

A structural examination of the lens capsule following manual and femtosecond laser-assisted capsulotomy

PhD Thesis



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INTRODUCTION

The crystalline lens of the eye is a principal component in the process of vision. To perform its role, the lens must be transparent and have the capacity to rapidly accommodate when focusing on near and distant objects. However, senile cataract is common, multifactorial age-related pathological change of the lens. As a consequence of metabolic disturbance of the lens its light passing ability decreases. The only solution for cataract is the surgical removal of the lens and the intraocular artificial lens implantation. Phacoemulsification is a conventional method of cataract extraction, which is one of the safest and most successful surgical procedures performed worldwide. Since the development of phacoemulsification, cataract surgery has undergone major improvements.

The first step is the removal of the anterior lens capsule (capsulorhexis, CCC) is a routine step of cataract surgery. Two kinds of CCC are known: the manual capsulorhexis with a continuous and circular opening of the anterior lens capsule (ALC), performed via manual pulling of the capsule, and the femtosecond laser-assisted capsulorhexis with continuous and circular opening of ALC using laser-beam. Today, the goals of cataract surgery is to achieve near emmetropia, rapid and significant improvement of visual acuity, to correct refractive errors, and in some cases to restore accommodation capacity. However, the standard cataract extraction method is safe, the femtosecond laser technology can deliver remarkable gains in reproducibility, centration, and safety in cataract surgery Posterior capsule opacification (PCO) is the most common postoperative complication following capsulotomy. Regarding the incidence of PCO, results of different studies are contradictory. Studies showed that laser-assisted surgery is slightly but not significantly better than manual capsulotomy, while others warn that femtosecond laser could increase the risk of this novel postoperative complication.

Secondary cataract develops by the migration and proliferation of residual lens epithelial cells after extracapsular cataract extraction. These residual lens epithelial cells regain their proliferating ability, and form clusters. The presence of crystallin-rich Elschnig pearls at the rear of the lens capsule interferes with vision due to light scattering.

Regarding the incidence of posterior capsule opacification, investigations showed that laser-assisted surgery is slightly better than manual capsulotomy, while others warn that femtosecond laser could increase the risk of this novel postoperative complication. Many clinical comparative studies focus on the cut surface, on the parameters of intraocular lens decentration, or on the parameters of continuous curvilinear capsulorhexis (CCC) that effect intraocular lens centration when manual or laser assisted methods are compared. Little is known

about the changes of the lens epithelial cells following capsulotomy using the two different methods. Epithelial cells on the cutting surface of the removed ALC were reported to be affected differently when femtosecond laser-assisted capsulotomy (FLACS) and manual capsulotomy (manual CCC) were performed. Detection of TUNEL-positive apoptotic epithelial cells at the cut surface of the capsule indicated that more cells have undergone apoptosis using FLACS when compared to manual CCC.

In addition to the cells on the cutting edge of the removed ALC, cells locating medially of the cutting may also be affected differently by the surgery.

It is plausible that the mechanical damage caused by the removal of the ALC may induce the formation of secondary cataract through the dedifferentiation and increased proliferation rate of the residual epithelial cells. The deleterious effect of the mechanical damage on epithelial cells due to surgery, and its consequence on the increased cell proliferation rate was also observed in other organs. Therefore, we hypothesize that the damage caused by FLACS and manual CCC can be different. However, the effect of the surgical methods on residual epithelial cells cannot be directly examined in the human, but, study of the epithelial cells of the removed ALC can be a useful method to get information about the changes that occur also in the residual epithelial cells. Therefore, in this work light microscopic and ultrastructural morphological alterations were detected in the epithelial cells of the removed ALC. In addition, expression of genes coding proteins related to cell proliferation and apoptosis was studied. Moreover, the expression of genes of cytokines, IL-6 and IL-8 which can participate in stress signaling due to the mechanical damage was examined and compared in the two different surgical methods, the manual and the femtosecond laser-assisted capsulotomy.

