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Apoptosis Factors of Lens Epithelial Cells Responsible for Cataractogenesis in Vitrectomized Eyes with Silicone Oil Tamponade

Authors' Contribution:
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Data Collection B
Statistical Analysis C
Data Interpretation D
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Literature Search F
Funds Collection G

CEG **Lili Zhu**
BF **Ke Zhao**
A **Dinghua Lou**

Department of Ophthalmology, The First Affiliated Hospital, Medical College, Zhejiang University, Hangzhou, Zhejiang, P.R. China

Corresponding Author: Dinghua Lou, e-mail: Julie8053@163.com
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Background: The aim of this study was to determine the expression of apoptotic factors Bax, Bcl-2, and Caspase-3 in lens epithelial cells (LECs) from cataracts secondary to pars plana vitrectomy with silicone oil (SO) tamponade. We also investigated the impact of SO emulsification on the expression of apoptotic factors.

Material/Methods: Anterior capsulotomy specimens of 20 eyes in 20 patients with cataract secondary to SO tamponade (Group 2), were collected. Another 20 eyes of 20 patients with age-related cataract (Group 1) were recruited as controls. The anterior capsule specimens were obtained from the patients during cataract surgery, frozen and later analyzed with respect to immunohistochemical stains of Bax, Bcl-2, and Caspase-3 using a confocal microscope.

Results: Age, sex, and laterality did not show any difference between the 2 groups. There was a greater increase in Bax and Caspase-3 expression in LEC in Group 2 than in Group 1 ($P_{\text{Bax}} < 0.0001$, $P_{\text{Caspase-3}} < 0.0001$). The Bcl-2 expression decreased in Group 2, although the difference was not significant ($P = 0.616$). The changes of apoptosis factors are not associated to SO emulsification ($P_{\text{Bax}} = 0.354$, $P_{\text{Bcl-2}} = 0.728$, $P_{\text{Caspase-3}} = 0.939$).

Conclusions: The caspase-3-dependent apoptosis of LECs increased in complicated cataract patients with SO endotamponade. The Bax played a critical role in regulating apoptosis of LECs in vitrectomized eyes with SO tamponade. The SO emulsification had no significant impact on the expression of apoptosis factors.

MeSH Keywords: **Apoptosis Regulatory Proteins • Cataract • Epithelial Cells**

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Background

Vitreotomy, which is the surgical removal of vitreous, has become a standard treatment for various vitreoretinal diseases. Vitreotomy surgery can significantly increase intraocular oxygen tension, and the abnormal exposure of the crystalline lens to high oxygen levels may lead to nuclear cataract formation [1]. Silicone oil (SO) is frequently used in vitreotomy. Vitreotomy with SO tamponade can produce a transient posterior subcapsular cataract postoperatively, which is resolved and followed by accelerated nuclear opacification [2]. Both direct contact by SO [3] and obstruction of normal metabolic exchange at the silicone-lens interface [4] can result in cataract formation. Accordingly, the cataract is one of the most common complications of pars plana vitrectomy with SO endotamponade, which is mainly caused by oxidative stress [3]. The occurrence or progression of a cataract can deteriorate visual acuity again after vitreoretinal surgery for phakic eyes and the incidence is about 60–98% within 2 years [5,6].

Lens epithelial cells (LECs) are the most active parts of the lens metabolism and their apoptosis is a common cellular basis for noncongenital cataract development in human and animals [7,8]. The relationship between oxidative stress and apoptosis has been widely studied [9,10]. Oxidative stress induced by H_2O_2 can cause cell viability decrease and apoptosis in human LECs [11]. Currently, there is no clear evidence of cataractogenesis after vitreotomy with SO endotamponade.

The aim of our study was to investigate the expression of apoptotic factors (Bax, Bcl-2, and Caspase-3) and to determine the effect of SO emulsification on them in the LECs of vitrectomized eyes with SO tamponade.

Material and Methods

A prospective study of patients who visited our medical center for phacoemulsification combined with SO removal from January to July 2013 was conducted. Twenty consecutive patients were included. Previous vitreoretinal surgery with SO instillation was performed for the rhegmatogenous retinal detachment (RRD) with proliferative vitreoretinopathy. Patients who had uveitis or intraocular hypertension after vitreoretinal surgery or who had coexisting ocular diseases were excluded from this study. A control group comprised 20 consecutive cases of age-related cataract (ARC) without history of systemic or other ocular diseases. The cataract grade was 2 to 3 according to LOCS III classification [12]. Demographic and clinical characteristics were collected from all case records.

