Research Article

Effect of Lignocaine Concentration on Human Fibroblasts Growth in Eyes Undergoing Trabeculectomy: An in vitro Study

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What Is It about?

Observational studies have suggested that subconjunctival local anesthetic may be associated with an increased risk of bleb failure or leakage in trabeculectomy, although there is no definite evidence to support this hypothesis. We performed this in vitro study to determine the effect of varying concentrations of lignocaine on human fibroblasts isolated from the tenon's capsule tissue of the patients undergoing trabeculectomy. The viability of cells decreased with increasing concentration of lignocaine and proliferation inhibition. Our findings suggest that lower concentrations of lignocaine (<1%) which prevent proliferation of fibroblasts without causing apoptosis are likely to be associated with lower rates of bleb failure or leaks. A definite in vivo study is required further to confirm this finding.

Keywords

Lignocaine · Tenon's fibroblast · Trabeculectomy

Abstract

Purpose: To evaluate the effect of lignocaine on growth and apoptosis indication of primary human Tenon's capsule fibroblast (HTFs) in an in vitro model. ***Patients and Methods:*** Tenon's capsule tissue obtained from patients undergoing trabeculectomy were grown in cell culture medium. The effect of different concentrations of lignocaine (0.5, 1.0, 1.5, and 2%) on the morphology and growth of the fibroblasts was studied using microscopy, cell viability, and proliferation assay, and apoptosis was detected using the FITC Annexin V Apoptosis Kit. ***Results:*** Morphological changes similar to those of apoptotic cells, including cytoplasmic vacuolation,

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shrinkage, and rounding were visualized in the cells treated with concentrations greater than 1.0% (i.e., 1.5, 2.0%). Though proliferation inhibition was found with all four concentrations (0.5–2.0%), the viability of cells decreased from 1.0% lignocaine. **Conclusion:** 0.5% lignocaine prevents proliferation of fibroblasts without causing apoptosis in vitro.

Introduction

Glaucoma is a group of diseases in which there is irreversible optic nerve damage. One tenth of all trabeculectomies performed to treat glaucoma fail due to fibrosis of Tenon's capsule. Excess scarring at the site of a filtering bleb is the most common cause of failure after glaucoma filtration surgery [1]. Tenon's fibroblasts are the main effector cells in the initiation and mediation of wound healing and fibrotic scar formation after trabeculectomy [2, 3]. Lignocaine is the main local anesthetic (LA) agent in trabeculectomy. LAs have several effects on wound healing. Two schools of thoughts and arguments exist on the effect of the anesthetic drug on the outcome of surgery. One school of thought believes that it suppresses the growth of fibroblasts and the other says it allows proliferation of the cells. The mechanisms by which LAs cause toxicity are not well understood, although it is known that LAs exert pharmacological actions primarily by inhibiting ionic flux through sodium ion channels of excitable tissues [4]. LAs cause adverse effects on the heart, blood vessels, and immune system [5]. Their effects on neutrophils include decreased chemotaxis, and a delayed onset of the inflammatory response. Reversible and irreversible local toxicity, including cell necrosis, has been observed when these drugs are injected into skeletal muscle [6]. The depressant effects of LAs on multiplication of culture cells have been demonstrated in human fibroblasts, human endothelial cells, human keratinocytes, type II pneumocytes, lung fibroblasts, and corneal epithelial cells [7–9]. The mechanisms by which LAs inhibit growth include mitochondrial damage, caspase activation, inhibition of tyrosine kinase, signaling by lysophosphatidate, and activation of p38 mitogen-activated protein kinase [2]. The adverse effects of lidocaine and other LAs on wound healing remain controversial [10–15]. Wound healing may be retarded due to reduction of mucopolysaccharide synthesis [14, 16]. This effect may be explained by changes in the stability of the cellular membrane and of sodium and calcium conductance [15]. Although some studies have shown that wound healing was impaired by LA, others have not been able to verify deleterious effects. Observational studies have suggested that subconjunctival LAs may be associated with an increased risk of bleb failure or leakage in trabeculectomy, but there is no definite evidence to support this hypothesis. With this background knowledge, the primary aim of our study was to determine the effect of different concentrations of lignocaine (0.5, 1.0, 1.5, and 2.0%) with respect to different time points (1, 2, and 24 h) on the growth of primary fibroblasts from patients' Tenon's capsule tissue. We performed this in vitro study to determine the effect of lignocaine on the growth of human fibroblasts. Thus, we aim to understand the effect of lignocaine on the growth of human fibroblasts and role of this anesthetic drug on glaucoma surgery.

