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Comparative study of inhibition of fibroblasts proliferation in vitro in the conjunctiva using mitomycin and ciclophosphamide

Estudo comparativo da inibição da proliferação de fibroblastos in vitro na conjuntiva utilizando mitomicina C e ciclofosfamida

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Abstract

Objective: *To evaluate the inhibition of fibroblast proliferation in vitro of conjunctiva obtained by excision of pterygium from patients using mitomycin (MMC) and cyclophosphamide (CF).* ***Methods:*** *Pterygiums were removed from 7 patients and subjected to cell culture. After cell cultivation, 3 fragments of equal dimensions of these tissues were collected from adjacent areas of each patient removed pterygium. They were randomly selected in such a way that one fragment of each patient was exposed to: the culture medium (group control), to MMC and to CF for an equal period of time at concentrations of 0,4 mg/dl and 10 mg/dl respectively. After this period, the fibroblast cell count of these groups were performed. Each group had seven fragments.* ***Results:*** *With the use of MMC we had a 95% rate of inhibition of fibroblast proliferation, while with CF 100%.* ***Conclusion:*** *Both drugs showed a high rate of inhibition of fibroblast proliferation, but CF showed greater inhibition than MMC.*

Keywords: *Fibroblasts proliferation; Healing; Mitomycin; Cyclophosphamide; Trabeculectomy; Pterygium; Cell culture; Antimitotics drugs*

Resumo

Objetivo: Avaliar a inibição da proliferação de fibroblastos in vitro das conjuntivas obtidas através de exérese de pterígios de pacientes utilizando mitomicina C (MMC) e ciclofosfamida (CF). **Métodos:** Os pterígios foram retirados de 7 pacientes e submetidos a cultivo celular. Após o cultivo, 3 fragmentos de dimensões iguais deste material foram colhidos de áreas adjacentes do pterígio removido de cada paciente. Eles foram randomicamente selecionados de tal forma que: um fragmento de cada paciente foi exposto: ao meio de cultura (grupo controle), a MMC e a CF por igual período de tempo nas concentrações de 0,4 mg/ml e 10 mg/ml respectivamente. Após este período realizou-se a contagem celular de fibroblastos destes 3 grupos. Cada grupo continha 7 fragmentos. **Resultados:** Com a utilização da MMC tivemos uma taxa de 95% da inibição da proliferação dos fibroblastos, enquanto com a CF 100%. **Conclusões:** Ambas as drogas apresentaram elevada taxa da inibição da proliferação de fibroblastos, porém a CF apresentou inibição maior que a MMC.

Descritores: Proliferação de fibroblastos Cicatrização; Mitomicina; Ciclofosfamida; Trabeculectomia; Pterígio; Cultura celular; Drogas antimitóticas

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Introduction

Fibroblast activity modulation in conjunctival healing from surgeries has the potential to improve outcomes after conjunctival surgeries, such as pterygium excision, fistulizing surgeries to treat glaucoma, and different eye disorders, including cicatricial pemphigoid and vernal keratoconjunctivitis.[1]

Healing has been mostly investigated in pterygium and filtering surgeries for glaucoma (trabeculectomy - TBC).[2]

Success in the aforementioned surgeries somehow depends on the fibroblast production modulation to reduce the chance of recurrences in the case of pterygium surgery and to decrease bleb site healing in the case of fistulizing surgeries, since it allows draining the aqueous humor from the anterior chamber (AC) to the subconjunctival space. Such process results in filtering bleb creation. Failure in glaucoma surgery usually happens because of excessive healing which closes the fistula- fibroblasts play significant role in such a process.[1,3]

Healing at subconjunctival and tenonian level in the fistula area is the main reason for trabeculectomy failure. In addition, two factors must be taken into account, namely: the vascularization necessary for oxygen supply and important nutrients for scar formation, and the migration and proliferation of Tenonian fibroblasts that synthesize collagen and make scar tissue contraction.[4]