It is known that every surgical intervention induces certain changes at cellular level. Following mechanical stress, various molecular pathways are activated. By these pathways, changes are induced in the cells including alteration of the cytoskeleton including the actin pattern. Thus, we can hypothesize that manual CCC and FLACS induce different cytoskeletal changes. Therefore, we compared the effect of the two surgical methods used for the removal of ALC (manual CCC and FLACS). Our main focus was to reveal cytoskeletal changes due to mechanical stress induced by capsulotomy. In the epithelial cells of the removed ALC, we examined the actin pattern and the intermediate filament glial fibrillary acidic protein (GFAP), which is characteristically expressed in the immature epithelial cells. However, it is well known, that due to deleterious effects such as mechanical damage, proteins, which characteristically appear throughout embryonic development show an increased expression level in cells of various organs including the eye. Therefore, we hypothesized that GFAP would be re-expressed

in ALC. In other cells, such as astrocytes, GFAP is colocalized with glutamine synthetase (GS). In the lens epithelial cells, a GS-like protein, lensin is present that can be detected with antibodies against GS. Lensin is highly expressed in normal lens epithelial cells, however, expression is decreased in age-related cataract. In order to get information about lensin expression, antibodies against GS were used.

AIMS OF THE STUDY

Epithelial cells of ALC removed by manual and femtosecond laser-assisted capsulotomy were studied. Our aim was to examine:

1. the morphological changes of the cells
2. the molecular changes in the cells including expression of various genes, which play a role in inflammation, cell death and cell survival.
3. the cytoskeletal changes of the cells following femtosecond laser-assisted and manually performed capsulotomy.

SUBJECTS AND METHODS

Materials

Samples of ALC ($n=110$) were taken during routine manual capsulotomy (CCC) and femtosecond laser-assisted capsulotomy (FLACS, VICTUS[®] Femtosecond Laser, Bausch+Lomb's, USA) with the following parameters: energy, 7.2 μJ ; spot spacing, 6 μm ; path spacing, 4 μm ; time, 400-550 femtoseconds. In all cases, we obtained written consent of the patients for further examination of the lens capsule. Investigations were carried out according to procedures approved by the Institutional Ethics Committee (University of Pécs 5426, Hungary.). Manually removed ALC were assorted in group 1 ($n=63$), while FLACS samples were classified in group 2 ($n=47$). Table 1 summarizes the number of ALC and the methods used in this study.

Table 1: Summary of the numbers of ALC and the methods used in this study

Methods	No. of laser assisted samples	No. of manual performed samples
Immunohisto-chemistry	$n=11$	$n=14$
RT-PCR	$n=10$	$n=10$
Cell culture	$n=5$	$n=5$
Histopathological examination	$n=16$	$n=29$
Examination of cytoskeletal changes	$n=5$	$n=5$

ALC: Anterior lens capsule; RT-PCR: Reverse transcription polymerase chain reaction.

Examination of morphology

Light Microscopy and transmission electron microscopy (TEM)

For routine light microscopy, ALC were fixed in a buffered solution (phosphate buffer 0.1 mol/L, pH=7.4) of 2% formaldehyde and 2.5% glutaraldehyde for 24h at 4°C. Specimens were post-fixed with 1% osmium tetroxide diluted in phosphate buffer for 30 minutes (min) at room temperature. Following dehydration with ethyl alcohol, samples were washed and placed in propylene oxide and then embedded in Durcupan resin (Sigma, Budapest, Hungary). Semithin sections and whole mount preparations were stained with toluidine-blue and studied with Olympus BX50 light microscopy.

Ultrathin sections were placed on mesh grids, and contrasted using solutions of lead-citrate and uranyl-acetate and examined in a JEOL JEM 1200EX transmission electron

microscope (TEM). Photographs were taken digitally with the iTEM software (Olympus, Japan).