Materials and Methods

Ethics Statement
The study was approved by the Vision Research Foundation Institutional Ethical Committee Board of Sankara Nethralaya, Chennai, India. All of the experiments were performed according to the Declaration of Helsinki. The study adhered to the Association for Research in Vision and Ophthalmology statement on human subjects.

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Establishment of Fibroblast Cell Culture from Tenon's Capsule

Primary human Tenon's capsule fibroblast (HTF) cell lines were established from Tenon's tissue obtained from patients undergoing trabeculectomies ($n = 8$). All the experiments were performed with fibroblasts established from patients between the ages of 23 and 45 years recruited from the ophthalmic clinic who were undergoing a trabeculectomy. The patients were healthy with no known systemic infection at the time of recruitment. Patients gave their written informed consent for the collection of conjunctival tissue specimens. Immediately after collection, the tissue was rinsed in 1× PBS (pH 7.4; product code: TL1101; 154 mM NaCl, 1 mM $KH_2 PO_4$, 5 mM $Na_2 HPO4$, HiMedia Laboratories, Mumbai, India) containing 100 units/ mL penicillin G and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA). The tissue was minced into 2-mm pieces and plated on a T25 tissue culture flask, and 4 mL of fibroblast growth medium (FGM) was added, supplemented with 20% FBS and the antibiotic mix. The tissue was incubated at 37 °C and 5–10% CO_2 until cells grew to cover the entire flask. The explants were monitored for outgrowth of cells periodically through a phase contrast microscope. Fibroblast cultures from each patient were treated separately. When confluence was achieved, cells were subcultured using trypsin-EDTA for dislodging cells and transferred to a tissue culture plate for cell growth and further experiments.

Treatment of Fibroblasts with Lignocaine

To evaluate the influence of lignocaine, its effect on the morphology of the HTF cells was estimated. Fibroblast cells were seeded onto a 6-well tissue culture plate and incubated overnight. The fibroblast monolayer was washed with phosphate-buffered saline (PBS; pH 7.4) and then treated with 0.5, 1.0, 1.5, and 2.0% of lignocaine (Loxicard; Neon Laboratories, India) for 1 and 2 h. Dilutions of lignocaine were prepared by diluting the commercially available stock 2% preservative free lignocaine with balanced salt solution. Control fibroblasts were treated with PBS for the same period. After treatment, cells were observed for morphological changes under a phase contrast microscope.

Characterization of Fibroblasts by Vimentin Staining

Cells were seeded on glass slides (coated with 0.025% sodium metasilicate) and treated with 0.5, 1, 1.5, and 2% lignocaine for 1 and 2 h prior to staining. After treatment, cells were fixed with ice-cold acetone. Cells were stained for vimentin using a primary mouse anti-vimentin antibody, and a secondary anti-mouse antibody labelled with Alexa 488, a derivative of FITC (fluorescein isothiocyanate). Cells were counterstained with DAPI (4′, 6-diamidino-2-phenylindole) for locating the cells and observed under fluorescence microscope.

Cell Viability and Cytotoxicity Assay

Once established, fibroblast cultures were trypsinized and plated in 5 sets of triplicates, one set for control and the others for test concentrations, onto a 96-well micro titer plate (10,000 cells/well) with FGM containing 10% FBS. After overnight incubation or when it reached 70–80% confluence, the medium was replaced with PBS overnight for cell starving. The cells were treated with varying concentrations of lignocaine (0.5–2.0%). The control wells were maintained with PBS. After 2 h of incubation, MTT assay was performed to analyze the cellular viability using MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; thiazolyl blue, Sigma-Aldrich, USA). The viability of cells was assessed at 2 h. After treatment, the cells were rinsed with 1× PBS and incubated with 0.5 mg/mL MTT diluted in complete DMEM for 2 h at 37 °C. After incubation, DMEM with MTT was removed, formazan crystals were solubilized in 100 μL dimethyl sulfoxide (DMSO), and absorbance was read at 570 nm using a 96-well ELISA plate reader (Power Wave HT Microplate Spectrophotometer).

