European Journal of Ophthalmology / Vol. 16 no. 1, 2006 / pp. 67-72

Effects of latanoprost and GLC756, a novel dopamine D_2 agonist and D_1 antagonist, on cultured normal human dermal fibroblasts

U.W. LAENGLE[1], R. MARKSTEIN[2], D. PRALET[1], B. GREINER[1], D. ROMAN[1]

[1]Department of Toxicology/Pathology
[2]Department of Ophthalmics, Novartis Pharma AG, Basel - Switzerland

PURPOSE. *Proliferation of subconjunctival fibroblasts plays a critical role in scarring and failure of glaucoma filtering surgery. Long-term topical glaucoma medications appear to increase fibroblast proliferation. In this study, the effects of topical antiglaucoma drugs latanoprost and GLC756, a novel dopamine D_2 agonist and D_1 antagonist, on cultured normal human dermal fibroblasts (NHDF) were examined.*
METHODS. *The NHDF cell line was incubated with latanoprost, prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$), GLC756, or 5-fluorouracil as a positive control at concentrations of 3 and 30 µM for 6, 18, and 24 hours. Fibroblast growth was measured by 5-bromodeoxyuridine (BrdU) uptake using laser scanning cytometry.*
RESULTS. *Latanoprost and $PGF_{2\alpha}$ had a biphasic response on the number of cultured NHDF positively stained with BrdU. A stimulating effect on proliferation occurred early, 6 hours after incubation, and an inhibitory effect 18 to 24 hours after incubation. GLC756, in contrast, revealed only inhibitory effects on BrdU uptake 18 to 24 hours after incubation. The pattern of GLC756 was similar to that of the positive control 5-fluorouracil.*
CONCLUSIONS. *Latanoprost seemed to have a biphasic response on the proliferation of cultured NHDF. First there was a stimulating thereafter a secondary negative modulating effect. GLC756 had a fully antiproliferative effect on the NHDF, indicating an additional potential of novel dopamine compounds for topical glaucoma medication. (Eur J Ophthalmol 2006; 16: 67-72)*

KEY WORDS. *D_2 agonist, D_1 antagonist,* In vitro *model, Latanoprost, Normal human dermal fibroblasts, Topical glaucoma medication*

Accepted: July 18, 2005

INTRODUCTION

Glaucoma designates a group of chronic eye diseases that are characterized by progressive atrophy of the optic nerve head and visual field loss and, in most cases, by elevation of intraocular pressure (IOP). The etiology of glaucoma is still unknown but elevated IOP and impaired perfusion of the optic nerve have been suggested to be important risk factors. Current medical treatment of glaucoma is focused on lowering IOP. Latanoprost, a prostaglandin$F_{2\alpha}$ ($PGF_{2\alpha}$) analogue, belongs to a new class of antiglaucoma drugs with IOP lowering properties (1, 2). Dopaminergic drugs are another class of compounds that lower IOP (3). In addition they can increase optic nerve blood flow (4) and may therefore be of particular value for the treatment of glaucoma (5-7). In some glaucoma patients who do not respond well to conventional antiglaucoma drugs, filtration surgery is an available alternative treatment. However, long-term topical medication with conventional antiglaucoma drugs has been suggested to have negative effects on the success of filtration surgery (8). A possible reason is that long-term treatment with some antiglaucoma drugs promotes the proliferation of fi-