Quantification of data and statistical analysis

Degenerating cell number was determined on toluidine-blue-stained semithin sections in both groups using light microscope (40× magnification). All the cells and those with degenerating morphology along the removed ALC were counted and the percentage of cells with degenerating morphology was determined. Data were presented as mean± SEM, and statistical significance ($P \leq 0.05$) was set using Student *t*-test analysis, using GraphPad Prism 5.03 program. Images were processed with Adobe Photoshop CS6.

Molecular biological examination

Gene expression analyses using real-time reverse transcriptase polymerase chain reaction

After removal, ALC were immersed into SAGMTM cell culture medium (Lonza, Basel, Switzerland) that contains all the ingredients that are necessary for the survival of human epithelial cells. From ALC total mRNA was isolated using NucleoSpin RNA Kit (Macherey-Nagel) within 24h following removal. cDNA was synthesized using High Capacity cDNA Kit (Life Technologies, Budapest, Hungary). Reverse transcription polymerase chain reaction (RT-PCR) was performed using a StepOnePlusTM and Real Time PCR System (Life Technologies, Budapest, Hungary) to quantify the PCR product once per cycle. Reaction consisted of 1 µl of each primer (in a final concentration of 500-500 nmol/L), 10 µl 2× SensiFAST SYBR Hi-ROX master mix (Bioline Reagents Ltd., USA) in a 20 µl final volume. The following pairs of primers were used: p53, Bcl-2, cyclin D1, IL-6, IL-8. Relative product quantities were determined using StepOne Software and the Livak analysis method. β-actin was used as endogenous control.

Cell culture

Obtained ALCs were maintained up to 14 days in SAGM tissue culture (Small Airway Cell Basal Medium and SAGMTMSingle Quots Supplement, Lonza, Basel, Switzerland). The epithelial cells were maintained in small Petri dish under standard cell culture conditions (37°C, 5% CO₂). Changes of gene expression levels were analyzed with qRT-PCR (StepOnePlusTM and Real Time PCR System). Quantification and melting curve analysis were performed using StepOnePlusTM and Real Time PCR quantification software 4.1

Immunohistochemistry

Pretreatment and fixation of the samples

Extracted tissue samples were immediately fixed in 4% paraformaldehyde (PFA; Sigma, Budapest, Hungary) diluted in phosphate buffered saline (PBS, 0.1 mol/L, pH 7.4) for two hours at room temperature. The fixed samples were washed in PBS and Triton-X (1:1000, Sigma, Budapest, Hungary), and incubated with the primary antibodies overnight at room temperature. Then, the samples were incubated with fluorophore-conjugated secondary antibody corresponding to the primary antibody. Finally, nuclear labeling was performed using 4',6-diamidino-2-phenylindol (DAPI, 1:10 000, Sigma, Budapest, Hungary) and preparations were coverslipped using fluoromount-G (Southern Biotech, USA).

The following antibodies were used for our work:

1. Primary antibody: anti-p53 polyclonal antibody produced in rabbit (1:50, Santa Cruz Biotechnology, USA), (1:50),
Secondary antibody: Alexa Fluor® 488 anti-rabbit, (1:100, Lifetechnology, Budapest, Hungary)
2. Primary antibody: anti-GFAP polyclonal antibody produced in rabbit (1:1000) (Sigma, Budapest, Hungary)
secondary antibody: Alexa Fluor® 568, anti-rabbit (1:1000, Lifetechnology, Budapest, Hungary)
3. Primary antibody: anti-GS mouse monoclonal antibody (1:100, Transduction Laboratories, USA)
Secondary antibody: Alexa Fluor® 488 anti-mouse, (1:1000, Lifetechnology, Budapest, Hungary)

In addition, GFAP immunoreaction was also examined with another, anti-human GFAP antibody (mouse monoclonal antibody, 1:750, DAKO, Glostrup, Denmark) on whole-mount preparations of ALC ($n=5$). Following binding of the primary antibody overnight at room temperature, anti-mouse biotinylated secondary antibody (1:100), then the avidin-biotin peroxidase detection system (Vector, Burlingame, CA) were used for 2h each. Binding sites were visualized with the chromogene 3-3'diamino-benzidine (DAB, Sigma, Budapest, Hungary). Following mounting on glass slides and dehydration, preparations were coverslipped

with DePeX (Sigma, Budapest, Hungary) and examined under light microscope (Olympus BX 50 microscope).

