Metformin activity in an *in vitro* model of posterior capsule opacification

**Abstract**

**Purpose:** To determine the activity of metformin in an *in vitro* model of posterior capsule opacification (PCO).

**Study Design:** Experimental laboratory research.

**Methods:** The HLE-B3 lens epithelial cell line was treated with PCO induction media (PCOM) supplemented with transforming growth factor-beta (TGF-β) and fibroblast growth factor (FGF). Different metformin concentrations (0-100 mM) were used. The following cellular parameters were assessed: (1) survival, using a viability assay; (2) morphology, via microscopy and image analysis; (3) migration, using the wound assay; (4) and expression of epithelial (Pax6, E-cadherin) and mesenchymal (α-smooth muscle actin or α-SMA, fibronectin) markers via Western blot. Expression of the uptake receptor SLC22A1 was evaluated in HLE-B3 and in human donor eyes with Western blot and immunohistochemistry, respectively. Statistical analysis of variance (ANOVA) with Tukey post-hoc test was done for analysis of cytotoxicity, morphology and migration data.

**Results:** Metformin was lethal to half (LC₅₀) of the cells at 30 mM, and a decrease in viability (P<0.05) was noted at 5 mM. LECs in PCOM treated with 1 mM metformin showed increased Pax6 and E-cadherin and decreased α-SMA and fibronectin expression. LECs in PCOM treated with metformin also maintained epithelial morphology. Migration was inhibited with 0.5 mM metformin (P<0.05). Both HLE-B3 and the lens epithelium in donor eyes were found to express SLC22A1.

**Conclusion:** Metformin decreased survival and migration in LECs, maintaining epithelial phenotype and reducing mesenchymal marker expression. Metformin therefore has potential as an adjunct in PCO prevention.

**Keywords:** metformin; lens epithelial cells; posterior capsule opacification; epithelial-to-mesenchymal transition.

**Introduction**

Posterior capsule opacification (PCO) causes a recurrence in visual decline in 20-40% of patients 2-5 years after cataract surgery.¹ PCO results from the proliferation, migration and secretion of extracellular matrix proteins by residual lens epithelial cells (LECs).² This collective response is called epithelial-to-mesenchymal transition (EMT).³

EMT is a key process in cancer proliferation and metastasis.⁴ The activity of metformin, a biguanide drug used primarily in the treatment of diabetes, has been observed against different cancers *in vitro* and *in vivo*. It requires an organic cation transporter for cellular uptake, the main receptor being the solute carrier family 22 member 1 (SLC22A1).⁵ Metformin has been noted to inhibit proliferation and EMT⁶ through a myriad of mechanisms. Among these are pathways that are also implicated in the development of PCO, such as PI3/Akt (mTOR), MAPK and Wnt.³

Literature on metformin and the eye is sparse. Metformin has been observed to be effective in preventing retinal angiogenesis⁷ and reducing inflammation in uveitis.⁸ Also, one large epidemiological retrospective cohort study of diabetic patients found that metformin was associated with a 25% reduced risk of developing open-angle glaucoma. The distribution of SLC22A1 in the eye
can explain the effect of metformin on specific tissues: this receptor has been demonstrated to be present in the cornea, in the iris and ciliary body, and in the blood-retinal barrier. To date, there has been no experiment performed to ascertain SLC22A1 expression in the lens.

Metformin has been associated with less non-enzymatic glycosylation of lens proteins in streptozocin-induced diabetes in rats. However, no studies have been done on the direct effect of metformin on LECs. Clinical studies on the risk of PCO formation in diabetic patients have shown conflicting results, due to the lack of consistent correlation with glucose control and the poor standardization of outcome measures.

This study was undertaken to determine the effect of metformin on LECs in an in vitro model of PCO. Four cellular parameters were analyzed: viability, morphology, migration, and expression of epithelial and mesenchymal markers. SLC22A1 expression in LECs and in human donor eyes was also evaluated.

Materials and Methods

Cell Culture

The human lens epithelial cell line HLE-B3 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained on Minimum Essential Media (MEM, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 20% fetal bovine serum (FBS) and 50µg/mL gentamicin (Thermo Fisher Scientific) and incubated (37°C, 5% CO₂). Cells were starved in serum-free (SF) media for 4 hours before addition of any treatment.

This in vitro model of PCO utilized the addition of transforming growth factor-beta (TGF-β) and fibroblast growth factor (FGF) to the culture media to induce proliferation and EMT (hereafter referred to as PCO media or PCOM). Both growth factors have been directly implicated in the formation of PCO and have been detected in the aqueous after cataract surgery. TGF-β and FGF were dissolved in SF at a concentration of 10 ng/mL and 40 ng/mL, respectively, as used previously.