In RRD cases, the patients underwent vitreoretinal surgery with 23-gauge standard sclerotomies, followed by injections with silicone oil (Oxane 5700; Bausch & Lomb, Rochester, NY, USA).

SO tamponade was performed to ensure intraocular pressure of about 20 mmHg when the procedure was finished, which ranged from 4 ml to 5 ml. SO removal and cataract extraction by phacoemulsification with intraocular lens implantation were accomplished in the same operation. Visible particles of SO observed via gonioscopy and slit-lamp biomicroscopy were regarded as SO emulsification. In ARC cases, the patients underwent cataract extraction and intraocular lens implantation. All the surgeries were uneventful and were performed by the same surgeon (DH Lou).

The anterior capsule epithelium specimens were obtained, immediately fixed in 4% paraformaldehyde (PFA), and stored at 4°C in a refrigerator. The PFA-fixed anterior capsules were washed with 0.1% Tween and phosphate-buffered saline (PBS) for 5 min 4 times. They were transfected with 0.5% TritonX100, followed by washing again with 0.1% Tween 20 and PBS. After that, they were blocked with Block Solution (PBS, 10% serum) for 25 min. Each anterior capsular was split into 2 pieces. One piece was incubated overnight with the primary antibodies (Bax and Bcl-2, Abcam) (1:100 dilution) and the other piece was incubated overnight with the primary antibody (Caspase-3, Abcam) (1:100 dilution) at 4°C. The capsules were incubated with their appropriate secondary antibody (Abcam) (1:100 dilution) at room temperature for 1.5 h. After they were washed with 0.1% Tween 20 and PBS for 15 min, they were stained with Hoechst33342 (Life technologies Corp.) to allow visualization of the nuclei. The capsules were mounted on glass slides and analyzed with a confocal microscope (Nikon A1).

The number of immunopositive cells was counted in 4 representative fields. An eyepiece-calibrated grid with $\times 10$ magnification and an objective calibrated grid with $\times 60$ magnification were used. At this magnification, the total number of cells in 1 microscopic field was counted and the percentage of positively stained cells was determined (positive cells/total cells $\times 100\%$). The average of the 4 fields was calculated for Bax, Bcl-2, and Caspase-3, and then analyzed for scoring. The scoring system was based on a scale of 0 to 4+ as follows [13]: 4+=very high (75% to 100% positive cells); 3+=high (50% to 75% positive cells); 2+=moderate (25% to 50% positive cells); 1+=low (<25% positive cells); 0=negative (0 positive cells).

All analyses were performed using the Statistical Package for Social Sciences (SPSS) for windows, Version 19.0. The Pearson's chi-squared test, Mann-Whitney *U* test, and independent-samples *t* test were used for statistical analysis. A *P* value of less than 0.05 was considered statistically significant. This study was approved by the hospital Ethics Committee and conducted in

Table 1. Characteristics of the patients in this study.

Variables	Group 1	Group 2	P value
Age (mean years \pm SD)	65.15 \pm 6.64	61.60 \pm 6.81	0.112
Gender (M/F)	9/11	7/13	0.519
Laterality (R/L)	7/13	11/9	0.204

Group 1 – age-related cataract; Group 2 – cataract with SO endotamponade; SO – silicone oil.

accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients. No patient information was extracted except for research purposes.

Results

There were 13 females (65%) and 7 males (35%) whose average age was 61.60 \pm 6.81 (SD) years (range, 50 to 78 years) in the cataract secondary to SO tamponade group. The left eye was affected in 13 cases and the right eye was affected in 7 cases. The duration of SO endotamponade ranged from 12 to 52 weeks (22.95 \pm 8.90 weeks). The ARC group consisted of 11 females (55%) and 9 males (45%) whose average age was 65.15 \pm 6.64 (SD) years (range, 56 to 76 years). The left eye was affected in 9 cases and the right eye was affected in 11 cases. There was no significant difference in age, sex and laterality between the 2 studied groups ($P>.05$) (Table 1).

Figures 1 and 2 show confocal immunofluorescent images of stained Bax and Bcl-2 in LECs of the 2 groups. Bax was weakly stained (1+) in the ARC group and strongly stained (3+) in the cataract with SO endotamponade group. Bcl-2 was weakly stained (1+) in both groups. Figures 3 and 4 show confocal immunofluorescent images of stained Caspase-3 in LECs of the 2 groups. Caspase-3 was weakly stained (1+) in the ARC group and strongly stained (3+) in the cataract with SO endotamponade group.