Quantification of data and statistical analysis

DAPI stained and the p53-immunoreactive cells were counted separately on the entire thickness of the whole mount preparation in both groups. The percentage of anterior lens epithelial cells expressing p53 protein was determined. In addition, anti-GFAP and anti-GS immunopositive cells as well the immunonegative cells were counted on the full thickness of the whole-mount preparations using a Fluoview FV-1000 Laser Confocal Scanning Microscope (40× magnification) and the percentage of immunoreactive cells was calculated. Data were presented as mean±SEM and statistical significance ($P\leq 0.05$) was set with Student's *t*-test analysis, using GraphPad Prism 5.03 program. Images were processed with Adobe Photoshop CS6.

Examination of the actin pattern

Samples were washed in phosphate buffered saline (PBS, 0.1 mol/L, pH 7.4), followed by fixation in 4% paraformaldehyde (PFA; Sigma, Budapest, Hungary) for 10 min at room temperature. Fixed tissues were incubated in permeabilization solution (diluted in PBS containing 0.1% Triton X-100, 0.1% Na-azide, 5% BSA) for 20 min at room temperature. Then capsules were stained with Alexa Fluor[®] 488-conjugated fluorescent phalloidin (Life Technologies, Budapest, Hungary) for 45 min, light protected at room temperature. Samples were washed in PBS and nuclear labeling was performed by propidium iodide (1:1000, Life Technologies, Budapest, Hungary), followed by coverslipping using VectaShield medium (Vector Laboratories, Burlingame, CA, USA).

Quantification of data and statistical analysis

Examination of the actin pattern was performed by analyzing 5 ALC samples from each group, taking 10 images from every sample in a Zeiss LSM 710 Microscope (63× magnification) and the number of cells was determined. Data were presented as mean±SEM and statistical significance ($P\leq 0.05$) was set with Student's *t*-test analysis, using GraphPad Prism 5.03 program. Images were processed with Adobe Photoshop CS6.

RESULTS

Results of morphological examination

Examination of toluidine blue-stained sections of the ALC using light microscopy clearly showed two types of cells in both groups.

One type of the cells had normal, lightly stained nucleus and cytoplasm. These cells were normal appearing cuboidal epithelial cells with round nuclei, and apparently, well-preserved junctions could be seen between the neighboring cells and between the cells and the capsule. The other cell type was characterized by darker nucleus and the cytoplasm. In many cases, loss of the cuboidal shape of cells and the shrinkage of the cytoplasm were seen. In addition, detachment of the lens epithelial cells from the capsule was observed. Shrunken dark cells frequently contained vacuoles in their cytoplasm, indicating the presence of swollen intracellular organelles. These morphological characteristics indicate severe cell damage. ALC removed by manual CCC and with FLACS contained epithelial cells with both normal and damaged morphology indicating that both methods induce certain level of cell damage. Apparently, more damaged (darker, shrunken) cells were seen in the ALC removed by manual CCC than after FLACS, although, the difference between the two groups was not significant.

In order to get more detailed information about the morphological differences caused by manual CCC and FLACS, TEM was performed. In the manually removed ALC, severe morphological changes of the nuclei were observed. In contrast to the round nuclei of the control ALC that mostly could be observed in group 2, the shape of the nuclei in group 1 were irregular due to shrinkage, while the nuclear envelope remained intact. In group 2, most of the cells contained well-preserved organelles in the relatively light cytoplasm, while in group 1, dark cytoplasm contained shrunken organelles. Due to the shrinkage of the cells, that is frequently observed in group 1, disruptions of cell-cell and cell-extracellular matrix junctions were observed. In contrast to the well-preserved, control-like junctions between epithelial cells of group 2, separation of cells by large gaps between them could be seen in group 1.

