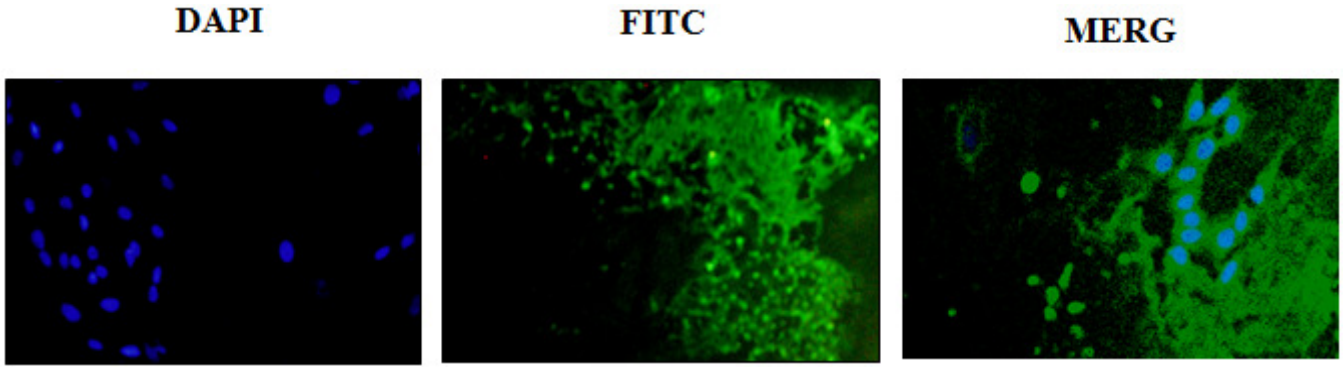

Fig. 1 : p63-stained HLECs. Left section: cells were stained with DAPI; middle section: p63-FITC; right section: merged pictures of two sections (inverted fluorescence microscope; 200x magnification).

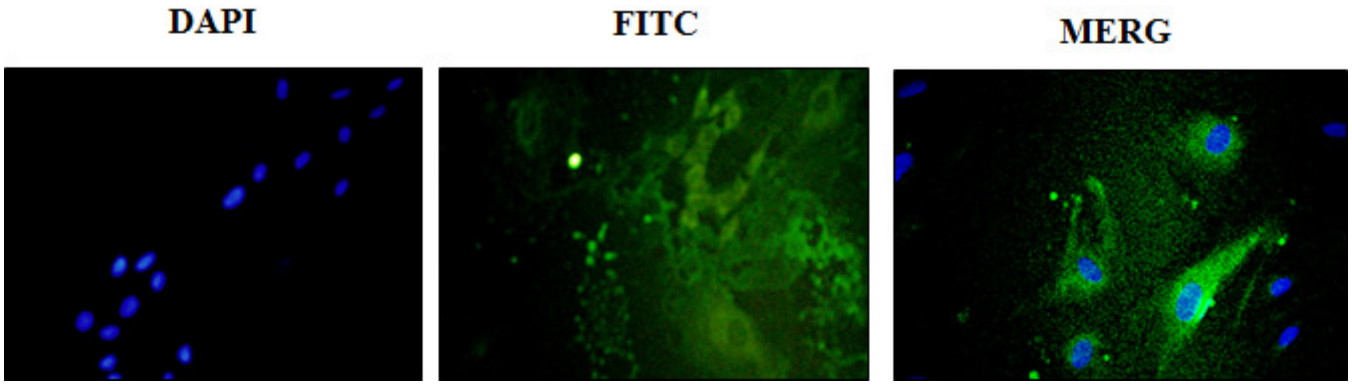

Fig. 2 : Vimentin-stained HLECs. Left section: cells were stained with DAPI; middle section: Vimentin-FITC; right section: merged pictures of two sections (inverted fluorescence microscope, 200x magnification).

aspirated and fixated with 3% formaldehyde for 15 minutes at room temperature. Subsequently, each well was washed in PBS 4 times, allowed to dry, and blocked with PBS containing 1% serum for 15 minutes at room temperature. Next, MMP-3 and TIMP-1 antibodies were added to each group andcell cultures were incubated at -4°C overnight. The results were then viewed using fluorescence microscope at 200x and 400x magnification whilethe expression levels were analyzed using ImageJ software version 1.52p. These were expressed in corrected total cell fluorescence (CTCF) determined through the formula: Integrated Density – (Area of selected cell × mean fluorescence background readings).

Statistical analysis

The statistical analyses were performed using SPSS version 25.0 software. Theses involved One-way ANOVA test, followed by Tukey HSD post hoc test, conducted to determine the statistical differences of TNF-á concentration as well as the fluorescence intensity of MMP-3 and TIMP-1 between groups at $p<0.05$.

