Subconjunctival amniotic membrane free graft in rabbit eyes: Effects on fibrovascular reaction

Transplantation d’une membrane amniotique libre sous la conjonctive chez les lapins : effets sur la réaction fibrovasculaire

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KEYWORDS
Amniotic membrane; Subconjunctival amniotic membrane; Subconjunctival fibrovascular reaction; Amniotic membrane transplantation

Summary
Purpose. – The purpose of our study is to investigate the effect of subconjunctival amniotic membrane free graft on subconjunctival fibrovascular reaction.

Methods. – Twelve healthy male white New Zealand rabbits were used for the study. The rabbits were divided randomly into two groups: Study Group (\(n = 6\)) and Control Group (\(n = 6\)). In the Study Group, a 4 mm limbal incision was made and a \(4 \times 4\) mm subconjunctival pocket was created with blunt dissection. A \(4 \times 4\) mm single layer of free amniotic membrane was placed in the pocket in an epithelium-up fashion without suturing. The limbal opening was secured with 10-0 nylon sutures on both sides. In the Control Group, a 4 mm limbal incision was made, a \(4 \times 4\) mm subconjunctival pocket was created with blunt dissection, and the limbal opening was closed with 10-0 nylon sutures on both sides. After the first month, sclero-conjunctival blocks were obtained from the operated area and sections were stained with hematoxylin and eosin, Masson trichrome, and Ki67, SMA and CD34 antibodies.

Results. – The number of fibroblasts, lymphocytes and macrophages was significantly higher in the Study Group than in the Control Group. The number of Ki67\textsuperscript{-} and SMA-positive cells, and CD34-positive vessels was also significantly higher in the Study Group. Amniotic membrane appeared to form folds in all the specimens.
Introduction

Any surgical intervention on the conjunctiva activates the wound-healing cascade in which fibroblasts play a significant role. Excessive fibrosis and scarring after trabeculectomy and glaucoma drainage device implantation leads to decreased success rates and elevated intraocular pressure which needs further medical or surgical intervention. Similarly, excessive scarring especially after repeated strabismus surgery is a major complication and compromises the effect of surgery [1]. To prevent these complications, mitomycin C and 5-fluorouracil have been widely used in glaucoma surgery [2,3].

Amniotic membrane (AM) modulates the multi-step healing and fibrosis cascade which includes the release of blood cells and plasma proteins into the damaged site; the activation of clotting and complement systems; release of growth factors; neutrophil, macrophage and lymphocyte migration to the wound site following by the fibroblast migration and proliferation and eventually wound contraction [4–6]. After the identification that the stromal matrix of AM suppresses DNA synthesis and subsequent differentiation of fibroblasts through suppressing the TGF-β signaling system [7,8], which is the most powerful stimulant of proliferation, migration and collagen synthesis of human Tenon’s fibroblasts [4,7–10], the AM has been also evaluated for the purpose of inhibiting postoperative fibroblastic proliferation. The anti-angiogenic, anti-fibrotic and anti-inflammatory effects of AM have been related to the action of covalent complex of hyaluronan and the heavy chain of inter-α-inhibitor [5,6].

The serious complications, which can occur with mitomycin C and 5-fluorouracil, when used to inhibit the excessive fibrovascular reaction after glaucoma and pterygium surgery, have created the need for safer options such as amniotic membrane [2,3].

The rational of this study was based on the immunologically inert nature, anti-angiogenic [6] and anti-fibrotic properties of AM [7,8]. The outcomes of clinical studies in which AM was used to prevent excessive wound healing and unwanted fibrosis in pterygium, glaucoma and strabismus surgeries were the additional starting point of this study [11–21]. The purpose of our study is to investigate the effect of subconjunctivally transplanted free amniotic membrane on subconjunctival fibrovascular reaction.

Methods

This experimental study was approved by the Local Ethics Committee of Animal Experiments (Ankara Training and Research Hospital, Ankara, Turkey), and conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Visual Research. The study was conducted in...
Ankara Training and Research Hospital Hüsnü Sakal Experimental and Clinical Practice Center.

