Ocular surface findings in impression cytology after interferon a2b or mitomycin C in rabbits

Achados em citologia de impressão da superfície ocular após uso de interferon alfa-2b ou mitomicina C: estudo em coelho

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ABSTRACT

Objective: To describe ocular surface findings in impression cytology obtained from healthy rabbit conjunctiva treated with interferon alpha-2b eyedrop, and compare them to findings after use of mitomycin C 0.02%.

Methods: An experimental study using a rabbit model was performed between September 2013 and October 2014 at the Faculdade de Medicina de Marília, Universidade Federal de São Paulo, Clínica de Olhos Moacir Cunha. Thirty New Zealand white rabbits were divided into 6 groups and received interferon alpha-2b or mitomycin C 0.02%. Impression cytology (IC) was performed prior to topical applications and at15, 30 and 60 days of use. The following variables were analyzed in impression cytology: goblet cells, cellularity, cell-to-cell adhesion, nucleus/cytoplasm ratio, nuclear chromatin, inflammatory cells keratinization, and cytomegaly.

Results: The major findings in impression cytology after use of interferon alpha-2b included loss of goblet cells (50.8%), reduced cell-to-cell adhesion (26.2%), abnormal nucleus/cytoplasm ratio (20%) and reduced cellularity (15.4%). After use of mitomycin C 0.02%, the most common changes included loss of goblet cells (46.2%), abnormal nucleus/cytoplasm ratio (25.6%), less cell-to-cell adhesion (23.1%), and reduced cellularity (20.5%). There were no significant differences in any variable when comparing impression cytology after interferon alpha-2b and after mitomycin C 0.02%. Goblet cell loss was more pronounced at days 30 and 60, as compared to impression cytology at day 15 for both drugs.

Conclusion: The loss of goblet cells, reduced cell-to-cell adhesion and cellularity, along with abnormal nucleus/cytoplasm ratio were the most common findings in impression cytology after use of interferon alpha-2b. These findings are similar to those described for use of mitomycin C 0.02%.

RESUMO

Objetivo: Descrever os achados em citologia de impressão de conjuntiva sadia de coelho submetida ao uso de colírio de interferon alfa-2b e compará-los ao que foi encontrado após uso da mitomicina C 0,02%.

Métodos: Estudo experimental realizado em modelo animal no período entre setembro de 2013 e outubro de 2014 nas dependências da Faculdade de Medicina de Marília, da Universidade Federal de São Paulo e da Clínica de Olhos Moacir Cunha. Trinta coelhos albinos da raça Nova Zelândia foram divididos em seis grupos e receberam interferon alfa-2b ou mitomicina C. A citologia de impressão foi realizada antes do início dos colírios e após 15, 30, 60 dias de seu uso. As seguintes variáveis foram analisadas na citologia de impressão: células caliciformes, celularidade, adesão intercelular, razão núcleo/citoplasma, cromatina, células inflamatórias, queratinização e citomegalia.

Resultados: Os principais achados na citologia de impressão após o uso do interferon alfa-2b incluíram perda de células caliciformes (50,8%), redução da adesão intercelular (26,2%), alteração da razão N/C (20%) e redução da celularidade (15,4%). Após uso da mitomicina C 0,02%, foram mais frequentes a redução das células caliciformes (46,2%), a alteração da razão N/C (25,6%), a adesão intercelular (23,1%) e a redução da celularidade (20,5%). Não houve diferença estatisticamente significante para nenhuma das variáveis estudadas quando se compararam as citologias de impressão após interferon alfa-2b com as citologias de impressão após mitomicina C 0,02%. Independentemente da substância utilizada, as citologias colhidas 30 e 60 dias após início das drogas apresentaram maior redução de células caliciformes quando comparadas com as citologias de impressão colhidas após 15 dias.