Table 2 shows the immunohistochemical scoring of Bax, Bcl-2, and Caspase-3 in the ARC group and the cataract with SO endotamponade group (Bax, 0: 0 vs. 0, 1+: 9 vs. 0, 2+: 10 vs. 5, 3+: 1 vs. 10, 4+: 0 vs. 5; Bcl-2, 0: 0 vs. 2, 1+: 17 vs. 17, 2+: 3 vs. 1, 3+: 0 vs. 0, 4+: 0 vs. 0; Caspase-3, 0: 0 vs. 0, 1+: 13 vs. 0, 2+: 7 vs. 7, 3+: 0 vs. 9, 4+: 0 vs. 4). As compared to ARC, there was a statistically significant increase of Bax and Caspase-3 expression in the cataract with SO endotamponade group ($P_{\text{Bax}}<0.001$; $P_{\text{Caspase-3}}<0.001$) (Table 3). There was no significant difference of Bcl-2 expression between the 2 groups ($P>.05$) (Table 3).

To further clarify the effect of clinical characteristics on apoptosis factors in LECs, we analyzed the differences in Bax, Bcl-2, and Caspase-3 distribution between the patients with emulsified SO and those without emulsified SO (Table 4). Eight patients

had intraocular SO emulsification in the cataract with SO endotamponade group. The distribution of apoptotic factors was: Bax, 0: 0 vs. 0, 1+: 0 vs. 0, 2+: 4 vs. 1, 3+: 2 vs. 8, 4+: 2 vs. 3; Bcl-2, 0: 1 vs. 1, 1+: 7 vs. 10, 2+: 0 vs. 1, 3+: 0 vs. 0, 4+: 0 vs. 0 and Caspase-3, 0: 0 vs. 0, 1+: 0 vs. 0, 2+: 3 vs. 4, 3+: 4 vs. 5, 4+: 1 vs. 3. However, no significant difference in the expression of the 3 apoptosis factors (Bax, Bcl-2, and Caspase-3) was found between patients with emulsified SO and those without emulsified SO ($P>.05$) (Table 5).

Discussion

The complicated cataract is the most common complication after vitrectomy, especially with intraocular tamponades. This kind of cataract not only leads to decreased vision, but also impairs the fundus observation. Injecting silicone oil into the human eye is likely to cause cataract formation [14]. It was reported that cataracts developed in all phakic eyes with silicone oil tamponade after vitrectomy within 6 months [15].

LEC apoptosis, which is a common cellular basis for noncongenital cataract development [7], can be induced by many factors, such as oxidative stress and hyperglycemia [16–19]. The increased apoptosis of hLECs and intracellular reactive oxygen species (ROS) were observed when the hLECs were incubated with high galactose [20]. Boyun Kim et al. [21] reported that the mean percentage of Bax-immunopositive cells was significantly higher in patients with DM than in those without DM, indicating that the apoptosis of LECs was higher in cataract patients with DM.

Vitrectomy surgery can markedly increase intraocular oxygen tension for prolonged periods after surgery, and this exposes the crystalline lens to abnormally high oxygen [22]. Studies have found that the oxidation of lens protein during vitrectomy was one of the reasons for nuclear cataract after vitrectomy [23], whereas adding antioxidants into the perfusion fluid during vitrectomy could inhibit the abnormal lens epithelial cell growth and lens fiber formation and thus prevent the cataractogenesis [24]. Our study shows that the expression of Bax and caspase-3 is significantly higher in cataracts secondary to SO tamponade than in age-related cataracts. To the best of our knowledge, this is the first study to describe