The optical density of the formazan product produced by metabolic activity of the cells is directly proportional to the number of live cells.

Cell Proliferation Assay

The HTF cells were plated in 5 sets of triplicates, one set for control and the others for test concentrations, onto a 96-well micro titer plate (10,000 cells/well) with FGM containing 10% FBS. After overnight incubation or when it reached 70–80% confluence, the medium was replaced with PBS overnight for cell starving. The cells were treated with varying concentrations of lignocaine (0.5–2.0%) at two time intervals (1 and 2 h). Proliferation of cells on response to lignocaine as well in controls were estimated using proliferation ELISA, BrdU Kit (Roche Applied Science) after 1 and 2 h as per the manufacturer's instructions. The optical density was measured at 370 nm using a 96-well ELISA phase reader (Power Wave HT Microplate Spectrophotometer).

Apoptosis Assay for Detection of Apoptotic Nuclei

The cells were grown on 11-mm coverslips in either 35-mm dishes on 12-well plates and treated with 0.5–2.0% of lignocaine for 2 h. At the end of treatment, the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min. The apoptotic nuclei were detected by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) assay by using a commercial kit (TACS® 2 TdT-Fluor In Situ Apoptosis Detection Kit) according to the manufacturer's protocol. In brief, the paraformaldehyde fixed cells were rinsed with PBS for 10 min, followed by incubation with proteinase K (1 mg/mL) for 15 min at room temperature. The cells were incubated with the reaction mix for 60 min in a humidity chamber at 37°C followed by Strep-Fluor labeling. The cover slips were mounted on to a glass slide and fluorescent images were captured by a Zeiss Axiovert microscope at 20× magnification.

Detection of Stages of Apoptosis in Treated Cells

Fluorescence-activated cell sorting, applied in flow cytometry, was performed using the FITC Annexin V Apoptosis Detection Kit II (BD Pharmingen™). The cells were grown in 6-well tissue culture plates and treated with 0.5–2.0% of lignocaine for 2 h. After 2 h of treatment the cells were washed with PBS. The cells were then spun at 2,000 rpm for 5 min and then resuspended in 200 μL of binding buffer. 2.5 μL of propidium iodide (PI) and 3 μL of Annexin V were added to the cells, and the cells were then incubated at 4°C 1 h in the dark. The cells were washed twice with 1× binding buffer and then analyzed by flow cytometry.
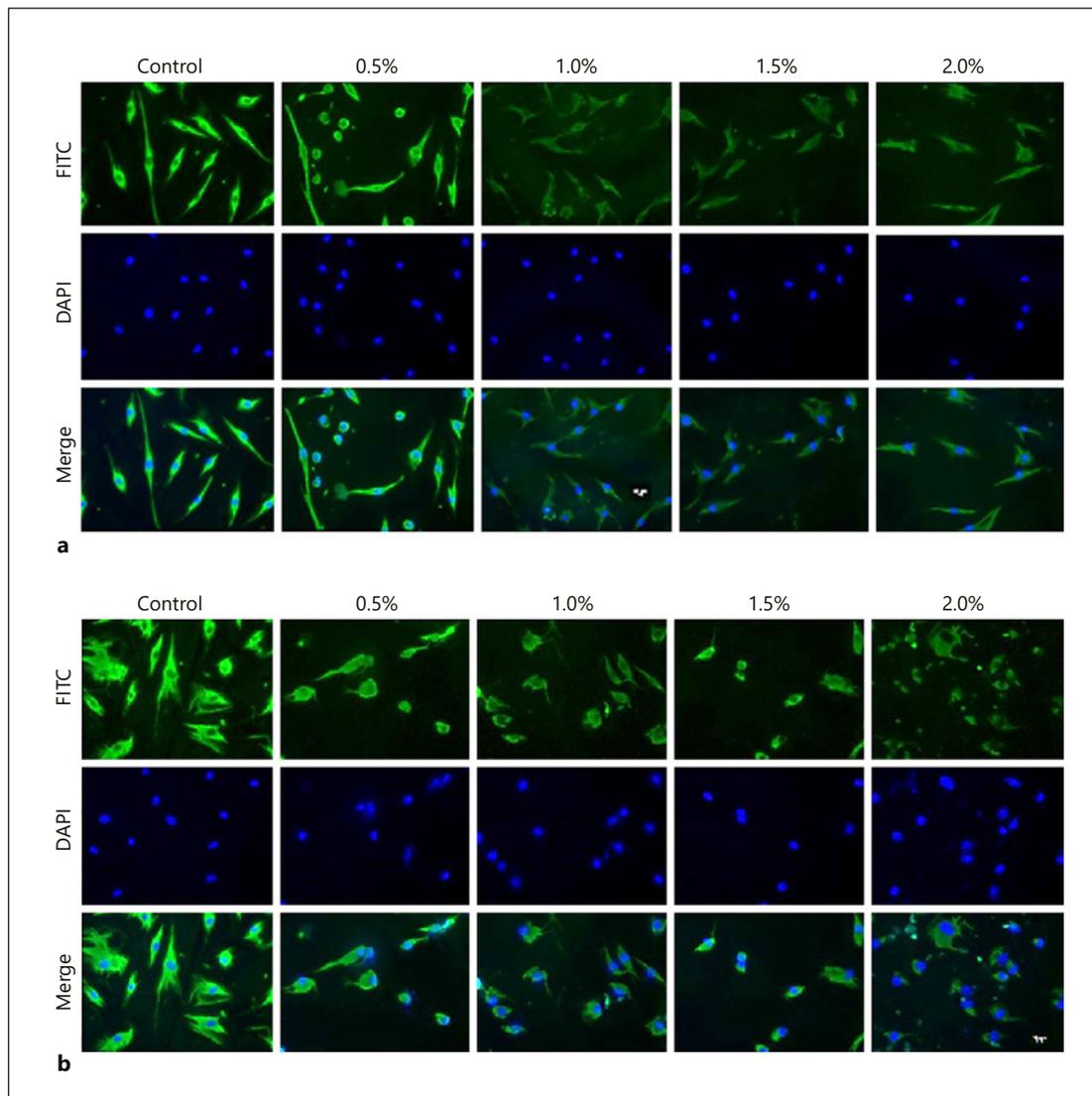
Statistical Analysis