 1120-6721/67-06$15.00/0

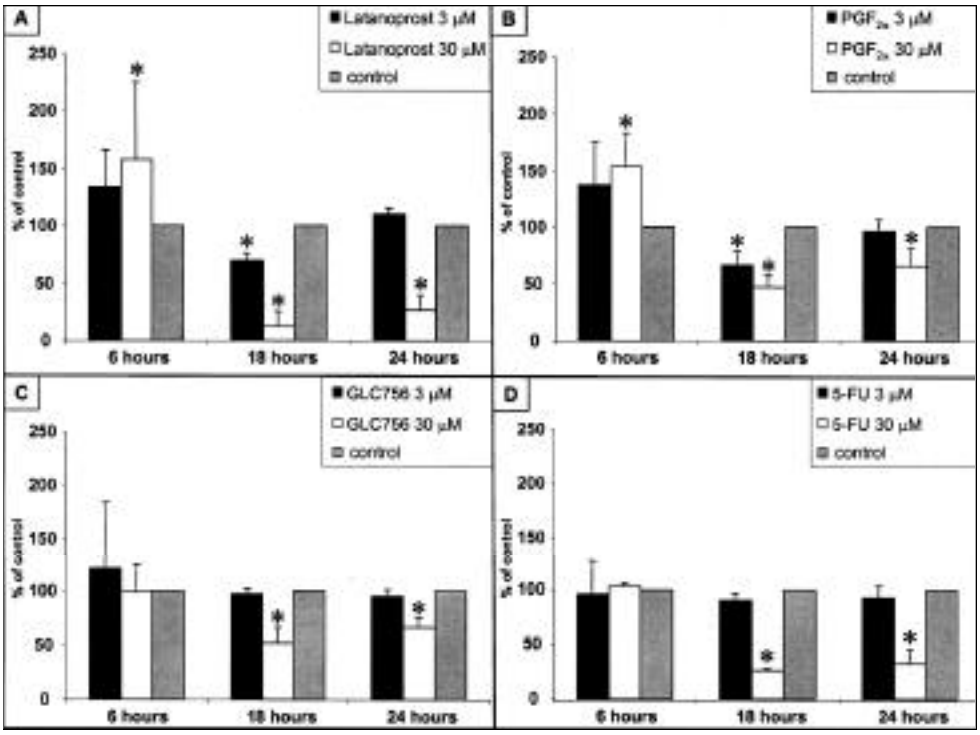

Fig. 1 - *BrdU uptake (% of control) of cultured normal human dermal fibroblasts incubated with latanoprost* **(A)**, *$PGF_{2\alpha}$* **(B)**, *GLC 756* **(C)**, *or 5-fluorouracil* **(D)** *for 6 to 24 hours. Each column is a mean of three values and the bars indicate SD. Each time point had a corresponding control group which was regarded as 100%. Values were considered significantly different from control at *p<0.05.*

broblasts and induces collagen deposition in the subconjunctival space which may result in scar formation, the most common cause of filtering surgery failure (9-12). Clinical observations indicated an association between decreased success rates for trabeculectomy and the duration of antiglaucoma medications (13, 14). Furthermore, exposure of rabbit conjunctival fibroblasts to timolol, epinephrine, or pilocarpine in vivo (15) was found to increase fibroblast cell count. Also an in vivo study demonstrated proliferation of subconjunctival fibroblasts after topical treatment with 0.005% latanoprost or 0.2% brimonidine (16). The direct effects of drugs used in the treatment of glaucoma on fibroblast growth have been investigated in various *in vitro* studies (17-20).

Interestingly, dopaminergic compounds have been reported to prevent proliferation of fibroblasts (21). The objective of this study was therefore to investigate the effects of latanoprost and GLC756, a novel dopaminergic compound, on proliferation of normal human dermal fibroblasts (NHDF) in vitro as well as to explore whether the use of dopaminergic compounds in glaucoma treatment may offer an advantage over presently used antiglaucoma drugs.

METHODS

Drug preparation

Latanoprost was commercially available from Cayman Chemical (Ann Arbor, MI, USA) as a solution in methyl acetate, purity ≥98%. The compound was used as a solution in ethanol after evaporation of the methyl acetate under a stream of nitrogen. $PGF_{2\alpha}$, as a solution in ethanol, was purchased from Fluka, Switzerland; purity 99%. GLC756, as a powder, received from Novartis Pharma AG (Switzerland), was reported to be 100% pure by certificate of analysis. The drug was dissolved in DMSO (Sigma, Switzerland). 5-fluorouracil (5-FU) was purchased from ICN Pharmaceuticals, Switzerland, in 5 mL ampoules of 250 mg each and dissolved in the Fibroblast Growth Medium-2 Bullekit® (FGM, Bio Whittaker, Belgium).

NHDF cell culture and treatment

NHDF were obtained from Bio Whittaker (Belgium) and cultured in 75 mL flasks (T-75, Costar) in FGM. Before each experiment, medium was discarded from

the cultures, and cells were washed with HEPES buffered saline solution (ReagentPack™, Bio Whittaker, Belgium), incubated in trypsin/EDTA solution (ReagentPack™) for 1.5 minutes at room temperature, centrifuged at 1300 rpm for 5 minutes after addition of FGM, resuspended in trypsin neutralizing solution (ReagentPack™), and recentrifuged. The supernatant was discarded and cells were resuspended in FGM. Cells were incubated in chamber slides (treated glass slides, Becton Dickinson, Switzerland) and the compounds were added at concentrations of 3 and 30 µM for 6, 18, and 24 hours.