Animals

Twelve healthy male white New Zealand rabbits (Saki Yenilli Experimental Animal Production Laboratory, Ankara, Turkey) weighing 2.0 to 2.5 kg, were used for the study. The rabbits were divided into two groups randomly: Study Group (n = 6, AM in the subconjunctival pocket) and Control Group (n = 6, conjunctival pocket only).

Preparation of amniotic membrane

Placenta was obtained from hepatitis B virus, hepatitis C virus, syphilis and human immune deficiency virus negative patients from elective cesarean sections. Amniotic membrane was separated from chorion by blunt dissection, washed with sterile saline and cleaned free of blood clot and stored in saline containing penicillin G (50 mg/mL), streptomycin (50 mg/mL), tobramycin (100 mg/mL) and amphotericin B (2.5 mg/mL) at 4°C for 24 hours. The AM was flattened over sterile cellulose paper in an epithelium-up position. Paper-amniotic-membrane-combined layer was cut in 3 × 3 cm pieces and placed in vials containing 1:1 Dulbecco modified Eagle solution and glycerol. The vials were stored at −80°C. Before use, the membranes were allowed to defrost at room temperature [22–24].

Surgical procedure

Prior to the surgical procedure, the animals were anesthetized with intramuscular ketamine hydrochloride 50 mg/kg (Ketalar, Parke-Davis Eczacibasi, Istanbul, Turkey) and xylazine 10 mg/kg (Rompun, Bayer, Istanbul, Turkey) supplemented with topical anesthesia (0.5% proparacaine hydrochloride, Alcain, Alcon-Couvreur, Puurs, Belgium). Eyes were prepared and draped. In all animals, dorso-temporal conjunctiva of the right eyes, which do not cover any extraocular muscle, was chosen. In the Study Group, a 4 mm limbal incision was made and 4 × 4 mm subconjunctival pocket was created with blunt dissection. A monolayer of free amniotic membrane of 4 × 4 mm dimensions was placed in the pocket in an epithelium-up fashion without suturing. The limbal opening was secured with 10-0 nylon sutures on both sides. In the Control Group, a 4 mm limbal incision was made and 4 × 4 mm subconjunctival pocket was created with blunt dissection and the limbal opening was closed with 10-0 nylon sutures on both sides. All the eyes were treated with topical ofloxacin 0.3% (Exocin, Allergan Pharmaceuticals Inc., County Mayo, Ireland) four times per day for one week. The animals were euthanized at the first month by intravenous injection of thiopental sodium (Ekipental, TÜM Ekip, Istanbul, Turkey). The operated conjunctival area was identified by the remaining sutures and excised with underlying sclera as a sclero-conjunctival block including only the operated area.

Pathologic examinations

The sclero-conjunctival blocks were fixed in 10% neutral buffered formalin (pH 7.2-7.4) and 4-μm-thick paraffin sections were stained with hematoxylin and eosine (H&E), Masson trichrome, and Ki67 (Novoceastra Ki67-MM1-CE-S, clone: MM1, Ig G1, Leica Biosystems Newcastle Ltd, United Kingdom), SMA (α-smooth muscle actin, Novoceastra SMA-R7-CE, clone: αsm-1, Ig G2a, Leica Biosystems Newcastle Ltd, United Kingdom) and CD34 (Endothelial cell marker, Novoceastra END-CE-S, clone: Q8End/10, Ig G1, Leica Biosystems Newcastle Ltd, United Kingdom) antibodies. Ki67 and SMA antibody stains were used to demonstrate the active fibroblasts. CD34 was used to demonstrate vessels. The specimens were evaluated using a light microscope (BX50, Olympus Corp., Tokyo, Japan) at 40- to 400-fold magnification in a masked fashion. The average of the cell counts taken from three randomly selected sections was accepted as the final cell count. The cells were counted in 10 randomly selected high power fields (×400 objective) by help of an eyepiece grid. Photographs were taken using a 5-megapixel camera (Olympus DP25, Olympus Corp., Tokyo, Japan).