All the experiments were performed three times independently unless otherwise stated. The Student t test was performed with variance as the standard error mean (SEM), and a statistical p value <0.05 was considered significant.

Results

Lignocaine at Higher Concentrations Disrupted Morphology of Fibroblasts

Once the cells formed a full sheath, the cells were initially tested for the expression of fibroblast marker using antibody targeting vimentin. The cells used for the experiments were from the 2nd to the 5th passage, and each time the expression of vimentin was confirmed with immunofluorescence staining (Fig. 1). After fibroblast cells were treated with lignocaine at different doses, it was found that the cells showed intolerance. The cells treated with a higher

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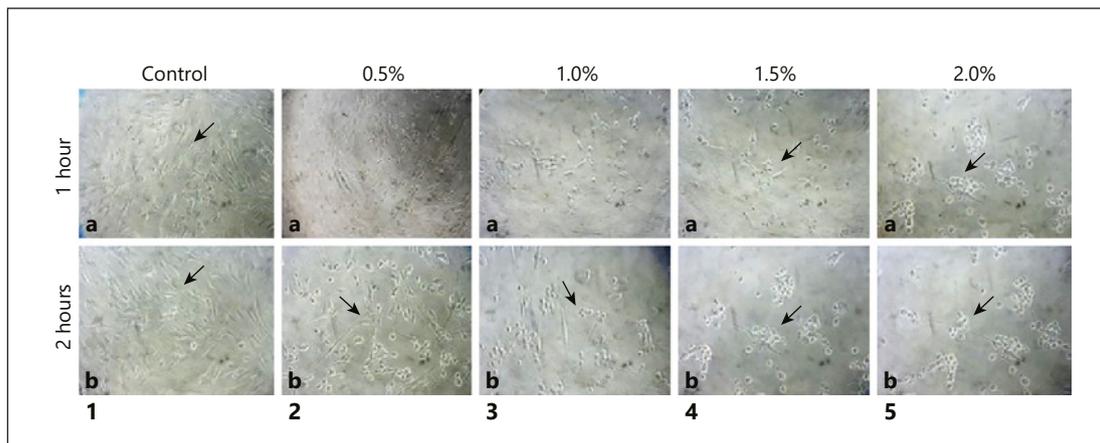Undergoing Trabeculectomy: An in vitro Study