Metformin Treatments

Metformin hydrochloride (Cayman Chemical, MI, USA) was dissolved to a concentration of 200 mM in SF or PCOM. The range of concentrations of metformin initially used in cytotoxicity studies were based on the minimum (0.1 mM) and maximum (100 mM) effective concentrations used against cancer cells. Subsequent studies utilized concentrations of 0.1-1 mM metformin, with SF and PCOM as controls.

Viability Assay

The Cell Counting Kit-8 (CCK-8) viability assay was used after (Dojindo Molecular Technologies, Kumamoto, Japan) 1.5x10⁴ cells were inoculated into each well in a 96-well plate (Falcon®, Franklin Lakes, NJ, USA) and after 24 hours of treatment. Spectrophotometry was done with the infinite M200 Pro plate reader and Tecan i-Control software (Tecan, Männedorf, Switzerland), and the survival rate for each treatment was calculated.

Brightfield Microscopy and Morphology

HLE-B3 cells (0.5x10⁵ cells/well) were seeded on a 96-well plate and grown to 50% confluence. After 48 hours of treatment, cells were fixed in methanol and stained with 25% crystal violet in methanol (Fisher Scientific, Nepean, ON, Canada). Wells were viewed under brightfield using the EVOS XL microscope (20X magnification). Digital processing using ImageJ was as follows: (1) images were made binary; (2) borders were dilated and t (3) eroded; (4) holes inside cells were filled; (5) cells were measured as particles in pixels, as optimized according to size in microns. Only particles with sizes from 500-15000 pixels were included to exclude edges, dirt and overlapping cells. Two morphological parameters on ImageJ were selected: circularity, which measured perimeter roughness, and roundness, which measured symmetry. Values were averaged for all particles measured.

Wound Assay

After seeding 2x10⁴ cells per well in 48-well plates (Corning, NY, USA), cells were grown to full confluence. A 200uL pipette tip was used to create a wound in the monolayer. Treatments were added after phase-contrast imaging of the wells with the EVOS XL microscope at 0 hours (wound induction). Imaging was repeated after 24 hours, and the wells were fixed and stained with crystal violet for brightfield microscopy with the Nikon Eclipse Ts2 (Tokyo, Japan). An ImageJ macro, the MRI Wound Healing Tool (MRI Redmine) was used for calculating the area of the cell-free gap at 0 and 24 hours after wound induction. Settings were optimized as follows: a variance filter radius of 10; a threshold of 50; a radius open of 4; and a minimum size of 5000 pixels. The percent cellular migration was calculated using the following formula: \([\frac{(area_{0hr} – area_{24hr})}{area_{0hr}}] \times 100\).

Data Analysis

At least three independent experiments with at least three technical replicates were performed for each assay. Data processing and statistical analysis was done using Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism (La Jolla, CA, USA). Analysis of variance (ANOVA) with the Tukey post-hoc test was done to compare viability, morphology and migration across treatments.
**Protein Extraction and Quantification**

For this study, 1x10^6 cells were seeded in 10-millimeter culture plates (Corning®) and grown to 80% confluence before treatment. After 48 hours, cell lysis was done with radioimmunoprecipitation (RIPA) buffer (Sigma-Aldrich, St. Louis, MO, USA) containing anti-phosphatase and anti-protease tablets (Roche, Basel, Switzerland). Cell lysis extracts were vortexed and centrifuged at 13,000g for 10 minutes at 4°C (IEC Micromax RF, Thermo Electron Corporation, Milford, MA, USA). The supernatant was isolated for protein quantification using a bicinchoninic acid (BCA) assay kit (Pierce BCA Protein Assay Kit, Thermo Fisher Scientific).

**Western Blot**

Bio-Rad TGX Stain-Free™ FastCast™ (Bio-Rad, Irvine, CA, USA) kits with 7.5%, 10% and 12% polyacrylamide were used to prepare gels. The Stain-Free™ gels were used to eliminate the need for loading controls, as these allowed for total protein normalization with the use of the Image Lab software (Bio-Rad). Protein samples were prepared by adding 4x Laemmli buffer (Bio-Rad) with β-mercaptoethanol (Sigma Aldrich) in a 3:1 ratio. Ten (10) ug of sample was loaded into each well. After electrophoresis, the gels were activated for 1 minute using the Chemidoc™ MP System. Proteins were transferred from the gel to the Trans-Blot Turbo™ nitrocellulose blots using the Trans-Blot Turbo™ Transfer System (Bio-Rad), with settings for efficient transfer optimized at 1.3 mA and 21-25V.