Results of molecular biological examinations

Gene expression analyses using real-time reverse transcriptase polymerase chain reaction

Slightly lower expression of cyclin D1 was detected following manual CCC compared to FLACS but the difference was not significant. Anti-apoptotic Bcl-2 expression levels were

reduced in the manual CCC group compared to FLACS. In harmony with this, we have found an increased level of pro-apoptotic p53 mRNA in the manual CCC specimens compared to those removed using FLACS, although the difference was not statistically significant. We found slightly higher expression of IL-6 and IL-8 mRNA in the manual CCC specimens of ALC compared to those removed using FLACS.

Results of cell culture experiments

Gene expression level of ALC epithelial cells in cell culture showed that the Bcl-2 expression levels in both groups were reduced compared to gene expression level of cells immediately after capsulotomy. Decrease of Bcl-2 expression was stronger following manual CCC compared to FLACS, but the difference was not significant. Gene expression level in FLAC cell culture showed increased level of p53 mRNA, compared to gene expression level of ALC epithelial cells immediately after capsulotomy. However the difference was not significant ($P=0.397$). Gene expression level of ALC epithelial cells immediately after CCC capsulotomy showed slightly increased level of p53 mRNA, compared to gene expression level in cell culture. Gene expression level in cell culture showed that the cyclin-D1 mRNA expression levels in both groups were significantly higher ($P = 0.0004$), compared to gene expression level of ALC epithelial cells immediately after capsulotomy. However, the difference of cyclin-D1 mRNA expression levels were not significant between CCC and FLACS.

Results of immunohistochemistry

We performed immunohistochemistry on whole mount preparation to detect p53 protein. In both groups, we detected intense staining of the epithelial cells in the peripheral area of the ALC, which gradually became weaker towards the central area. Following manual CCC, 48.5% of the cells showed p53 immunoreactivity. In contrast, in case of FLACS, p53 positivity was present only in 31.42% of the cells, and the difference between the two groups was significant ($P=0.019$).

Immunohistochemical examinations performed in tissue samples obtained by different capsulotomy methods showed intense GFAP and GS immunopositivity of the epithelial cells. The immunoreaction, especially following manual CCC, was stronger in the peripheral area of the ALC, and became patchy and gradually weaker towards the central area. The nuclei of the

epithelial cells remained unstained, but the cytoplasm showed GFAP and GS immunoreactivity. Many GFAP-immunoreactive cells contained also GS. Following manual CCC of the ALC, 48.07% of the epithelial cells were positive to GFAP immunostaining, 45.18% of them showed GS immunoreactivity and 28.85% of the cells displayed colocalization of GFAP and GS. In contrast, samples obtained from FLACS showed only 26.38% GFAP immunoreactivity, while the GS immunoreactivity was observed in 46.78% of the cells. Colocalization of GFAP and GS was found in 19.07% of epithelial cells. Apparently, no meaningful difference could be detected in the GS-immunoreactive cell density between FLACS and manual CCC.

Comparing the results of the quantification, significant differences were found between the two groups (manual CCC and FLACS) in the expression of GFAP ($P=2.24 \times 10^{-6}$), as well as GFAP and GS coreactivity ($P=0.003$). In conclusion, GFAP expression can be an indicator of mechanical damage in case of manual CCC and FLACS.

The morphology of GFAP-immunoreactive cells following manual CCC was examined with anti-human GFAP antibody, using DAB as chromogene. In addition to the normal morphology of GFAP-positive ALC epithelial cells, cells with altered shape and long thin processes were observed that supported robust change of their cytoskeleton proposed on the base of toluidine blue staining. Therefore, another, a more dynamic component of the cytoskeleton, microfilaments were also studied.