5-Fluorouracil (5-FU) and mitomycin C (MMC) are powerful and effective antiproliferative drugs that have been used to improve TBC success for years. However, these substances have side effects such as corneal toxicity, hypotonia, cystic and avascular bubble formation, blebitis and endophthalmitis.[5-7]

Several other agents, besides MMC and 5-FU, have been proposed to decrease episcleral healing after TBC. However, only few of them have been assessed in clinical trials and none of them have become well-accepted or widely used.[8]

The aim of the current study was to test the ability of cyclophosphamide (CF) to inhibit fibroblasts' proliferation. CF is an antimitotic, antiproliferative and antineoplastic drug widely used to treat neoplasms. It is already described as promising antifibrotic agent by several studies carried out in vivo and in vitro.[9] Different from other drugs, it is not vesicant

Methods

Patients:

Seven patients (3 men and 4 women) aged 30 to 60 years had their pterygiums removed through the bare sclera technique. All pterygiums were primary and did not have any associated eye disease. Patients did not use any local or systemic immunosuppressive drugs. All participants signed the informed consent

Pterygium epithelial cell culture

Protocol for the performed cell culture was previously established by Almodin et al. (2013)[12], based on Kria et al. (1998). The pterygium epithelial tissue was removed, washed, fragmented and divided into three groups: control (without medication), mitomycin C and cyclophosphamide. Fragments were cultivated in Eagle medium (GIBCO, Grand Island, NY, USA) added with 10% fetal bovine serum (Nutricell, Campinas, SP, Brazil) and antibiotics, and incubated at 37º C in atmosphere with 5% CO2.

Testing the effect of drugs on cells

Cells were incubated after their first layer was formed in 0.25% trypsin solution (GIBCO, Grand Island, NY, USA) for 1 minute, at room temperature. After trypsinization, cells were washed and resuspended for counting. Cells were then taken to plates filled with 1 ml of Eagle medium and 10% fetal bovine serum at density of 1x104 cells/ml to be subcultured. Drugs were added to the cell culture plates after 5 days at the following concentrations: 500mg/m2 or 10 mg/ml of CF (Baxter Oncology Gmbh, Frankfurt, Germany), and 20mg/m2 or 0.4 mg/ml of MMC (Bristol-Myers Squib Brasil S.A, Santo Amaro, SP, Brazil). Drugs were diluted in Eagle medium added with 10% serum and kept in incubator for 12 hours before its use to stabilize pH. Plates were taken back to the incubator right after the drugs were added to the cells. Cells were observed in inverted phase-contrast microscope to assess cell proliferation after 24-hour exposure to the drugs. Then, cell viability was determined through 0.1% trypan blue dye exclusion test (Ophthalmos, Jabaquara, SP, Brazil). Viable cells are impervious to this dye since their penetration into cells indicates integrity loss of their membrane. The entire culture medium of each plate added with the drugs was removed and placed in tubes. The plates were washed several times to prevent any cell to remain on the bottom - cleaning was confirmed through observation under a microscope. Eagle medium added with 10% serum was taken to the tubes for cell washing through centrifugation for 5 minutes. The supernatant was removed and 100µl of culture medium was added to the tube. Trypan blue (0.4%) was added to the cells (1:1

Figure 1: Control culture – MO 400x

volume), which were incubated for 5 minutes at room temperature. After that time, the quantitative assessment of cytotoxicity was performed by counting the stained and unstained cells in a Makler chamber in order to determine the viability index:

$$\text{Viability index (\%)} = \frac{\text{(total of cells– stained cells) X100}}{\text{Total of cells}}$$

Negative control was performed when no drug was added (Figure 1). Experiments were repeated 3 times.