Conclusão: A redução das células caliciformes, a diminuição da adesão intercelular, a alteração da razão N/C e a diminuição da celularidade foram as alterações mais frequentes na citologia de impressão colhida após o uso de interferon alfa-2b. Os achados em citologias de impressão após o uso de interferon alfa-2b são semelhantes àqueles encontrados após o uso da mitomicina C 0,02%.
INTRODUCTION
Topical chemotherapy is a treatment option for benign, premalignant and malignant conjunctival lesions. The most common drugs used in this therapy are interferon alpha-2b (INFα2b), mitomycin C (MMC) and 5-fluorouracil (5-FU), which can be used as monotherapy when treating intraepithelial lesions, or as adjuvant treatment for invasive neoplasms.1

Chemotherapy agents
Mitomycin C is an antibiotic originally isolated from Streptomyces caespitosus, in 1958. It is an alkaline agent that produces cytotoxic free radicals, which induce DNA damage, inhibit cell migration and restrict extracellular matrix production.1 Topical administration has demonstrated better tolerability and reduces the side-effects of intravenous use, such as nausea, vomiting, bone marrow suppression, fever and malaise.2,3 Mitomycin C was first introduced to treat ocular surface squamous neoplasia (OSSN), in 1994,8 at concentrations ranging from 0.002% to 0.04%.9 Although this treatment has been effective, side effects can include conjunctival hyperemia, ocular allergy, punctate keratitis, ocular pain, epiphora, recurrent corneal erosion, limbal stem cell deficiency, punctal stenosis and surrounding skin toxicity.1,3

Interferon is a family of glycoproteins first discovered in 1957. Interferon alpha-2b is classified as an antineoplastic agent due to a combination of antiproliferative, antiangiogenic and cytotoxic effects.1,3 Interferon alpha-2b was used to treat hairy cell leukemia, 10 years after its discovery,10 and subsequently was trialed to treat Kaposi’s sarcoma in HIV patients, melanoma, multiple sclerosis, renal carcinoma and other malignancies.10 In the 1980’s, it was used to treat ocular diseases, including conjunctival carcinoma(1) and herpetic keratitis.11 Interferon alpha-2b has also been used to treat non-Hodgkin lymphoma.12 As treatment for OSSN, INFα2b can be administered topically (dose ranging from 1 to 3 M UI/mL) or in the subconjunctiva (dose ranging from 3 to 10 M UI/mL).11,14

Although this is an important agent in the treatment of a variety of neoplasms, several side effects have been related to INFα2b. Local side effects include conjunctival hyperemia, ocular allergy and punctate keratitis.11,13 but these adverse events are less frequent when MMC is used.1 Systemic side effects are more likely to occur with subconjunctival administration and include malaise, fever and arthralgia.14

Ocular surface squamous neoplasia
Ocular surface squamous neoplasia is a group of conjunctival and corneal neoplasms. These neoplasms include intraepithelial neoplasia and invasive squamous cell carcinoma (SCC); the former is a precursor of SCC. Ocular surface squamous neoplasia is associated with sunlight exposure, HIV infection, human papilloma virus (HPV), cigarette smoking, and immunosuppression, including that secondary to neoplastic treatment.15-17 It is more often observed in the exposed conjunctiva, near the limbus and can be a pigmented or non-pigmented lesion.1

Histopathology is the gold standard for diagnosis and subclassification of OSSN. It can be used to distinguish OSSN dysplasia (mild, moderate and severe), carcinoma in situ and invasive SCC.18

Historically, excisional biopsy with a large margin was the initial treatment for OSSN. Adjuvant therapies, such as cryotherapy, lowered the recurrence rate from 40% to 6%.19 Topical chemotherapy has also been used as a primary treatment for intraepithelial tumors, and as a neoadjuvant treatment in large lesions.1,20 With topical therapy, sometimes surgical biopsy is not be performed, but a definitive diagnostic method that can replicate the accuracy of histopathology is required. Impression cytology (IC) is a less invasive method that may be able to provide accurate diagnostic information on OSSN.21

Impression cytology
Impression cytology (IC) was first described in 1977,22 as a method to analyze goblet cells of patients with Stevens-Johnson syndrome, pemphigoid diseases and keratoconjunctivitis sicca. In 1985, IC techniques were modified to analyze the cytological aspects of OSSN.23 Unlike surgical biopsy, IC is a minimally invasive approach that allows clinicians to identify the location of the lesion and analyze the cell characteristics.23 With appropriate technique and analysis, the correlation with histopathology results is 80%,21 making IC is an important method to identify OSSN in patients who are unable to undergo biopsy.