To reach the end concentrations serial dilutions of each drug were carried out. The same cell line with the same number of passages under identical experimental conditions was used in three independent assays. The control group received culture medium or culture medium plus corresponding vehicle DMSO or ethanol at 0.1%.

Cell proliferation assay

Cell proliferation was quantified by 5-bromodeoxyuridine (BrdU) uptake into DNA. BrdU (*In situ* Cell Proliferation Kit, FLUOS; Roche, Switzerland) was added at 1/1000 to the medium 1.5 hours before the end of treatment. At the end of the incubation time, medium was removed and slides were allowed to dry and were kept at –70°C until immunofluorescence staining and analysis.

Detection of BrdU incorporation by immunofluorescence

Detection of BrdU was performed as follows: cells were fixed with 70% ethanol for 10 minutes, incubated with HCl 4N for 20 minutes at room temperature, washed once in distilled water, neutralized in sodium tetraborate (Merck, Switzerland) 0.1 M for 1 minute and finally washed once for 5 minutes in phosphate-bufferedsaline (PBS, Gibco, Switzerland). The slides were then incubated with Alexa 488-conjugated anti-BrdU antibody (Molecular Probes, Switzerland) at 1/100 in the buffer from the *in situ* proliferation kit (*In situ* Cell Proliferation Kit, FLUOS; Roche, Switzerland) for 2 hours at room temperature. After two washing steps in PBS, propidium iodide (PI; Sigma, Switzerland) at 10 µg/mL in PBS was added for 30 minutes at room temperature. The slides were then briefly washed in PBS and coverslips were applied using GelMount (Biomeda, Switzerland), followed by analysis using Laser Scanning Cytometry (LSC).

LSC evaluation

The fluorescence emission of the stained cells was measured on the laser scanning cytometer interfaced to a Compaq computer equipped with the WinCyte 3.6 software (Compucyte, Cambridge, MA, USA). A detailed description of the LSC methodology has been previously published (22). The scan area was adjusted to the entire coverslip area and all cells in each chamber were acquired. Slides were scanned using a 20x objective. For the purpose of this study, the contouring parameter was PI fluorescence and cells expressing BrdU were gated on green max pixel fluorescence. Overlapping nuclei were automatically excluded from counting by special statistical filters.

Statistical analysis

Log-transformed data were analyzed with one-way analysis of variance. The control group, which received only culture medium or culture medium plus corresponding vehicle DMSO or ethanol, was regarded as 100%. Each group was compared to the control group using linear contrasts. Values were considered significantly different from control at $p<0.05$, with no correction for multiplicity.

RESULTS

NHDF incubated either with DMSO at 0.1% in FGM or ethanol at 0.1% in FGM showed a BrdU uptake similar to that of NHDF cultured in FGM alone for 6 to 24 hours (not shown). Furthermore, there was no cytotoxicity seen on NHDF at any concentration with any compound tested in this study.

Latanoprost induced a marked dose-related increase of the BrdU uptake at 3 and 30 µM already 6 hours after incubation when compared to the vehicle control ethanol, which reached statistical significance at the high-dose level. After 18 hours of incubation, the early increase in the BrdU uptake changed into a dose-related statistically significant decrease at 3 and 30 µM. At 24 hours, a decrease, statistically signifi-

cant, of the BrdU uptake could still be observed at the 30 µM concentration (Fig. 1A).

$PGF_{2\alpha}$ had a pattern in the BrdU uptake similar to that of latanoprost. For both concentrations there was also an early dose-related increase in the BrdU uptake, 6 hours after incubation, which also reached statistical significance at the high-dose level. As was the case for latanoprost, there was as well a statistically significant drop below the vehicle control ethanol after 18 hours of incubation at 3 and 30 µM. After 24 hours, a statistically significant decrease in the BrdU uptake was still present at 30 µM, whereas at 3 µM a BrdU uptake could be measured similar to that of the corresponding control (Fig. 1B).

GLC756 revealed a statistically significant decrease of the BrdU uptake 18 and 24 hours after incubation at a concentration of 30 µM. In comparison, at 3 µM BrdU uptake was similar to that of the vehicle control DMSO. At the early time point of 6 hours, both the low and high concentration of GLC756 showed no difference in BrdU uptake compared to that of the corresponding control (Fig. 1C).