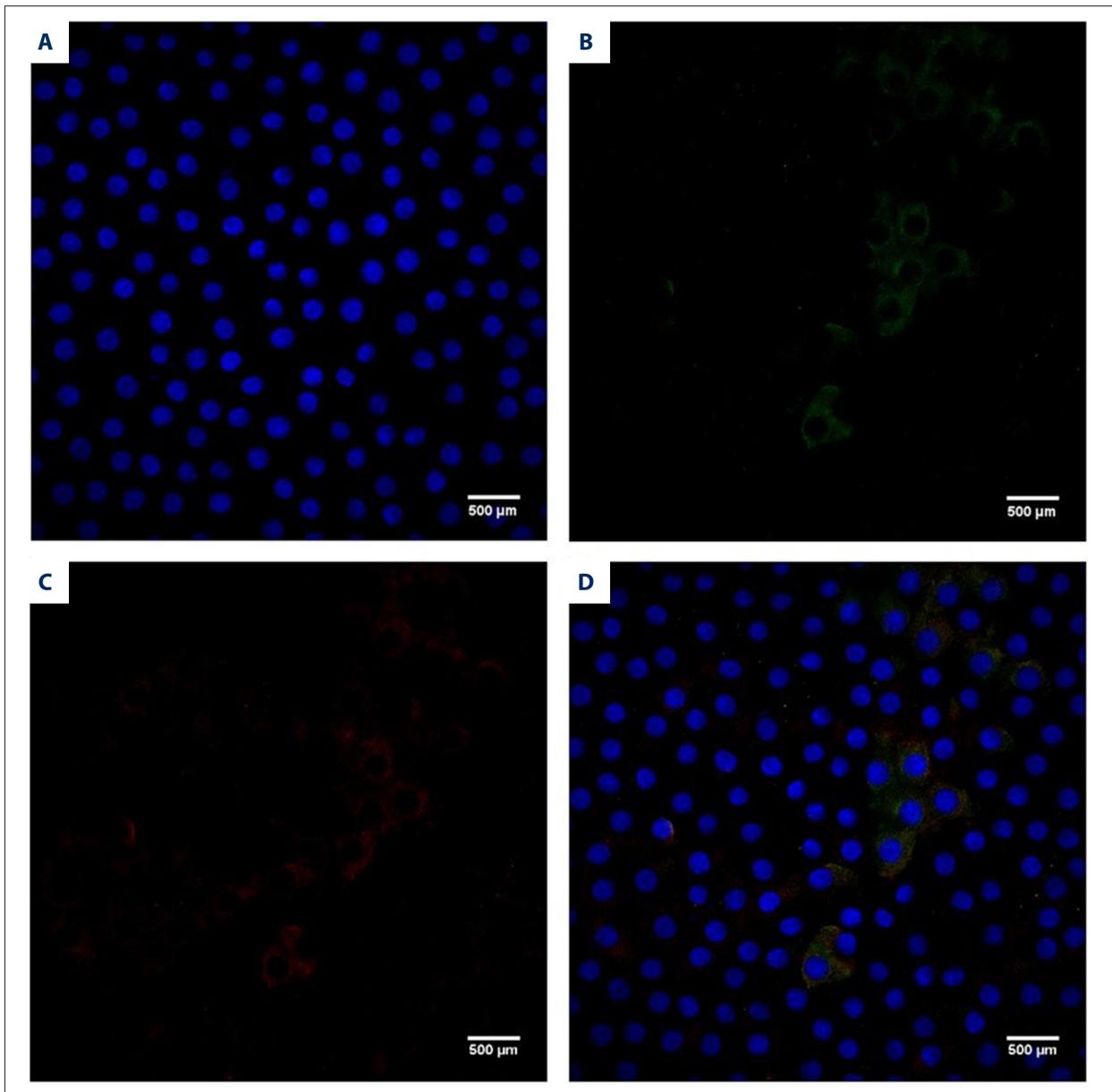


Figure 1. Confocal immunofluorescent image of LECs in Group 1 (age-related cataract group). (A) Blue nuclei visualized using Hoechst stain. (B) Bax is weakly stained (1+) (green). (C) Bcl-2 is weakly stained (1+) (red). (D) Merged image shows weak (1+) Bax and Bcl-2 expression (original magnification $\times 600$).

the Bax/Bcl-2 and caspase-3 distribution of *in vivo* hLECs in vitrectomized eyes with SO tamponade.

The Bcl-2 family comprises antiapoptotic proteins (e.g., Bcl-2, Bcl-XL, and Mcl-1) and proapoptotic proteins (e.g., Bax, Bak, and Bad) [25]. Bax/Bcl-2 are 2 important pro- and anti-apoptotic proteins that influence cell fate. Overexpressed Bax counters the death repressor activity of Bcl-2 and accelerates apoptotic death induced by cytokine deprivation [26]. During apoptosis, Bax is activated and translocated from the cytosol into mitochondria, which can be better inhibited by R21A [27]. Our

study demonstrated that the expression of Bax increased significantly in complicated cataracts secondary to SO tamponade. Hence, inhibition of Bax overexpression might be used to prevent this type of cataract formation.