Barros et al. developed a modification of the Bethesda cervical cytology score to the human ocular surface to differentiate invasive from non-invasive lesions,24 using IC. From a logistic regression analysis of 11 cytological parameters, 7 were found to be predictive of malignancy: nuclear size, chromatin, nucleoli, syncytial-like groupings, nucleus-cytoplasm (NC) ratio,
cytoplasmic stain and cytoplasmic borders. Barros score has a 95% positive predictive value and 93% negative predictive value.\(^{(21)}\)

The application of MMC to normal conjunctival cells can result in changes that mimic neoplastic cells when evaluated using IC. These changes include reduced cell-to-cell adhesion, cytomegaly, hyperchromatic nuclei, and keratinization.\(^{(24)}\) However, it is not clear whether similar changes can be observed in normal conjunctival cells exposed to INFα\(^{2b}\).

This study is justified since understanding cell changes attributed to treatment can be a great ally for pathologists and clinicians to differentiate between changes associated with a disease process, and those related to a beneficial treatment effect.

The objectives of this study were to describe changes observed via IC in healthy conjunctiva cells that have been exposed to INFα\(^{2b}\), and to compare these findings to changes observed when healthy conjunctival cells are exposed to MMC.

**METHODS**

This study was approved by the Ethics in Animal Research Committee from Faculdade de Medicina de Marília (FAMEMA; CEUA-1206/12) and Universidade Federal de São Paulo (Unifesp; CEUA-319094). The studies were conducted between October 2013 and September 2014.

**Drugs**

The following drugs were used: MMC 0.02% (Ophthalmos S/A, São Paulo, SP, Brazil) was received lyophilized and reconstituted by adding distilled water; INFα\(^{2b}\) 1 million UI/mL (Ophthalmos S/A) was received as a 10 mL solution and the vehicle was phosphate buffer (distilled water in a 10-mL sterile ampoule, and phosphate buffer in a 10-mL bottle).

**Animals**

Thirty male, white New Zealand rabbits (age: 6 months; weight: range from 2.5 to 3 kg) were included in this study. The right eye (OR) was treated and the left eye (OS) received the vehicle. The animals were divided into six groups (I to VI) and received drops as described in Table 1. Groups V and VI received no vehicle solution in the OS. All animals used in this study were maintained in the FAMEMA Animal Facility. Time of light and dark environment, air flow and room temperature were controlled. Light/dark cycle maintained light from 6:00am to 6:00pm.

### Table 1. Drops received by the rabbits of each group on right and left eyes

| Groups  | OR       | Drugs                  | OS       |
|---------|----------|------------------------|----------|
| I       | MMC 0.02%| 15 days Distilled water| Distilled water |
| II      | INFα\(^{2b}\) | 15 days Phosphate buffer| Phosphate buffer |
| III     | INFα\(^{2b}\) | 30 days Phosphate buffer| Phosphate buffer |
| IV      | INFα\(^{2b}\) | 60 days Phosphate buffer| Phosphate buffer |
| V       | INFα\(^{2b}\) | 15 days No vehicle| No vehicle |
| VI      | MMC 0.02%/15 days No vehicle| No vehicle |

OR: right eye; OS: left eye; MMC: mytomycin C; INFα\(^{2b}\): interferon alpha 2 beta.

**Impression cytology**

All IC strips were collected at FAMEMA and analyzed at Clínica de Olhos Moacyr Cunha and at Unifesp. The samples were taken at four different time points after the initial application (Table 2).

### Table 2. Times of specimen collection for impression cytology

| Groups | Time of specimen collection |
|--------|----------------------------|
|        | T₀ | T₁  | T₂  | T₃  |
| I      | X  | x   | x   | x   |
| II     | X  | x   | x   | x   |
| III    | X  | x   | x   | x   |
| IV     | X  | x   | x   | x   |
| V      | X  | x   | x   | x   |
| VI     | X  | x   | x   | x   |

T₀: before treatment; T₁: day 15; T₂: day 30; T₃: day 60.