Fig. 1. Immunofluorescence staining of vimentin and nucleus on fibroblast cells treated with 0.5, 1.0, 1.5, and 2.0% lignocaine at two different time intervals. **a** At the 1-hour time point, vimentin fluorescent intensity decreased in the 1.0, 1.5, and 2.0% lignocaine-treated cells compared to the untreated control. **b** At the 2-hour time point, the fluorescent signal for all four concentrations of lignocaine-treated cells was similar and decreased to that of the control cells. Data are representative of three independent experiments.

dosage of lignocaine exhibited morphological changes similar to those of apoptotic cells, including cytoplasmic vacuolation, cellular shrinkage, turning round, cell death, and loss of vimentin expression. The extent of morphological changes of fibroblast cells exposed to lignocaine was dose dependent, indicating that lignocaine induces toxicity on HTF at higher doses (Fig. 2).

Lignocaine at Higher Concentrations Decreased the Viability of Fibroblast Cells
MTT assay for cells treated with various concentrations of drugs gave an increasing trend in toxicity levels of cells as the concentration of anesthetic drugs increased. This was inferred by calculating the percentage viability using the optical density data obtained for cell control

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Fig. 2. Morphology of cells after treatment with lignocaine at varying concentrations (0.5–2.0%) at two different time intervals (1 and 2 h): arrows point toward individual cells. **1a**, **b** Healthy untreated fibroblasts, spindle in shape and are present in a layer. **2a**, **b** Change in cellular morphology, and gaps in uniform layer. **3a**, **b** Cytoplasmic vacuolation and rounding of cells. **4a**, **b** Cellular shrinkage, cells turning round. **5a**, **b** Cells rounded and plaques due to decrease in number of cells.
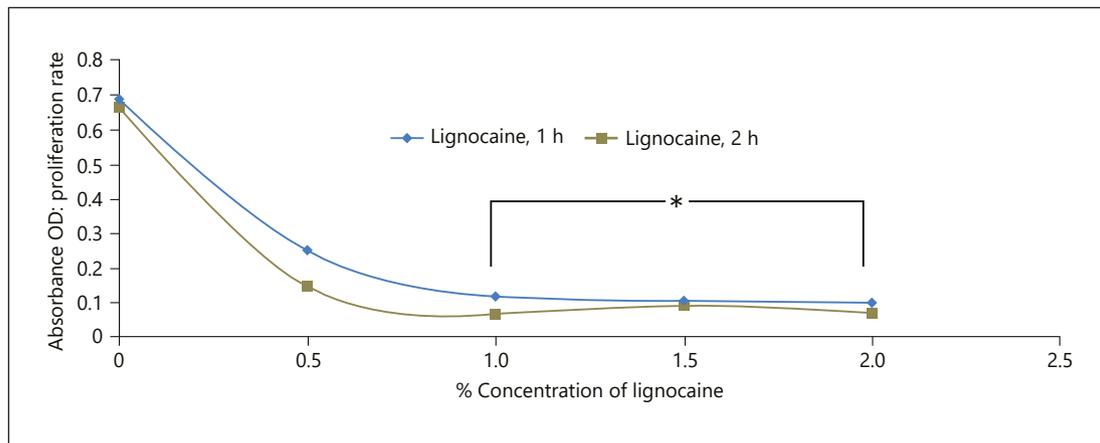

Fig. 3. Percentage viability of the cells upon 2 h of treatment with 0.5, 1.0, 1.5, and 2.0% lignocaine. Dose-dependent effect of lignocaine on viability was determined by MTT assay at 2 h with various concentrations. Data represent means ± SD of three independent experiments (* $p < 0.05$; ** $p < 0.01$).
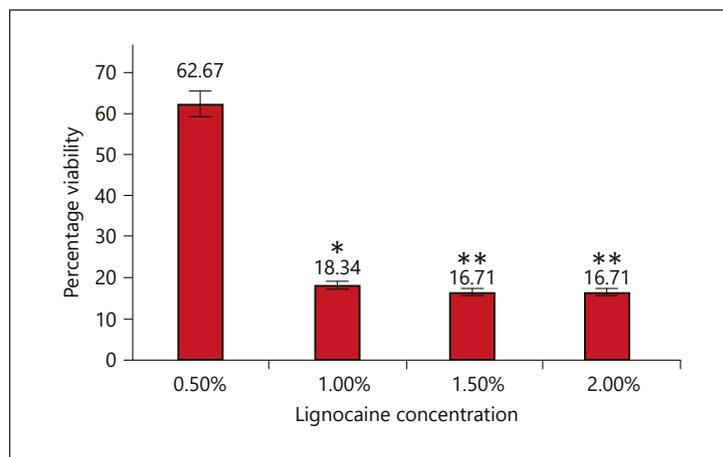
and cells treated with various concentrations of drugs. Cells treated with 0.5% lignocaine showed 62.67% viability (MTT). Treatment with higher doses of lignocaine, 1.0, 1.5 and 2.0%, showed cytotoxicity with viability of 18.31, 16.71, and 16.71%, respectively (Fig. 3).