Caspases are important signs of apoptosis and caspase-3 is a major downstream effector caspase in the apoptotic process of LECs [28,29]. Andersson et al. [29] proposed that posterior subcapsular cataracts result from defective differentiation, in which caspase-3-dependent apoptosis may play an important role. In our studies, the protein levels of caspase-3 were

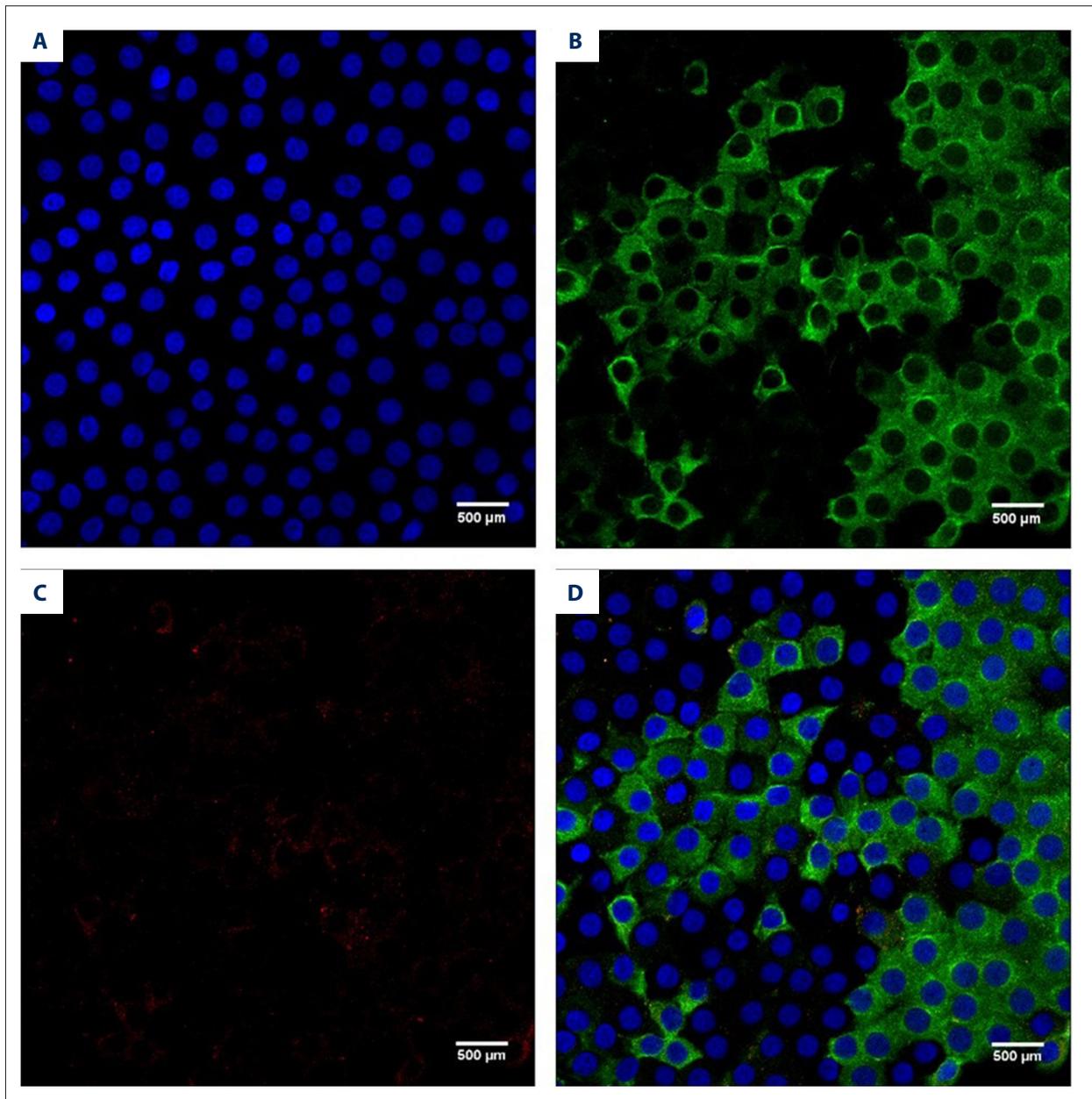


Figure 2. Confocal immunofluorescent image of LECs in Group 2 (cataract with SO endotamponade group). (A) Blue nuclei visualized using Hoechst stain. (B) Bax is strongly stained (3+) (green). (C) Bcl-2 is weakly stained (1+) (red). (D) Merged image shows strong (3+) Bax and weak (1+) Bcl-2 expression (original magnification $\times 600$).

elevated after vitrectomy with silicone oil tamponade, suggesting that caspase-3 is involved in apoptosis of LECs after pars plana vitrectomy.