The positive control 5-FU exhibited a pattern of BrdU uptake similar to that of GLC756 at 6 to 24 hours. There was also a statistically significant decrease of the BrdU uptake 18 and 24 hours after incubation only at the high concentration of 30 µM. At the early time point of 6 hours, there was also no difference in BrdU uptake for the low and high dose of 5-FU compared to the corresponding control (Fig. 1D).

DISCUSSION

Previous in vivo and in vitro studies suggested that conventional glaucoma medication increases the number of fibroblasts in the subconjunctival space (16, 20). In addition, increased numbers of inflammatory cells were found in humans who have been treated with antiglaucoma drugs (8, 23, 24). Both effects may contribute to the failure of glaucoma filtering surgery (8). It is unclear whether the proliferation of the fibroblasts is a direct effect of the medication, or mediated by mediators of inflammatory response (16). To address this question, we performed an in vitro study with NHDF.

In this study, latanoprost, $PGF_{2\alpha}$, and GLC756, a novel dopamine D_2 receptor agonist and D_1 receptor antagonist, were assessed for their direct effect on the growth of NHDF. BrdU was used as an indicator of proliferating fibroblast cells (25). The effects of the antiglaucoma drugs were compared to the antiproliferative effect of 5-FU, a cytostatic drug that has been used as an adjunctive treatment to improve the results of glaucoma filtering surgery (26, 27).

Latanoprost induced a dose-related increase of proliferation of the cultured fibroblasts already 6 hours after incubation. After 6 hours, the mean percentage of cells stained with BrdU was about 34% and 58% higher for the 3 and 30 µM concentrations, respectively. The increase was statistically significant at the high-dose level of 30 µM, representing a drug concentration about four times lower than in commercially available 0.005% latanoprost eye drops. The same effect was confirmed with $PGF_{2\alpha}$, where a mean increase of BrdU positive cells of about 38% and 54% for the 3 and 30 µM concentrations, respectively, was observed 6 hours after incubation when compared to the control. The increase was also statistically significant at the high-dose level of 30 µM. This stimulating effect on fibroblasts after exposure to latanoprost might explain the results of an in vivo study in rabbits where topical treatment of 0.005% latanoprost, twice daily, caused a proliferation of subconjunctival fibroblasts during a relatively short treatment period of 10 weeks (16).

Based on this in vivo finding, it might be of no relevance for the in vivo situation that latanoprost had after the initial proliferating effect a relatively high inhibitory effect on BrdU uptake as shown in our study. Therefore, it seems that the histopathologic manifestation observed in the in vivo study might be due to an initial proliferating effect of latanoprost on fibroblasts. A positive and negative modulation of fibroblast proliferation can be seen during wound healing and might be due, in part, to the high prostaglandin concentration of the inflammatory exudates (28). Generally, it was described that prostaglandins exert in vitro a balanced effect on fibroblast growth with a stimulatory action of $PGF_{2\alpha}$ (29) and a negative action of PGE_2 (30, 31). Various types of fibroblasts secrete in vitro different prostaglandins (32, 33). It was also described that exogenous addition of $PGF_{2\alpha}$ significantly enhanced the production of PGE_2 (28). Therefore, in the present study, one can assume that the exogenous addition of $PGF_{2\alpha}$ has a primary stimulating effect on fibrob-

last proliferation and that an induction of endogenous prostaglandin synthesis (e.g., PGE_2, at least in part) may lead to a secondary negative modulation of the fibroblast proliferation. In addition to the induction of fibroblast proliferation, latanoprost is suggested to play a role in the stimulation of inflammatory cells and pathways (16). In fact, there is evidence linking uveitis and cystoid macular edema to latanoprost exposure (34).

GLC756, a novel dopamine D_2 agonist and D_1 antagonist, appears to exert an antiproliferative effect on NHDF growth.

A clear dose relationship was observed and the statistically significant effect occurred after 18 and 24 hours of incubation. In contrast to latanoprost, GLC756 had no stimulating effect on fibroblast growth at the early phase of incubation. The results obtained in this study are in accordance with previous findings (21) where antiproliferative effects were detected when cultured human tendon fibroblast cells were treated with several dopamine antagonists such as domperidone, haloperidol, moperone, and loxapine and the dopamine agonist bromocriptine. However, the mechanism of action of these dopaminergic effects is, to our knowledge, unknown.

It is of interest to compare the antiproliferative effect obtained after GLC756 treatment with that obtained using 5-FU, an antimetabolic drug which has been used to minimize scarring of filtering blebs by inhibiting the proliferation of wound fibroblast cells (35, 36). In the present study, 5-FU showed in general a similar pattern on fibroblast growth during the incubation periods of 6 to 24 hours, however, with a higher antiproliferative effect at 18 and 24 hours.