Data analysis

The sample size calculation indicated that 12 rabbits were required to detect a 10cells/mm² difference in the mean fibroblast counts between the groups at a two-sided significance level of 0.05 and 80% power, assuming that standard deviation is 5cells/mm². Data analysis was performed using Statistical Package for Social Sciences for Windows software (SPSS version 16.0, SPSS Inc. Chicago, USA). All the evaluated variables were continuous. Dependent on the small sample size, the descriptive statistics were expressed as median (minimum-maximum) and the groups were compared by Mann Whitney U test.

Results

Amniotic membrane appeared to form folds in all the specimens (Figs. 1 and 2). Number of fibroblasts (P=0.002), lymphocytes (P=0.002) and macrophages (P=0.002) were significantly higher in the Study Group than in the Control Group both with H&E and Masson trichrome stains (Table 1).
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Number of Ki67- positive cells (P = 0.002) (Figs. 3 and 4) and SMA-positive cells (P = 0.002) (Figs. 5 and 6), and CD34-positive vessels (P = 0.002) (Figs. 7 and 8) were also significantly higher in the Study Group (Table 1).

Discussion

Excessive subconjunctival fibroblastic reaction is a problem in glaucoma [25], pterygium [11–13] and strabismus surgery [26–28]. In glaucoma surgery, excessive fibrosis leads to failure necessitating additional surgeries or medications. In strabismus surgery, fibrosis compromises the effect of the surgery, which is a challenging complication and difficult to manage with repeated surgeries. Recurrent pterygium with visual and cosmetic unwanted appearance is another result of excessive fibrosis.

As the quiescent subconjunctival fibroblasts convert to active fibroblasts that gain capacity to produce extracellular matrix and lead to fibrosis, most of the efforts to inhibit subconjunctival scarring have been directed toward the fibroblasts that play key role in fibrosis.

For this purpose, after the establishment of anti-fibroblastic effects of 5-fluorouracil [29] and mitomycin C [30], they gained wide clinical use. Although these agents

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Histopathologic characteristics of Study group (subconjunctival amniotic membrane transplantation) and Control Group.</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Study Group</td>
</tr>
<tr>
<td></td>
<td>Median (min-max)</td>
</tr>
<tr>
<td><strong>Hematoxyline and eosine</strong></td>
<td></td>
</tr>
<tr>
<td>Fibrocyte/mm²</td>
<td>87.5 (72-96)</td>
</tr>
<tr>
<td>Active fibrocytes (%)</td>
<td>35 (30-35)</td>
</tr>
<tr>
<td>Inactive fibrocytes (%)</td>
<td>65 (65-70)</td>
</tr>
<tr>
<td>Lymphocytes/mm²</td>
<td>33 (19-44)</td>
</tr>
<tr>
<td>Macrophages/mm²</td>
<td>7 (2-12)</td>
</tr>
<tr>
<td><strong>Masson trichrome</strong></td>
<td></td>
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<tr>
<td>Fibrocyte/mm²</td>
<td>89 (74-98)</td>
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<tr>
<td>Active fibrocytes (%)</td>
<td>35 (30-35)</td>
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<tr>
<td>Inactive fibrocytes (%)</td>
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<tr>
<td>Lymphocytes/mm²</td>
<td>33 (19-44)</td>
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<tr>
<td>Macrophages/mm²</td>
<td>7 (2-12)</td>
</tr>
<tr>
<td><strong>Ki67-positive cells</strong></td>
<td>12 (11-14)</td>
</tr>
<tr>
<td><strong>SMA-positive cells</strong></td>
<td>11.5 (10-12)</td>
</tr>
<tr>
<td><strong>CD34-positive vessels</strong></td>
<td>9 (8-11)</td>
</tr>
</tbody>
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P values denote the results of Mann Whitney U test between the Study Group and the Control Group.
increased the success of glaucoma and pterygium surgeries, complications also increased [2,3]. Thus, the need for a solution with fewer side effects became apparent.