Emulsification is a clinically significant complication of using SO tamponade [30]. To the best of our knowledge, there have been no studies on the effect of SO emulsification on complicated cataracts; therefore, we cannot ascertain whether complicated cataracts are related to SO emulsification in vitrectomized eyes or to SO tamponade. One study revealed that

emulsified silicone may potentially enhance opacification of residual anterior capsule following pars plana vitrectomy by silicone oil deposition and subsequent activation of LECs [31]. However, the present study is the first to show no significant difference in the expression of 3 apoptosis factors (Bax, Bcl-2, and Caspase-3) between patients with emulsified SO and those without emulsified SO.

Although we found no difference in apoptosis factors expression in LECs between the 2 groups, several known complications

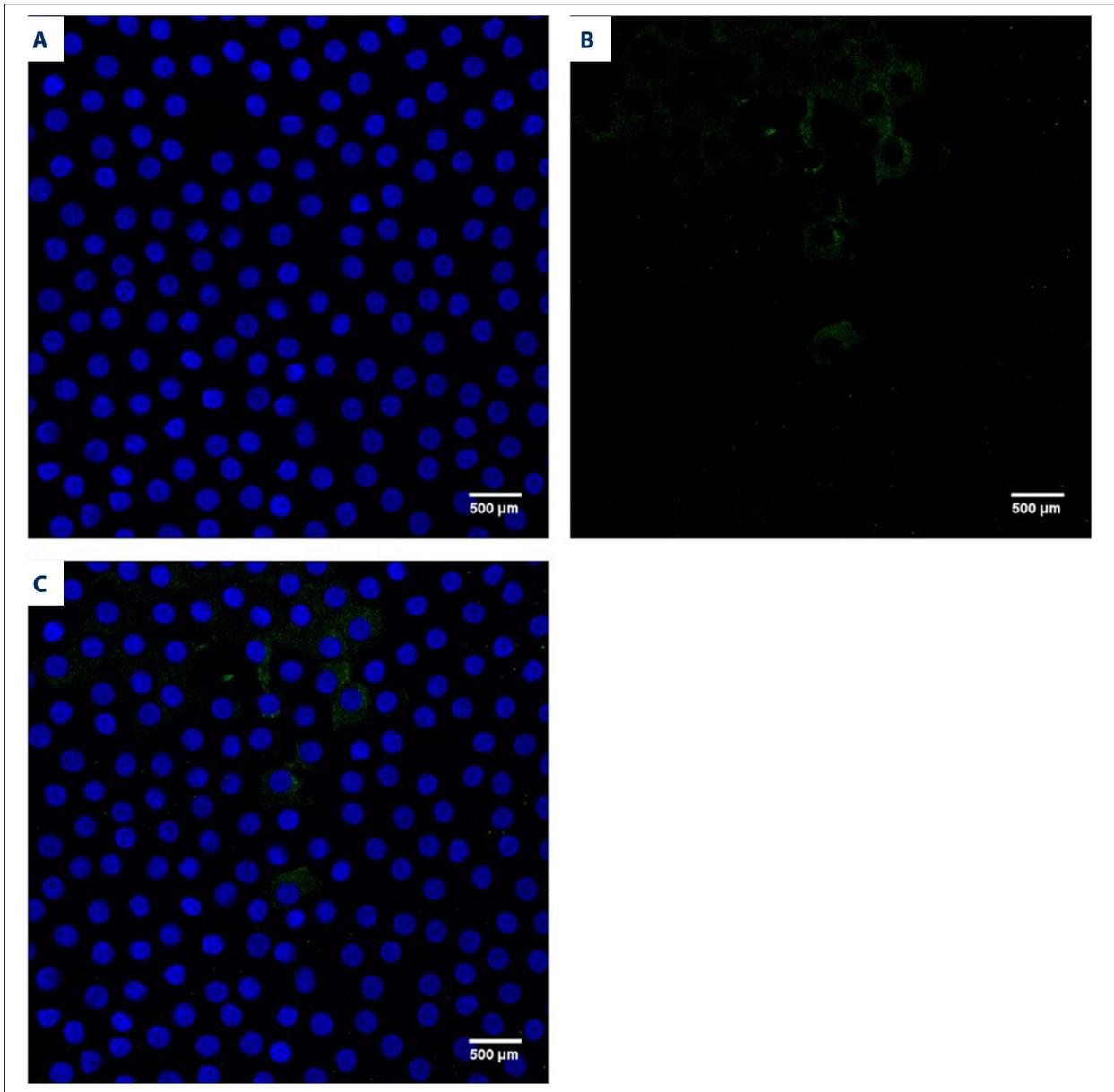


Figure 3. Confocal immunofluorescent image of LECs in Group 1 (age-related cataract group). (A) Blue nuclei visualized using Hoechst stain. (B) Caspase-3 is weakly stained (1+) (green). (C) Merged image shows weak (1+) Caspase-3 expression (original magnification $\times 600$).