In summary, in our in vitro study on cultured NHDF, latanoprost seemed to have a biphasic response on proliferation of fibroblasts.

First there was a direct stimulating, thereafter a negative modulating effect on fibroblast growth. GLC756, a novel dopaminergic compound, showed only antiproliferative effect on fibroblast growth, indicating an additional potential of novel dopamine compounds for topical glaucoma medication.

ACKNOWLEDGEMENTS

The authors thank Dr. Wolfgang Seewald, Novartis Animal Health Inc. Development, for the statistical evaluation of the data.

Supported by Novartis Pharma AG, Basel, Switzerland

Reprint requests to:
D. Roman, MD
Novartis Pharma AG
Department of Toxicology/Pathology
MUT-2881.133
CH-4002 Basel, Switzerland
danielle.roman@novartis.com

REFERENCES

1. Ziai N, Dolan JW, Kacere RD, Brubaker RF. The effects on aqueous dynamics of PhXA41, a new prostaglandin F2 alpha analogue, after topical application in normal and ocular hypertensive human eyes. Arch Ophthalmol 1993; 111: 1351-8.
2. Alm A, Stjernschantz J. Effects on intraocular pressure and side effects of 0.005% latanoprost applied once daily, evening or morning. A comparison with timolol. Scandinavian Latanoprost Study Group. Ophthalmology 1995; 102: 1743-52.
3. Prünte C, Flammer J. The novel dopamine D_1 antagonist and D_2 agonist, SDZ GLC 756, lowers intraocular pressure in healthy human volunteers and in patients with glaucoma. Ophthalmology 1995; 102: 1291-7.
4. Prünte C, Flammer J, Markstein R, Rudin M. Quantification of optic nerve blood flow using MR-imaging. Invest Ophthalmol Vis Sci 1995; 36: 247-51.
5. Chiou GC, Chen YJ. Effects of dopamine agonist, bromocriptine, and some dopamine antagonists on ocular blood flow. J Ocul Pharmacol 1992; 8: 285-94.
6. Markstein R, Gull P, Rudeberg C, et al. SDZ GLC 756, a novel octahydrobenzo[g]quinoline derivative exerts opposing effects on dopamine D_1 and D_2 receptors. J Neural Transm 1996; 103: 17-30.
7. Prünte C, Nuttli I, Markstein R. Topical SDZ GLC-756, a novel dopamine D-1 antagonist and D-2 agonist, lowers intraocular pressure in conscious rabbits. Jpn J Ophthalmol 1996; 40: 167-73.
8. Sherwood MB, Grierson I, Millar L, Hitchings RA. Long-term morphologic effects of antiglaucoma drugs on the conjunctiva and Tenon's capsule in glaucomatous patients. Ophthalmology 1989; 96: 327-35.
9. Maumenee AE. External filtering operations for glaucoma: the mechanism of function and failure. Trans Am Ophthalmol Soc 1960; 58: 319-28.
10. Flaxel JT, Swan KC. Limbal wound healing after