We performed the modified Barros technique to collect and process the strips. Briefly, for this technique, the procedures were performed at 2:00 pm, and topical anesthesia with 0.5% proxymetacaine hydrochloride (Anestalcon® 0.5%, Alcon, São Paulo, SP, Brazil) was applied to the eye. A strip of acetate cellulose filter paper with a pore size of 0.45 mm (Millipore HAWP 304F0, Bedford, United States) was then placed onto the superior conjunctiva of rabbits’ OR. This paper was gently pressed for 5 seconds and then removed. The filter was immediately fixed in a solution containing glacial acetic acid, formaldehyde 37% and ethyl alcohol in a 1:1:20 volume ratio. A second IC sample was obtained over the same area. This process was repeated for the OS (Figure 1). Then, all strips were processed for periodic acid-Schiff (PAS), Gill’s hematoxylin and Papanicolaou stain, according to a technique\(^{(23)}\) adapted from Tseng.\(^{(23)}\) The longest time between fixation and strip process was 48 hours. All strips were analyzed.

Two different observers analyzed the IC specimens. They were blinded as to the used drops, and to reports of each other.

The two samples obtained from each animal were compared under light microscope, at a very low magnification (4x objective lens, total magnification 40x), and
only the sample with the highest cellularity was considered for analysis.

Four fields of the chosen sample were checked under magnifications of 100x, 200x and 400x. Periodic acid-Schiff-positive goblet cells were assessed under magnification x200 and their number was estimated.

Eight variables were analyzed and defined as normal or abnormal. The following variables were defined as abnormal: abnormal cellularity, if the strip was under one-third filled with cells; abnormal cell-to-cell adhesion, if cells were not attached too adjacent; abnormal NC ratio, when it was 2:3; abnormal chromatin, if hyperchromasia was present; abnormal goblet cell density for reduced or absent, in at least one field; abnormal keratinization, if present; abnormal inflammation, if present; and abnormal cytomegaly, if present.

After this classification we applied a score similar to that used by Aragona. Since a binary scoring system was used for each variable, a score between zero and three was considered normal and scores >3 were considered abnormal (Figure 2).

Statistical analysis

Since the OR and OS data were obtained from the same rabbit, all analyses presumed dependence of the eyes. Generalized estimating equations (GEE) were used with Bonferroni-corrected post-hoc tests in the presence of an interaction effect between groups and eyes. Generalized linear mixed models were also used. In this analysis, the group and time of specimen collection were considered the random effect, to prioritize comparisons between INFα2b and MMC 0.02%. For all comparisons, the significance level considered was p>0.05. All analyses were performed using Statistical Package for Social Sciences (SPSS), version 19.0.

RESULTS

A total of 58 eyes from New Zealand white rabbits (29 OR and 29 OS) were studied. One animal from Group IV died and was therefore excluded from the analysis.
Impression cytology findings after interferon alpha-2b application
Considering all collection times, 65 IC samples were obtained from 20 eyes (OR) for the INFα2b groups. Goblet cell loss was found in 50.8% of all specimens, reduced cell-to-cell adhesion was present in 26.2%, abnormal NC ratio was found in 20%, and cellularity was reduced in 15.4%. Less frequent abnormalities included cytomegaly (4.6%), presence of inflammatory cells (3.1%) and keratinization (1.5%). There was no evidence of hyperchromasia in any specimen.

Impression cytology findings after mitomycin C 0.02%
As for all collection times for the MMC groups, 36 IC samples from nine eyes (OR) were analyzed. The most frequent findings were reduced goblet cells in 46.2% of specimens, abnormal NC ratio in 25.6%, reduced intercell adhesion in 23.1%, and reduced cellularity in 20.5%. Less frequent abnormalities included cytomegaly (8.3%), keratinization (5.1%), and the presence of inflammatory cells (3.1%). There was no abnormal hyperchromasia in any of the specimens.

Comparisons between interferon alpha-2b and mitomycin C 0.02% using impression cytology
For each variable, the drug (INFα2b/MMC 0.02%), treatment duration (15d/30d/60d), eye (OR/OS), and times of collection (T0, T1, T2, T3) were compared. When comparing IC results collected at day 15, Groups I and VI were compared to Groups II and V. When comparing IC results collected at day 30, Groups I and III were compared. Groups I and IV were compared to study IC results at day 60 (Table 3).

Table 3. Groups considered to compare interferon alpha-2b and mitomycin C 0.02% on each time of specimen collection

| Time (days) | Groups | Groups |
|-------------|--------|--------|
|              | I – MMC | II – INF 15 | III – INF 30   | IV – INF 60 | V – INF 15 | VI – MMC |
| 15          | x       | x       | x    | x      | x       |
| 30          | x       | x       | x    | x      | x       |
| 60          | x       | x       | x    | x      | x       |

MMC: mitomycin C, INF: interferon.