Results

Extensive inhibition of cell proliferation was observed in plates whose MMC and CF were used in the first 24 hours in comparison to the control plate. Cells detached from the plate

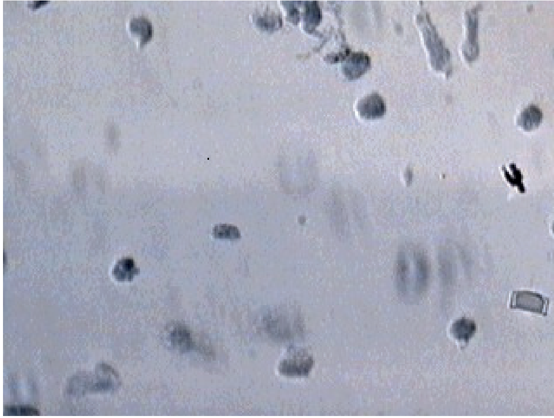

Figure 2: Stained cells after the CF using – MO 400x

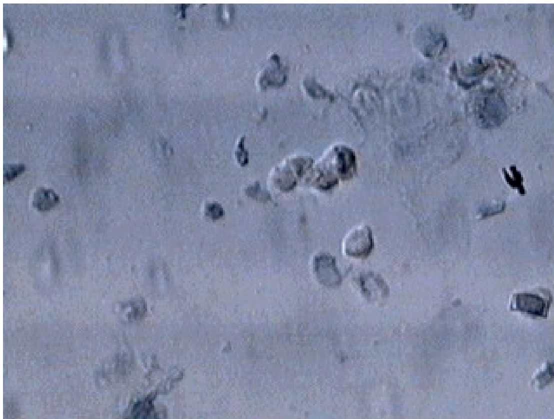

Figure 3: Stained cells after MMC using - MO 400x

added with MMC in almost its entire length. Similar effect was observed when cyclophosphamide was used. The control plates remained with all cells adhered to the bottom of the plate.

In total, 100% of cells subjected to CF were stained (Figure 2) when 0.1% Trypan blue staining was performed. In other words, the viability test was zero (0) after 24-hour exposure to the drug, whereas 95% of cells subjected to MMC were stained (Figure 3); thus, they presented 5% viability test.

Discussion

Pterygium is featured by fibrotic subconjunctival connective tissue growth and hypertrophy of the overlying conjunctival epithelium.[9] The occurrence of this disease does not seem to be related to exposure to ultraviolet light, but to accelerated fibroblast proliferation produced by surgical trauma.[10] The current research was performed through experiments conducted in vitro to assess pterygium fibroblasts growth inhibition, due to CF suing. It aimed at finding a therapeutic alternative to inhibit fibroblasts proliferation during fistulizing surgery. CF is an antimitotic, antiproliferative and antineoplastic drug widely used to treat neoplasms. It has been described as promising antifibrotic agent by several studies carried out in vivo and in vitro.[11,12] CF was chosen because it is a non-vesicant drug, whereas assumingly, the unwanted effects of MMC on the eye are due to such an effect caused by this drug. Vesicant drugs cause severe irritation, form vesicles and account for tissue destruction when they accumulate outside blood vessels and lead to necrosis.[13]

MMC outcomes in inhibiting pterygium fibroblasts proliferation in the current research were already expected to be found; however, CF was also very effective. The inhibitory action of CF in fibroblastic proliferation in vitro suggested that it can be used to improve scar modulation in surgeries. CF is a cyclic phosphamide ester of mechlorethamine which prevents primarily cell division by crosslinking DNA strands.[14] CF dosage was higher than the MMC doe, since MMC concentrations used in the current research were already used in ophthalmology, whereas CF concentrations was used to treat neoplasms.

CF can be used as adjuvant therapy to replace MMC in surgeries to inhibit fibroblast proliferation, such as pterygium surgeries or other surgeries in the conjunctival region, like TRC. However, further research carried out in animals and humans, based on different surgery types are necessary to assess the efficacy and safety of this drug.

Conclusion

Cell culture subjected to CF showed the highest fibroblast proliferation inhibition (100%) rate, whereas those under MMC action showed 95% inhibition.

CF has the advantage of being a non-vesicant drug; thus, it does not induce vesicle formation and tissue degradation; moreover, it can be managed with greater safety and account for lower hypothetical scleral offense rate and for less complications.

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