Proliferation Inhibition of HTFs by Lignocaine

The effect of lignocaine on fibroblast proliferation was measured with a 5-bromo-2-deoxyuridine (BrdU) assay that quantitates BrdU uptake into newly synthesized DNA of replicating cells. A statistically significant decrease in fibroblast proliferation was observed with increasing concentration of lignocaine treatment. Proliferation rates were significantly reduced measured using BrdU incorporation on cells upon treatment with an increased time interval of lignocaine treatment (2 h). This demonstrated significant inhibition at 1 and 2 h with lignocaine with increasing concentration compared with control ($p < 0.05$; Fig. 4).

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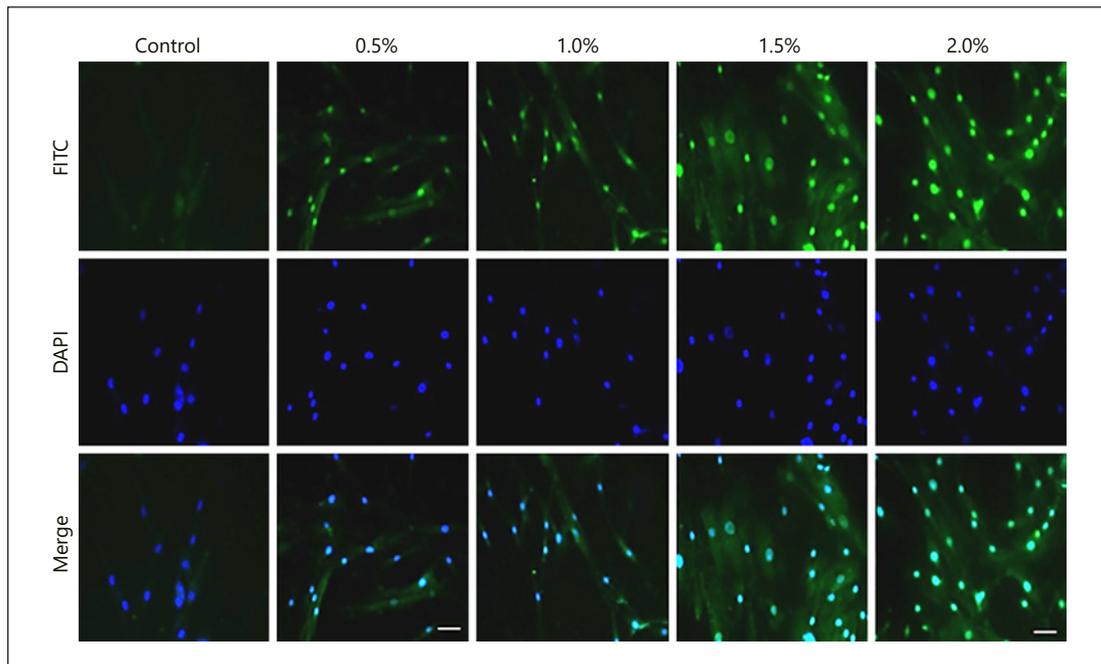Undergoing Trabeculectomy: An in vitro Study

Fig. 4. HTF proliferation as measured with BrdU assay. Significant inhibition at 1 and 2 h with increasing concentrations of lignocaine, in relation to control group. Values plotted are means ± SD ($n = 3$). * $p < 0.05$ with respect to untreated cells of three independent experiments.