When comparing time of collection and treatment group, we found greater reduction in cellularity for the INFα2b group when compared to the MMC group, at day 60 (p<0.001). For all other variables, there was no statistical difference between groups at any time point. There was also no difference between the treated eye and control eye results, with p-value greater than 0.2 on all analysis made for each variable.

As previously mentioned, a group comparison was made between INFα2b and MMC 0.02% (MMC Groups I and VI) versus (INFα2b Groups II, III, IV and V) for the treated eye alone. Groups and time collection of specimens were considered random events in the model. There was a significant interaction effect for time, with a greater reduction in goblet cells at day 30 and day 60 as compared to day 15, for both groups. There was no significant difference between INFα2b and MMC 0.02% for any variable in this analysis (Table 4).

Table 4. Global comparison between interferon alpha-2b and mitomycin C 0.02% groups of each variable analyzed and classified as abnormal

| Variable | Drug | p-value |
|----------|------|---------|
|          | INFα2b (n=65) | MMC 0.02% (n=36) |
| Goblet cell density | 50.8 | 47.2 | 0.816 |
| Cell-to-cell adhesion | 26.2 | 22.2 | 0.692 |
| NC ratio | 20 | 25.0 | 0.638 |
| Cellularity | 15.4 | 22.2 | 0.392 |
| Cytomegaly | 4.6 | 8.3 | 0.455 |
| Inflammation | 3.1 | 5.6 | 0.579 |
| Keratinization | 1.5 | 5.6 | 0.286 |
| Chromatin | -- | -- | -- |

Results expressed as %.

INFα2b: interferon alpha 2 beta; MMC: mitomycin C; NC: nucleus-to-cytoplasm ratio.

DISCUSSION
The use of topical chemotherapy to treat OSSN has been increasing in the last decades. Although MMC, INFα2b and 5-FU have been all used for this goal, our study focused on the first two drugs.

One of the benefits of using topical chemotherapy is to provide a less invasive treatment to high surgical risk patients. It also allows a combination of drugs and routes of administration (drops and subconjunctival) enabling a customized treatment to meet the needs of each patient. Interferon alpha-2b has been preferred among the three drugs for its lower frequency of side effects. However we must consider some aspects that can impair its use: it must be used for a long time, with an inconvenient dosing schedule (every 6 hours), and the cost may be too high for patients in developing countries.

Previous studies have shown a 54% positive correlation between clinical and histopathological diagnoses. Non-invasive diagnostic methods, such as IC, may help making diagnosis of this condition when biopsy is not feasible, which occurs when neoadjuvant treatment has been initiated or when topical chemotherapy is the only therapeutical choice. Impression
cytology has an 80% positive correlation to histopathology, and can also differentiate invasive and non-invasive lesions with 95% sensitivity and 93% specificity.

It is important, though, to highlight some adverse outcomes of IC, such as short-lived epithelial defects and conjunctival hyperemia at the collection site. These may lead to foreign body feeling that recovers using artificial tear eyedrops.

Topical chemotherapy as single treatment is recommended for non-invasive lesions, and as adjuvant treatment for invasive lesions, because the depth of penetration is limited in topical treatments. Impression cytology may play an important role in treatment planning. It can also be used to identify and track relapses by identifying atypical cells, and allowing for immunohistochemical study with no repeated invasive procedures.

Mitomycin C application is well-known to induce changes to healthy cells that can be mistaken for atypical cells. Understanding how MMC affects normal tissue is essential to avoid confusion when OSSN is assessed by IC. Previous studies found that MMC can induce cytoplasm vacuoles, cytomegaly, multinucleated cells, irregularly contoured nucleus and hyperchromatic nucleolus, but no changes were noted in the N/C ratio. The application of MMC in our study revealed fewer goblet cells, decreased cell count and less cell-to-cell adhesion were the primary abnormalities identified by IC analysis. Although these changes are not fully unexpected, we also found an abnormal N/C ratio of 25%, unlike a previous study reporting no abnormalities in the N/C ratio. Cytomegaly, keratinization and presence of inflammatory cells were less common abnormalities in our sample. Some differences between the two studies may be attributed to the animal model. We used a rabbit model in our experiments, while a previous study used a human model.