of emulsified SO, such as keratopathy [32], glaucoma [33] and retina toxicity [34], have been reported. According to the literature [35], the duration of SO tamponade remains the most significant risk factor for SO emulsification. The mean silicone oil removal time in our cases (22.95 ± 8.90 weeks) was longer than that of a previous study (4.4 ± 1.1 months) [36]. Three patients lost to follow-up with longer duration (52, 35, and 30 weeks) prolonged SO removal time in our cases. Follow-up visits and timely treatment were hampered by poverty and lack of health awareness in these 3 patients, who were all low-income people. More extensive education for patients who undergo

vitreoretinal surgery with SO tamponade is necessary, especially in developing countries.

Our results may have been affected by the fact that this was a non-randomized, small case series in patients with advanced age. Tang et al. [37] found that activation of the unfolded protein response-induced ROS production suppressed the antioxidant status and triggered the apoptotic pathway, ultimately leading to the formation of age-related cataracts. We found that Bcl-2 was expressed weakly in all these cataract cases. It is possible that advanced age was a limitation of our study, but

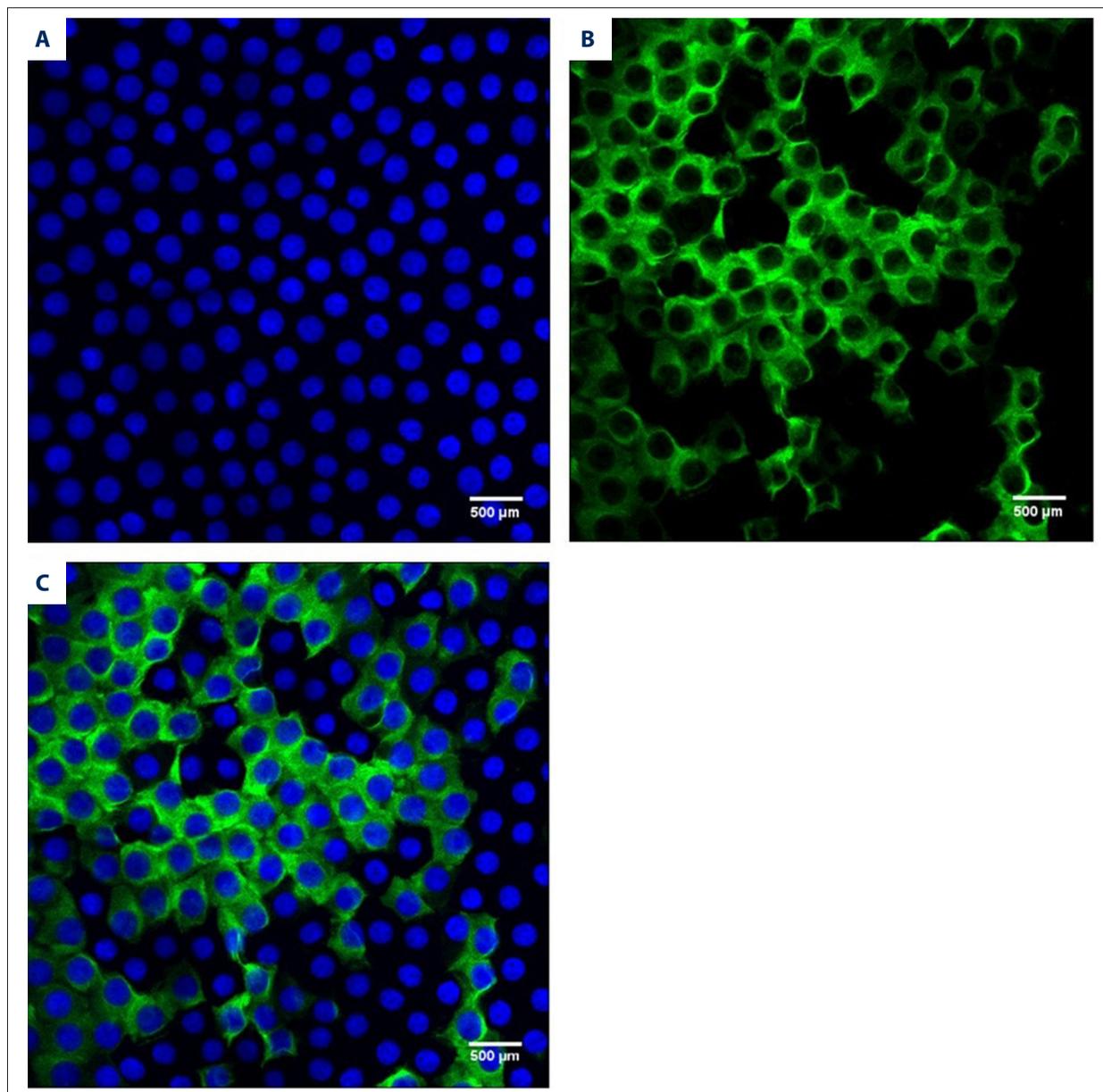


Figure 4. Confocal immunofluorescent image of LECs in Group 2 (cataract with SO endotamponade group). (A) Blue nuclei visualized using Hoechst stain. (B) Caspase-3 is strongly stained (3+) (green). (C) Merged image shows strong (3+) Caspase-3 expression (original magnification $\times 600$).

fortunately there was no significant difference in age between the 2 studied groups ($P > .05$). In our study, particles of silicone oil were observed via gonioscopy and slit-lamp biomicroscopy by the same ophthalmologist. The Coulter counter can detect emulsified SO droplets below $2 \mu\text{m}$ size, which are difficult to see in slit-lamp biomicroscopy [38]. And meanwhile, the study also found a correlation between the number of droplets between 7 and $30 \mu\text{m}$ and the number of droplets less than $2 \mu\text{m}$ in diameter [38]. We speculate that the large visible droplets reflected the extent of SO emulsification. Hence,

we believe that these limitations did not significantly affect the major findings of this study.