Membranes were rinsed thrice with 1X Tris buffered saline with 0.1% Tween-20 (TBST) for 5 minutes, blocked with 5% non-fat dry milk in TBST for 1 hour, and incubated overnight and for 1 hour with the primary and secondary antibodies, respectively. Chemiluminescent substrate detection (ECL Prime Western Blotting System, Amerhsam, GE Healthcare, Chicago, IL, USA) was used, and the blots were visualized using the Chemidoc™ system under auto-exposure.

Using Image Lab, a multi-channel image of both the membrane and the blot was created. The molecular weight of the bands of interest were determined using this overlay of images. Total protein normalization was automatically performed by the software, negating the need for probing with a housekeeping protein. Relative quantitation of the amount of target protein in each treatment, in comparison to the first lane (SF), was then obtained using the measured densitometric volumes.27

**Primary Antibodies**

The following primary antibodies were used in the following optimized concentrations: the mouse monoclonal anti-Pax6 (1:500, clone 1C8, ThermoFisher Scientific); anti-E-cadherin (1:500, clone 4A2, Cell Signaling Technology, Danvers, MA, USA; anti-α-SMA (1:500, clone 1A4, Dako, Santa Clara, CA, USA); and anti-SLC22A1 (1:1000, clone 2C5, Novus Biologicals, Littleton, CO, USA); and a rabbit monoclonal anti-fibronectin (1:500, ab32419, Abcam, Cambridge, UKs);

**Secondary Antibodies**

For mouse anti-Pax6, anti-αSMA and anti-SLC22A1 antibodies, the goat anti-mouse IgG-HRP linked secondary antibody (1:1000, sc-2005, Santa Cruz) was used. The anti-E-cadherin primary antibody was probed with the horse anti-mouse IgG HRP-linked antibody (1:2000, #7076, Cell Signaling). The goat anti-rabbit HRP-linked IgG (1:1000, #7074, Cell Signaling) was used to probe for fibronectin.

**Immunohistochemistry**

Four anterior segment sections of normal donor human eyes, obtained from an eye bank and routinely processed and embedded in paraffin, were taken from the Ocular Pathology Laboratory Archive (Montreal, Quebec, Canada). Automated immunohistochemistry was done using the Ventana Benchmark machine per the manufacturer’s protocol (Ventana Medical Systems, Tucson, AZ, USA) after baking, solvent-free deparaffinization and CCl (Tris-EDTA buffer pH 8.0)-based antigen retrieval. The slides were incubated with the mouse monoclonal IgG primary antibody against SLC22A1 (2C5, Novus Biologicals) at a dilution of 1:4000 for 30 minutes at 37°C, followed by the addition of an avidin/streptavidin enzyme conjugate complex. The ultraView Universal Detection Kit (Ventana Medical Systems) was utilized as a chromogenic substrate, with hematoxylin as counterstain. The cornea was used as internal positive controls, and uterus sections were used as negative controls. Slides were evaluated by two ocular pathologists in the laboratory and were scanned using the Aperio AT2 Turbo (Leica Biosystems, Wetzlar, Germany).

**Results**

**Effect of Metformin on LEC Viability**

Increasing concentrations of metformin were cytotoxic to LECs (Figure 1), whether grown in SF or in PCO media. After 24 hours, there was an increase in the number of cells grown in PCOM versus SF. Compared to SF and PCOM, there was a significant decrease (P<0.05) in the survival rate of cells treated with 20 mM and 5 mM
Lasiste JM et al. Metformin and posterior capsule opacification

Figure 1. Metformin was cytotoxic to LECs.

Figure 2. Metformin maintained lens epithelial circularity and roundness.

Figure 2. (a) Circularity was decreased in cells grown in PCOM. At 1 mM metformin, however, the circularity of the cells approximated that of SF. (b) Roundness was significantly decreased (P<0.05) with PCOM. Cell roundness was maintained with metformin, although measurements never attained those of untreated cells (SF). (c, 20X) Cells in PCOM became slightly more elongated and spindle-shaped. Treatment with metformin for 48 hours resulted in HLE-B3 cells more closely resembling their original phenotype.
metformin, respectively. To prevent the confounding effect of decreased viability, all subsequent experiments determining the effect of metformin on HLE-B3 cells in PCOM utilized a maximum concentration of 1 mM.

**Effect of Metformin on LEC Morphology**

Metformin was also noted to maintain the morphology of LECs amid exposure to FGF and TGF-β. Upon treatment with PCOM, LECs were expected to lose symmetry and acquire a more elongated shape, as was observed during experimentation (Figure 2a, 2b). With the addition of metformin, however, circularity was observed to be greater than PCO medium-treated cells at a concentration of 1 mM (Figure 2a). Roundness was also greater in LECs treated with 0.1-1 mM metformin (Figure 2b) than that of PCOM alone. However,
Lasiste JM et al. Metformin and posterior capsule opacification

Measurements of both circularity and roundness—which corresponded to shape and symmetry, respectively—only approximated that of untreated cells (SF, Figure 3a).