Changes due to MMC have been previously assessed, but we are unaware of any investigation describing the changes in normal conjunctiva cells after application of INFα2b. Our results revealed normal rabbit conjunctiva cells treated with INFα2b changed similarly to MMC use, including a reduction in goblet cells, lower cell count, less cell-to-cell adhesion, and altered N/C ratio. As to MMC, cytomegaly, keratinization and presence of inflammatory cells were less frequent changes.

These descriptive findings support the statistical analysis, which found no differences between INFα2b and MMC, with the exception of lower total cell count in the INFα2b group, at day 60. However, this difference may be explained by diverse topical application and data collection techniques. Since the number of cells on the IC paper depends on the rigor of collection technique, the animal must be immobilized for accurate results. Rabbits in the INFα2b group received drops over 60 days and were more stressed at the time of IC collection, compared to rabbits in the MMC group that only received drops for 15 days. Immobilization proved more difficult in the INFα2b group, which may have reduced the number of cells acquired at the time of data collection.

There were no statistically significant differences between the treated and control eyes. Abnormalities in the control eyes were also present in the contralateral treated eyes. Even though groups V and VI received no vehicle in control eye, abnormalities were similar in the treated eye, in these rabbits. It is possible that systemic absorption caused changes in the control eye. The doses used in this study were equivalent to those recommended in human trials. However, the weight of rabbits ranged from 2.5 kg to 3 kg, which is much lower than human weight, and the rabbit epithelium is ten-fold more permeable than human epithelium. One study demonstrated systemic absorption of MMC 0.02% after topical vesical application. Despite these differences in animal models and the potential for systemic changes, we maintained a similar human dose because the focus of this study was on local cell changes. The ocular surface is comparable to that of human eyes, hence, a human-equivalent dose was tested in this study.

As few statistical differences were found between groups and eyes, the results from all IC analyses were considered and solely compared between drugs, with the times of collection and groups considered random events in the model. This provided a larger number of samples and better statistical power to detect any real differences, should they exist. Despite the larger sample, no differences were observed between groups, making us confident in the validity of our findings.

One of the strengths of this study was the possibility to show ocular surface changes in rabbits submitted to topical chemotherapy by the relatively simple noninvasive IC method. Otherwise, it could be monitored only by performing repeated surgical biopsies, which are invasive and harmful procedures. Another strength was the advantage of a standardized animal model with precise exposure conditions, which detected early signs of ocular surface changes, or subclinical disease that could not be
detected by clinical examination. This may be beneficial for early therapeutic intervention and research purposes. The major strength of this study is it was the first to evaluate possible cell changes on the ocular surface after topical interferon application.

Although this study identified important aspects related to cell changes in chemotherapy application, there are some limitations. On medical observation, it is possible to detect side effects during drop administration, such as conjunctival hyperemia, chemosis, and follicular or allergic conjunctivitis being most frequently associated to MMC than to INFα2b. However, these side effects are related to stromal changes, and would not be detected by conventional IC analysis that collects cells from superficial epithelium. Considering this is an animal model study, the results may not be generalized to human application. Further studies should replicate our aims in human models and could check if the loss of goblet cells would lead to mucin deficiency and development of dry eye disease in the long-term pathogenesis, or if their loss would represent a nonspecific indication of ocular surface subclinical alteration.

This study focused on cell changes due to topical chemotherapy in healthy conjunctiva. Future research has been planned to compare these changes in tumor cells.

Different time prescription for MMC and INF was chosen. Mitomycin C was given for 15 days corresponding to one cycle. INF can be prescribed for months, non-stop if necessary, and we chose 60 days as the longer time. For IC may lead to superficial epithelial defects, it could allow changes in deeper cells if taken from the same animal, at different times. Therefore, we divided INF animals into three groups (15, 30 and 60 days of INF drops). However different time prescription may also interfere in the results.

**CONCLUSION**

The most frequent findings in impression cytology after interferon alpha-2b drops were reduction in goblet cells and cell-to-cell adhesion, nucleus-to-cytoplasm ratio abnormality, and decrease in total cell number. The least common changes were cytomegaly, inflammatory cells, and keratinization. No chromatin changes were found. Impression cytology findings from conjunctiva submitted to interferon alpha-2b drops were similar to those observed after use of mitomycin C 0.02%.

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