RESULTS

Isolation and characterization of HLECs

The HLECs were isolated though the explant technique. On supplementing the cellcultures with bFGF and NEAA, confluency of 85-90% were reached in day 14. Vimentin and p63 were characterized and visualized under microscope fluorescence on passage 3 as shown in Figs. 1 and 2.

TNF-á expression

Six hours after treatment, the supernatantsfrom all the groups were tested for TNF-á expression. The results showed that all the groups have low concentration of TNF-á expressions with the least concentration was found in FBS 10% control group (5.9896 pg/mL; with One-way ANOVA test ($F(3,20) = 7.067$, $p=0.002$). The Tukey HSD post hoc test showed a statistically significant difference result between the between the control group with FBS 10% compared with the metformin 0.1 mM ($p=0.002$) and 1.0 mM ($p=0.013$) groups as shown in Fig. 3.

MMP-3 and TIMP-1 expression

The MMP-3 expression decreases after the administration of metformin 0.1 mM (3.0102×10^5 pixels± 2.0813×10^5 pixels), 0.5 mM(2.5817×10^5 pixels± 1.0759×10^5 pixels) and 1.0 mM(1.8069×10^5 pixels ± 1.2715×10^5 pixels). Also, the Tukey HSD post hoc test revealed significant reduction of MMP-3 expression in 1.0 mM concentration compared to control group ($p=0.012$).

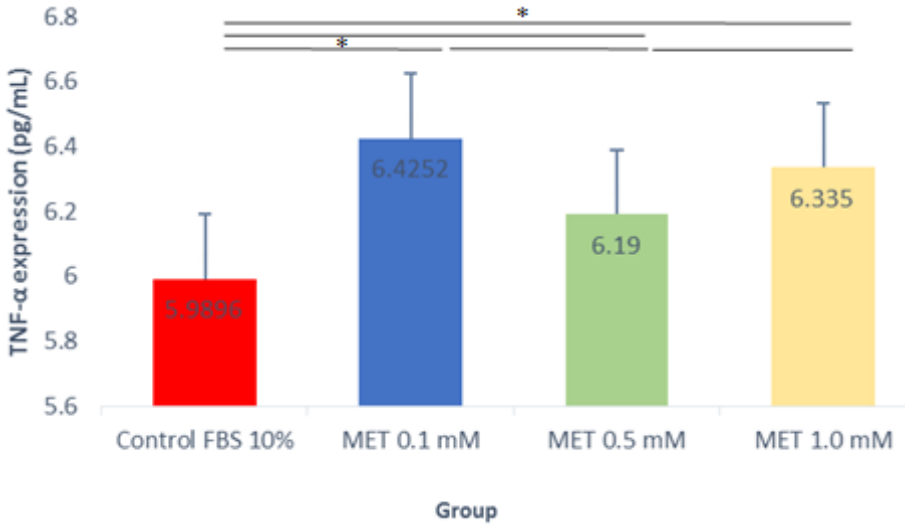

Fig. 3 : Average TNF-á expression (post hoc Tukey HSD, *p<0.05).

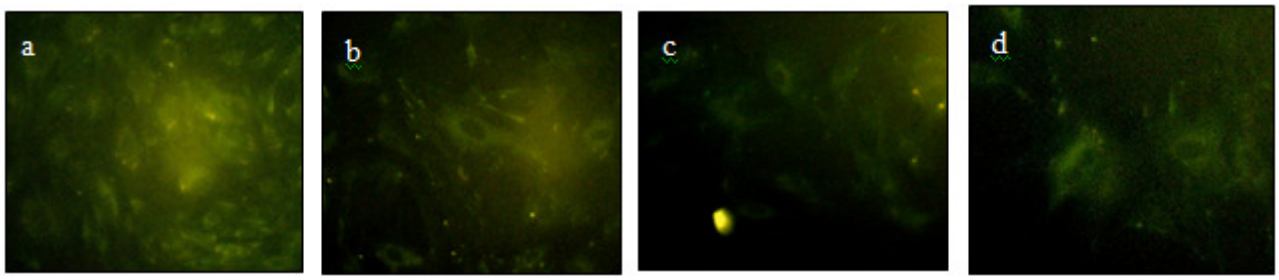

Fig. 4 : MMP-3 expression in control FBS 10% group (a), metformin 0.1 mM group (b), metformin 0.5 mM group (c), and metformin 1.0 mM group (d) (inverted fluorescence microscope, 200x magnification).