cataract extraction. Arch Ophthalmol 1969; 81: 653-9.
11. Miller MH, Grierson I, Unger WI, Hitchings RA. Wound healing in an animal model of glaucoma fistulizing surgery in the rabbit. Ophthalmic Surg 1989; 20: 350-7.
12. Addicks EM, Quigley HA, Greene WR, Robin AL. Histologic characteristics of filtering blebs in glaucomatous eyes. Arch Ophthalmol 1983; 101: 795-8.
13. Broadway DC, Grierson I, O'Brien C, Hitchings RA. Adverse effects of topical antiglaucoma medication. II. The outcome of filtration surgery. Arch Ophthalmol 1994; 112: 1446-53.
14. Lavin MJ, Wormald RP, Migdal CS, Hitchings RA. The influence of prior therapy on the success of trabeculectomy. Arch Ophthalmol 1990; 108: 1543-8.
15. Young TL, Higginbotham EJ, Zou XL, Farber MD. Effects of topical glaucoma drugs on fistulized rabbit conjunctiva. Ophthalmology 1990; 97: 1423-7.
16. Lark KK, Pasha AS, Yan X, Edward DP. The effect of latanoprost and brimonidine on rabbit subconjunctival fibroblasts. J Glaucoma 1999; 8: 72-6.
17. Williams DE, Nguyen KD, Shapourifar-Tehrani S, Kitada S, Lee DA. Effects of timolol, betaxolol, and levobunolol on human Tenon's fibroblasts in tissue culture. Invest Ophthalmol Vis Sci 1992; 33: 2233-41.
18. Cunliffe IA, McIntyre CA, Rees RC, Rennie IG. The effect of topical cholinergic medications on human Tenon's capsule fibroblasts in tissue culture. Graefes Arch Clin Exp Ophthalmol 1995; 233: 507-12.
19. Cunliffe IA, McIntyre CA, Rees RC, Rennie IG. Pilot study on the effect of topical adrenergic medications on human Tenon's capsule fibroblasts in tissue culture. Br J Ophthalmol 1995; 79: 70-5.
20. Wu KY, Hong SJ, Wang HZ. Effects of pilocarpine and other antiglaucoma drugs on cultured human Tenon's fibroblast cells. J Ocul Pharmacol Ther 1997; 13: 13-20.
21. Wu KY, Hong SJ, Chung YM. Effects of dopamine antagonists and agonist on cultured human Tenon's fibroblast cells. Kaohsiung J Med Sci 1996; 12: 388-93.
22. Kamentsky LA, Burger DE, Gershman RJ, Kamentsky LD, Luther E. Slide-based laser scanning cytometry. Acta Cytologica 1997; 41: 123-43.
23. Ariturk N, Oge I., Baris S, Erkan D, Sullu Y, Koc F. The effects of antiglaucomatous agents on conjunctiva used for various durations. Int Ophthalmol 1997; 20: 57-62.
24. Broadway DC, Grierson I., O'Brien C, Hitchings RA. Adverse effects of topical antiglaucoma medication. I. The conjunctival cell profile. Arch Ophthalmol 1994; 112: 1437-45.
25. Kellett M, Potten CS, Rew DA. A comparison of in vivo cell proliferation measurements in the intestine of mouse and man. Epithelial Cell Biol 1992; 1: 147-55.
26. Heuer DK, Parrish RK, Gressel MG, Hodapp E, Palmberg PF, Anderson DR. 5-fluorouracil and glaucoma filtering surgery. II. A pilot study. Ophthalmology 1984; 91: 384-94.
27. Rockwood EJ, Parrish RK, Heuer DK, et al. Glaucoma filtering surgery with 5-fluorouracil. Ophthalmology 1987; 94: 1071-8.
28. Durant S, Duval D, Homo-Delarche F. Effect of exogenous prostaglandins and nonsteroidal anti-inflammatory agents on prostaglandin secretion and proliferation of mouse embryo fibroblasts in culture. Prostaglandins Leukotrienes Essential Fatty Acids 1989; 38: 1-8.
29. De Asura LJ, Clingan D, Rudland PS. Initiation of cell proliferation in cultured mouse fibroblasts by prostaglandin F2?. Proc Natl Acad Sci USA 1975; 72: 2724-8.
30. Ko SD, Page RC, Narayanan AS. Fibroblast heterogeneity and prostaglandin regulation of subpopulations. Proc Natl Acad Sci USA 1977; 74: 3429-32.
31. Korn JH, Halushka PV, Leroy EC. Mononuclear cell modulation of connective tissue function. Suppression of fibroblast growth by stimulation of endogenous prostaglandin production. J Clin Invest 1980; 65: 543-54.
32. Ali AE, Barrett JC, Eling TE. Prostaglandin and thromboxane production by fibroblasts and vascular endothelial cells. Prostaglandins 1980; 20: 667-88.
33. Durant S, Duval D, Homo-Delarche F. Mouse embryo fibroblasts in culture: characteristics of arachidonic acid metabolism during early passages. Prostaglandins Leukotrienes Essential Fatty Acids 1988; 32: 129-37.
34. Warwar RE, Bullock JD, Ballal D. Cystoid macular edema and anterior uveitis associated with latanoprost use. Experience and incidence in a retrospective review of 94 patients. Ophthalmology 1998; 105: 263-8.
35. Anand N, Sahni K, Menage MJ. Modification of trabeculectomy with single-dose intraoperative 5- fluorouracil application. Acta Ophthalmol Scand 1998; 76: 83-9.
36. Khaw PT, Doyle JW, Sherwood MB, Smith MF, McGorray S. Effects of intraoperative 5-fluorouracil or mitomycin C on glaucoma filtration surgery in the rabbit. Ophthalmology 1993; 100: 367-72.