In summary, the expression of Bax and Caspase-3 was remarkably higher in cataracts secondary to SO tamponade vs. ARC. These results suggest that caspase-3 dependent apoptosis contributes to complicated cataracts with SO tamponade after vitrectomy, which is regulated by Bax/bal-2.

Table 2. Immunohistochemical scoring for the apoptotic factors Bax, Bcl-2 and Caspase-3 in LECs from two groups.

Score		0	1+	2+	3+	4+
Bax	Group 1 (n)	–	9	10	1	–
	Group 2 (n)	–	–	5	10	5
Bcl-2	Group 1 (n)	–	17	3	–	–
	Group 2 (n)	2	17	1	–	–
Caspase-3	Group 1 (n)	–	13	7	–	–
	Group 2 (n)	–	–	7	9	4

Group 1 – age-related cataract; Group 2 – cataract with SO endotamponade; SO – silicone oil.

Table 3. Immunohistochemical analysis for the apoptotic factors Bax, Bcl-2 and Caspase-3 in LECs from two groups.

Factor	Mean positive cells (%) \pm SD		P Value
	Group 1	Group 2	
Bcl-2	11.80 \pm 9.17	10.05 \pm 7.88	0.616
Bax	25.35 \pm 11.91	63.30 \pm 16.92	<0.0001
Caspase-3	21.15 \pm 10.49	58.10 \pm 19.90	<0.0001

Group 1 – age-related cataract; Group 2 – cataract with SO endotamponade; SO – silicone oil.

Table 4. Emulsification of SO and immunohistochemical scoring for Bcl-2, Bax and Caspase-3 in LECs from patients with and without emulsified SO.

Score		0	1+	2+	3+	4+
Bax	With emulsified SO(n)	–	–	4	2	2
	Without emulsified SO (n)	–	–	1	8	3
Bcl-2	With emulsified SO(n)	1	7	–	–	–
	Without emulsified SO (n)	1	10	1	–	–
Caspase-3	With emulsified SO(n)	–	–	3	4	1
	Without emulsified SO (n)	–	–	4	5	3

SO – silicone oil.

Table 5. Immunohistochemical analysis for the apoptotic factors Bax, Bcl-2 and Caspase-3 in LECs from patients with and without emulsified SO.

Factor	Mean positive cells (%) \pm SD		P Value
	With emulsified SO	Without emulsified SO	
Bcl-2	11.25 \pm 9.19	9.25 \pm 7.20	0.728
Bax	58.88 \pm 22.35	66.25 \pm 12.37	0.354
Caspase-3	58.75 \pm 20.43	57.67 \pm 20.46	0.939

SO – silicone oil.

Conclusions

Although the exact pathogenesis in vitrectomized eyes with SO tamponade is unclear, our results raised the possibility that use of antioxidant would be beneficial for preventing cataractogenesis. Further studies will be required to explore the abnormal signaling in LECs and to investigate the potential

antiapoptotic targets for the development of complicated cataracts in vitrectomized eye with SO tamponade.

Conflict of interest

No conflicting relationship exists for any author.

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