Examination of the actin pattern revealed a marked cortical network as well as regular actin filaments both in manual CCC and in FLACS capsulotomy samples. However, a difference was found between the two groups when the compactness of the actin pattern was studied. In case of FLACS capsulotomy, the compactness of the actin pattern was similar that seen in control epithelial cells. In contrast, the epithelial cells after in manual CCC showed a reorganization of the actin pattern. Instead of compact actin filaments, actin islands appeared and gap formation was observed between them. In these gaps, tubular elements connecting epithelial cells were visible. The tubular elements appeared only after in manual CCC of the ALC.

Quantitative measurements using analysis of variance from whole ALC resulted in a significant difference ($P<0.0001$) between the two capsulotomy methods when the presence of tubular elements was examined.

DISCUSSION

Due to the introduction of femtosecond laser technology, the critical points of cataract surgery have become automated. Several mild injuries including mechanical damage cause stress to the cells. We suppose that all surgical interventions have an influence on the affected cells. The goal of our study was to compare cell death and survival as well as cytoskeletal changes associated with CCC and FLACS capsulotomy.

Morphological examination and quantification of cells with degenerating morphology revealed that manual CCC induces stronger cell damage than FLACS. The majority of cells with signs of degeneration revealed the morphological features of apoptosis, such as the shrinkage and darker staining of the nucleus and the cytoplasm. These light and electron microscopic observations were in harmony with the molecular biological results and the p53 immunolabeling.

Although the differences detected were not significant, the expression of the pro-apoptotic p53 gene was higher in manual CCC samples than in FLACS. The increased expression of p53 gene was associated with an increased level of p53 protein in the ALC epithelial cells after manual CCC compared to that after FLACS. Previous studies have shown TUNEL positive ALC epithelial cells after cataract surgery, indicating apoptosis. Two of these studies have shown lower number of apoptotic cells after manual CCC than after FLACS, and their goal was to demonstrate that cell death depends on the energy used for the FLACS. Their results are apparently in contradiction with to our observations. The plausible explanation for this can be the following: being a transcription factor, p53 can induce several processes, not only apoptosis. Depending on the severity of the damage, it can stop the cell cycle or can stimulate DNA repair, and, finally, when it is not avoidable, it induces apoptosis. We suppose that epithelial cells of our samples were mildly affected, and the mild damage does not induce immediate apoptosis, only arrest of the cells in G1 phase of the cell cycle, or DNA repair. Since we could observe only the higher level of p53 mRNA and the presence of p53 protein product in the cytoplasm, most probably during translation, the exact role of p53 in our samples (whether it induces apoptosis or repair) is not clear.

We have to emphasize that the time-window needed for the removal of the ALC and the time that lasts until mRNA is isolated from the cells may be too short for the entire apoptotic process. However, this time is enough for transcription and translation of p53. According to a

study performed in rat lenses, thirty minutes after the injury mRNA and protein product of another transcription factor were already expressed. In harmony with our results, it indicates a very fast and robust change in the gene expression of ALC following capsulotomy.

GFAP is one component of intermediate filaments that is found in astrocytes of the central nervous system, which expression is increased due to various damages. In addition to astrocytes, GFAP can also be detected in many non-glial cells under normal conditions and it was observed in the lens during the embryonic development. Following differentiation, it is not detected in lens epithelial cells. However, due to damage, proteins being characteristically present during embryonic development show an increased expression in adults in many organs including the eye. Our results revealed that after ALC removal, the intermediate filament GFAP can be detected in epithelial cells. We used two different antibodies to verify the presence of GFAP and both indicated the expression of it. Our immunohistochemical study revealed that the rate of GFAP-immunoreactive epithelial cells were significantly higher after CCC removal of the ALC compared to FLACS capsulotomy. Repair mechanism following cell injury involve recapitulation of developmental processes in various organs including the eye and induce re-expression of those genes that are expressed during embryonic development. Some of protein products of these genes such as growth factors and their receptors can promote recovery processes. Others, like cytoskeletal proteins (such as GFAP, vimentin or nestin) indicate the presence of cell damage.