*Apoptotic Effect of Lignocaine on HTFs*

Exposing HTFs to lignocaine led to a time- and dose-dependent increase in the number of dead cells, as assessed by MTT assay (Fig. 2). A single exposure to lignocaine at the concentration of 1.0–2.0% resulted in a statistically significant effect on cell death. Next, to determine the nature of the cytotoxicity of lignocaine in HTFs, the phenotypic characteristics of apoptosis were examined. Cells were treated with 0.5–2.0% lignocaine for 1 and 2 h. Then the apoptotic nuclei was detected by TUNEL assay and visualized under a fluorescence microscope (Fig. 5a, b). Apoptotic cells were detected at 2 h of exposure to lignocaine (Fig. 5). The expression of apoptotic nuclei was increased with increasing concentration of lignocaine. To analyze the apoptosis further, FITC Annexin V/PI staining was performed to detect the externalization of membrane phospholipid phosphatidylserine (PS) and the loss of membrane integrity. Upon 2 h of treatment of different concentrations of lignocaine with HTF, the viability of cells was found to be not reduced with a lower concentration (0.5%) of lignocaine (Fig. 6a), while cells treated with higher concentrations of lignocaine (1.0 and 1.5%) underwent apoptosis at a later stage, with Annexin V staining showing the loss of membrane integrity (Fig. 6b, c). 8% of cells treated with 2.0% lignocaine were gated in necrosis, with induced apoptosis resulting in a reduction in the number of viable cells (Fig. 6d).

Discussion

In the present study, we demonstrated that lignocaine at higher concentrations induces cytotoxicity of HTF through a significant reduction in fibroblast viability, inhibition of HTF proliferation, induction of cell death, and apoptosis in vitro. Excessive postoperative scarring is the most common cause of failed glaucoma filtration surgery [17]. Intraoperative MMC and 5-FU are clinically used to inhibit fibrosis and improve surgical outcome [18]. These chemotherapeutic agents increase the success of glaucoma-filtering surgery by preventing fibroblast proliferation and excessive scar formation [19–22]. It has been suggested that the antiproliferative effects of these agents are mediated by activation of the apoptosis-signaling pathway in conjunctival fibroblasts [23]. However, despite their use, a significant failure rate persists, with an associated increase in other postoperative complications.

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Fig. 5. Lignocaine-induced apoptosis and cell death. HTFs were exposed to 0.5, 1.0, 1.5, and 2.0% of lignocaine for 2 h. TUNEL assay was performed to detect apoptosis. Cells stained positive for apoptotic nuclei at all concentrations of treatment, with an increasing pattern in intensity coinciding with increased drug concentration, which was absent in the control cells. Data are representative of three independent experiments. Scale bar = 50 μm.
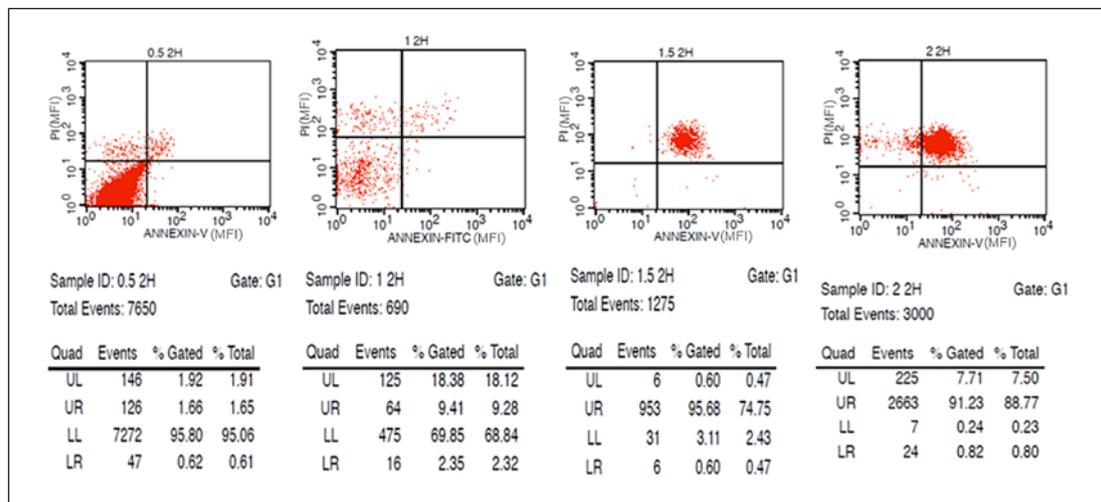
Fig. 6. Flow cytometry analysis of fibroblast cells treated with 0.5–2.0% lignocaine for 2 h in relation to control group. Lignocaine-treated cells were examined for apoptotic cells using an Annexin V-FITC apoptosis detection kit. LL: both Annexin V- and PI-negative cells were viable cells. LR: Annexin V-positive/PI-negative cells were in early stages of apoptosis. UR: Double-positive cells were in late apoptosis. UL: Annexin V-negative/PI-positive cells were necrotic. Upon 2 h of treatment of different concentrations of lignocaine with HTF, the viability of the cells was found not to be reduced with a lower concentration (0.5%) of lignocaine (**a**), while cells treated with higher concentrations of lignocaine (1.0 and 1.5%) underwent apoptosis at a later stage with Annexin V staining showing the loss of membrane integrity (**b**, **c**). 8% of cells treated with 2.0% lignocaine were gated in necrosis, with induced apoptosis resulting in reduction in number of viable cells (**d**).