**Effect of Metformin on LEC Migration**

Metformin had the greatest impact on migratory ability (Figure 3a, top). Treatment with PCOM significantly increased migration (P<0.05). The lowest concentration of metformin (0.1 mM) was able to markedly decrease the ability of cells to migrate, with the migration rate approximating cells grown in SF. Inhibition of migration was significant at 0.5 mM and at higher concentrations, so that the gap induced in the wound assay was maintained (Figure 3b).

**Effect of Metformin on Protein Expression**

**Lens Epithelial Markers**

Consistent with the loss of epithelial phenotype, a decrease in Pax6 and E-cadherin expression was observed in LECs treated with PCOM (Figure 4). An increase in both markers was noted with treatment of 1 mM metformin, although at levels less than observed in cells cultured in SF.

**Mesenchymal Markers**

The mesenchymal markers α-SMA and fibronectin were increased after induction in PCOM and decreased with metformin treatment (Figure 5). This decrease was proportional to increasing concentrations of metformin.
**SLC22A1 Expression**

Western blot revealed a 40-kDa band that corresponded to the SLC22A1 receptor (Figure 6a). There also was increased SLC22A1 expression in response to increasing concentrations of metformin.

**SLC22A1 Expression in the Lens Tissue**

Immunohistochemistry revealed that the ocular lens epithelium expressed SLC22A1 in the cytoplasm (Figure 6b-6c).

**Discussion**

The experimental data obtained demonstrated that, in an *in vitro* model of PCO, metformin reduced LEC viability; preserved epithelial marker expression and morphology; and reduced mesenchymal marker expression and migration. These effects were evident at a concentration of 1 mM metformin.

This is the first study to utilize quantitative measures of cell morphology in LECs *in vitro*. Previous studies have qualitatively noted the acquisition of a spindle shape after exposure to TGF-β, both *in vitro* and in histological sections of the eye.28-31 Parameters of circularity and roundness, as automated and standardized by the image analysis software, can definitively characterize the changes in LEC morphology and can be used in future studies.

It was observed that untreated HLE-B3 cells had an average circularity and roundness of 0.37 ± 0.01 and 0.45 ± 0.01 (mean ± SEM), respectively *(Fig. 2B, 2C)*. This correlated with the elongated and asymmetrical shape of HLE-B3 cells *in vitro* but contrasted with the cuboidal shape of LECs *in vivo*. A similar observation was noted for the ileal colorectal adenocarcinoma cell line HCT-8 which, despite its epithelial morphology, had a circularity and roundness of approximately 0.37 and 0.27.32 Recognition of baseline morphological characteristics enables a more robust analysis of treatments on LECs.

The results of this study are encouraging for three reasons. First, this is the first study to investigate SLC22A1 expression in the ocular lens epithelium *(Figure 7B-7C)*. The presence of SLC22A1 in the lens epithelium suggests that it is highly probable that LECs *in vivo* can respond to metformin. Second, metformin has an excellent pharmacologic profile: it is inexpensive, readily available, and has a wide therapeutic index. An ophthalmic preparation of metformin has been utilized to study the ocular pharmacokinetics of organic cation transporters, and a concentration of 7.85 mM metformin does not adversely affect the cornea33. No clinical trial has been conducted on the association of systemic intake of metformin on PCO. Considering the effect of metformin on metabolism and inflammation, such a study is worthwhile.

Metformin was not lethal until a concentration of 80 mM *(Figure 1)*. Nevertheless, it will be difficult to administer this supraphysiologic concentration without causing damage to proximal tissues. There is also the debatable issue of the risk of lens dislocation with early elimination of LECs.34 Due to differences in pharmacokinetics between cell culture systems and living organisms, varying doses, schedules and routes must be tested to determine the optimum conditions for metformin to produce the desired outcome.

Finally, it is reasonable to hypothesize that inhibition of LEC proliferation and EMT will translate into PCO prevention. The efficacy of metformin in other models of PCO, as in explant cultures and rabbits, must be verified.

In conclusion, metformin inhibits proliferation and EMT in an *in vitro* model of PCO. Given the findings of this study and its intrinsic pharmacological properties, metformin can be a therapeutic adjunct to prevent or reduce the incidence of PCO.

**Acknowledgments**

The authors would like to thank Dr. Patrick Logan for his valuable comments on the manuscript and Dr. Ana Beatriz Dias for her help with immunohistochemistry.