Part of the GFAP-positive cell population were GS-immunoreactive that indicates the presence of lensin in ALC cells. No meaningful difference could be detected in GS-immunopositive cell density between FLACS and manual capsulotomy. Since expression of lensin decreases in age-related cataract, the similarity in the rate of GS-immunoreactivity of epithelial cells indicates similar lensin expression, and consequently, similar severity of cataract. Thus, it shows the homogeneity of our cataract patients' groups treated by the two different surgical methods and even highlights the difference found in GFAP expression.

In harmony with the strongly significant difference in GFAP-immunopositive cell density, the GFAP and GS double-labeled cell density revealed significant differences between the groups. The higher density of GFAP-positive cells detected after manual CCC compared to FLACS suggests that manual CCC induces stronger mechanical stress on the epithelial cells than FLACS.

The alteration of the cytoskeleton of epithelial cells around capsulorhexis showed alteration in actin pattern that may indicate a change of the cells in contractility and in ability to migrate. Due to stress, direct cell-cell communication can also be changed. Direct cell-cell communication was supposed to occur via gap junctions and different paracrine mechanisms. Recently, a new actin-containing tubular structure has been described that provides continuity between distantly positioned cells. These tubular structures have been reported in many cell types (astrocytes, various tumor cells, immune cells, etc.). By comparing the two surgical methods, we focused onto the examination of the actin pattern in our investigations.

It is known that stress leads to the reorganization of the actin pattern. The change in actin cytoskeleton can trigger the expression of different signaling proteins playing a role in the epithelial cells' response and survival. Examination of the actin pattern revealed similar cortical networks in the cells after manual CCC and FLACS. By contrast, we have found marked difference in the compactness of the actin pattern and remodeling of the actin cytoskeleton of epithelial cells following CCC capsulotomy.

In manual CCC, actin "islands" appeared instead of the compact actin filaments, and between the actin "islands" gap formation was found. Gaps were not visible after FLACS capsulotomy. Gap formation can be explained with the higher mechanical effect of manual CCC capsulotomy, which is supported with our s light and electron microscopic results and the increased GFAP expression. Between the gaps, actin-containing tubular elements could be seen. These structures were observed after manual CCC.

Our results suggest that FLACS capsulotomy causes milder mechanical stress on epithelial cells, than manual CCC. The robust morphological and cytoskeletal changes, as well as the altered expression of genes detected following manual CCC indicate the possibility of altered cell-cell communication and increased ability to migration that may result in epithelial-mesenchymal transformation and, consequently, development of secondary cataract. The milder morphological and cytoskeletal changes following FLACS allows us to suggest that following laser-assisted method fewer residual epithelial cells undergo epithelial-mesenchymal transformation, which may decrease the risk for the formation of posterior capsule opacification.

SUMMARY

We revealed that manual capsulotomy results in stronger damage on epithelial cells, however, we have to emphasize that the difference found in the changes of the morphology was insignificant. The expression level of genes, which are playing a role in cell death and cell survival, was higher after manual than laser-assisted surgery. We observed that the conventional removal of the ALC means stronger mechanical stress and induces changes of the cytoskeleton of the epithelial cells. We detected that following laser-assisted capsulotomy, the epithelial cell's cytoskeleton actin pattern is similar to the normal cytoskeleton actin pattern. In contrast, in manual capsulotomy, we found the presence of tubular elements that connect epithelial cells. We conclude that manual capsulotomy results in stronger damage on epithelial cells due to the pulling and stretching of the capsule during surgery.

Taken everything together, we can state that both methods are fully suitable for the removal of the ALC in age-related cataract, however, femtosecond laser-assisted capsulotomy causes milder damage on the epithelial cells of the ALC than the removal of the lens capsule performed manually.

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