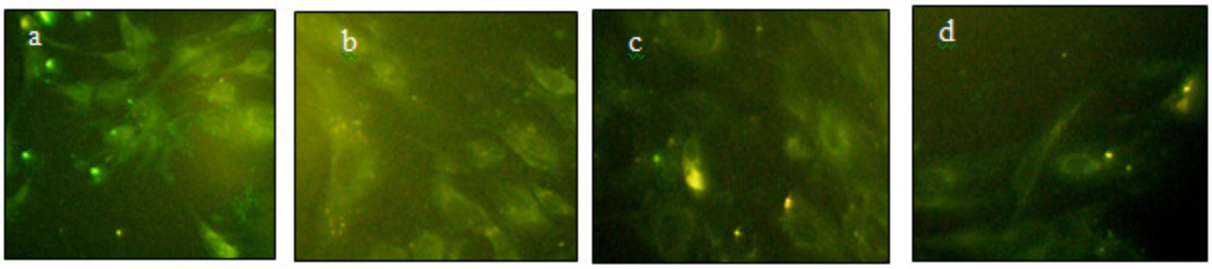

Fig. 5 : TIMP-1 expression in control FBS 10% group (a), metformin 0.1 mM group (b), metformin 0.5 mM group (c), and metformin 1.0 mM group (d) (inverted fluorescence microscope, 200x magnification).

Similarly, the fluorescence staining of TIMP-1 lessens after treatment of 0.1 mM(6.3759×10^5 pixels± 3.3324×10^5 pixels), 0.5 mM(2.6662×10^5 pixels± 1.6261×10^5 pixels) and 1.0 mM(3.2540×10^5 pixels± 1.6244×10^5 pixels) metformin. The Tukey HSD post hoc test result also revealed statistically significant decrease in 0.5 mM (p=0.003) and 1.0 mM (p=0.008) metformin groups compared with the FBS 10% group as shown by its fluorescence intensity in Figs. 4 and 5.

DISCUSSION

Mechanical stress-activated HLECs play role in EMT initiation associated with PCO development by decreasing gene transcription, which maintains the epithelial cell phenotype within the first 24 hours and upregulating gene that promotes fibrosis and mesenchymal cell survival (Jiang *et al*, 2018). This study revealed that metformin-treated HLECs expressed less MMP-3 and TIMP-1 (Figs. 4 and 5) without downregulation effect on TNF-á (Fig. 3). However, scratch woundingstimuli in other studies showed induction of aldose reductase pathway and increased expression of toll-like receptor (TLR)-4 mRNA, which results in phosphorylation of p38 MAPK, JNK, and ERK 1/2 followed by the activation of proinflammatory and profibrogenic cytokines as well as proteolytic enzymes (Zhao *et al*, 2017). Also, a study the found expression of MMP-3 and MMP-9 by inhibiting phosphorylation of NF-êB transcription and the activators of STAT3 pathway by TLR-4 (Eslani *et al*, 2014; Zhang *et al*, 2019). Attenuation of TLR-4 in the lens results in decelerated cellular proliferation (Yu and Lu, 2015). NF-êB phosphorylation is responsible for the expression of

TIMP-1 and MMP-3. Aside of modulation through TLR-4, upregulation of MicroRNA-26b by metformin inhibits NF-êB translocation into nucleus of cells. In a study conducted by Lisboa *et al* (2013), there were no increase inTNF-á and IL-1â after TLR was activated by mechanical force stimulus, which is in concordance with the result of this study, which showed low expression of TNF-á in all the groups. On the other hand, increased expression of TNF-á among treatment groups in this study may be associated with its activation by metformin-induced lipocalin-2 (LCN2). In an injured lens, Jiang *et al* (2018) discovered that LCN2 regulates proinflammatory cytokines including TNF-á within the first 24 hours and subsides in 48 hours. However, this multifunctional protein shows protective capability in somestudy in other cells which in turn counter their proinflammatory nature (Kang *et al*, 2017; Kang *et al*, 2018). Regarding EMT in PCO, metformin works through SLC22A1 receptor in HLECs in vitro with optimum dose of 0.5 mM and has the capacity to penetrate trans-corneally *in vivo* as shownin the study conducted by Nirmal *et al* (2013). The results of this study showed effective dose of 0.5-1.0 mM which were correlated to the downregulation of TIMP-1 and MMP-3.

Our study has several limitations. This study is a post-test only design, thus, the baseline levels of each parameter in primary HLEC culture were not assessed. TNF-á expression was only evaluated in protein level, hence localization of intracellular TNF-á could not be confirmed with this method. We did not evaluate the downstream for MMP-3 and TIMP-1 particularly such as E-cadherin shedding and extracellular matrix changes which wereobserved to be fibrosis marker in PCO.

CONCLUSION

Metformin has an anti-fibrotic role by downregulating MMP-3 and TIMP-1 regardless the expression of TNF-á.

Conflict of interests

None

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