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Understanding the basic mechanisms that underlie apoptosis will point to potentially new targets of therapeutic treatment of disease that shows an imbalance between cell proliferation and cell loss. Microscopic observation of HTF cells, treated with lignocaine at doses above 1.0%, showed dramatic morphological changes such as cytoplasmic vacuolation, cellular shrinkage, detachment, and cell death with time and concentration, which were similar to those of apoptotic cells [24]. This implies that lignocaine might have an apoptosis-inducing effect on HTF cells. To validate the apoptosis-inducing hypothesis, loss of membrane integrity of HTF cells was then examined by vimentin staining since loss of membrane integrity is one of the characteristic features of apoptotic cells [25]. Vimentin staining revealed an inverse relationship with intensity of fluorescence to concentration of drug. This may be due to caspase-mediated degradation of vimentin in apoptotic cells and is expected as cytoskeleton degradation [5]. To verify this, MTT and TUNEL assays were performed on cells treated with lignocaine. A drastic decrease in viability was observed in HTF cells after treatment with lignocaine at doses above 0.5% in a dose-dependent manner by MTT assay. DNA fragmentation is one of the most remarkable features of apoptotic cells [26]. HTF cells were treated with lignocaine to identify nuclear DNA fragmentation prior to the detection of an apoptotic peak. Endogenous endonuclease is activated by apoptotic cells and is responsible for the fragmentation of nDNA. This activation of endogenous endonuclease can be visualized on cells using the in situ nick translational assays. In this study, expression of apoptotic nuclei increased with increasing concentration of the drug in a dose-dependent manner. Thus, we postulate that lignocaine probably has an apoptosis-inducing effect on HTF cells.

Loss of plasma membrane is one of the earliest features of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35- to 36-kDa Ca^{2+}-dependent phospholipid-binding protein with a high affinity for PS and binds to exposed apoptotic cell surface PS [26]. PS translocation precedes the loss of membrane integrity, which accompanies the later stages of cell death resulting from either an apoptotic or necrosis process when used in conjunction with vital dye such as PI [22, 27]. In the current study, HTF treated with a lower concentration (0.5%) of lignocaine did not disrupt the viability or morphology in a major way. Higher concentrations of lignocaine induced apoptosis and necrosis at a later stage in HFT.

We conclude that lignocaine has an apoptosis-inducing effect on HTF cells in a dose- and time-dependent manner. At higher concentrations (≥1.0%) of lignocaine, apoptotic changes were seen and a noncytotoxic inhibitory effect was observed only with 0.5% lignocaine. 0.5% lignocaine prevented proliferation of fibroblasts without causing apoptosis and this solution is likely to be associated with lower rates of bleb failure or leaks. Whether this data is relevant to actual clinical application of lignocaine or not needs to be further investigated in an in vivo study.

The study does have some limitations, which include the effect of lignocaine was not compared with other anesthetic drugs that are currently in use and the apoptosis and proliferation assays were not measured with varying time periods since the cells were not able to withstand longer exposure. Therefore, we confined all experiments to 1 and 2 h.

Further studies elucidating the apoptosis-inducing mechanism of lignocaine in HTF cells would be intriguing.

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Disclosure Statement

The authors declare no conflicts of interest.

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