Amniotic membrane has been shown to have anti-fibrotic effects by suppressing TGF-β activity, DNA synthesis and differentiation of fibroblasts [7,8]. Depending on this anti-fibrotic effect, the AM has been investigated experimentally and used clinically to prevent excessive wound-healing and unwanted fibrosis.

The first article reporting the beneficial effects of subconjunctival amniotic membrane transplantation (AMT) in pterygium treatment was published in 1998, reporting 4 patients with recurrent pterygium and symblepharon [31]. The authors placed a small limbal autograft over a large AM leaving the peripheral portions of the membrane uncovered. Since then, other articles reporting a small number of pterygium cases were published [11–13,32,33]. Promising results of limbal autografts transplanted over AM in five cases with total limbal deficiency have also been reported [34].
The beneficial effects of subconjunctival AMT placed under or over the scleral flap in trabeculectomy have been reported both in animals and humans. Less scar formation and less fibroblasts have been demonstrated in amniotic membrane transplanted rabbits than in controls [15,35,36]. Subconjunctival AMT has also been used to increase the success of trabeculectomy [14—20] and tube implantation [21] in eyes at high risk for failure, and promising results have been obtained.

The subconjunctival fresh AM has been studied in experimental strabismus surgery and its anti-fibrotic effect was demonstrated [37]. Four of six human case reports treated with AM wrapped around extraocular muscles have been reported to have good results [26—28,38].

Controversially, there are also some studies that show histopathologically more inflammatory reaction after the use of AM. It has been reported previously that conjunctival inflammation occurred more frequently in pterygium patients-treated with AM than those treated with conjunctival surgery alone [39]. Similarly, Barton et al. demonstrated marked granulomatous inflammation after trabeculectomy covered by cryopreserved AM compared to conjunctival flap between 2 to 4 weeks [15]. Wang et al. also showed increased inflammation in rabbits after AM-assisted trabeculectomy (AM placed under scleral flap), compared to the mitomycin C-assisted trabeculectomy and trabeculectomy eyes [40]. In a rabbit strabismus model, it has been shown that lyophilized AM insignificantly decreases postoperative adhesions and fibrosis after strabismus surgery [1]. In another strabismus model, fresh AM caused more inflammation than control eyes at the second month [37]. Two of six human cases who were treated with AM wrapped around extraocular muscles have been reported to develop extensive adhesions [26—28,38].

The effect of subconjunctival AM on subconjunctival fibrosis was studied in glaucoma and strabismus rabbit models. However, its effect has not been studied in a simple conjunctival surgery. We evaluated the effect of cryopreserved AM placed in a subconjunctival pocket as a free monolayer without suturing. Our results demonstrated that one month postoperatively, fibrovascular reaction was greater in the Study Group than in the Control Group and AM formed folds rather than remaining as a straight layer. Our results are not consistent with the results of clinical studies that report favorable results after amniotic membrane-assisted conjunctivolimbal autografts [11—13,32,33].

The authors of the studies that have found more inflammation after AMT than without AMT stated that the reason is unclear [15,37,39]. The possible reasons that have been postulated by these authors were the longer suture stay in AMT group [39] and possible xenograft reaction [15,37].

The possible reason for the higher inflammation in our study could be the technique of amniotic membrane transplantation. In previously published amniotic membrane-assisted conjunctivolimbal autografts [11—13,32,33], the AM was sutured to the episclera as a tight monolayer; however, we transplanted it in the subconjunctival pocket as a free layer without sutures. In histopathological sections, this unsutured AM appeared to form folds. The stronger fibrovascular reaction proven by our histopathological evaluation may have occurred due to free AMT that formed folds. These folds may have led to decreased contact surface between AM’s substantia propria and recipient episclera. Thus, anti-inflammatory and anti-fibrotic potential remain entrapped between these folds and did not show its anti-fibrotic and anti-inflammatory actions. Another hypothetical reason may be the possible movement of the AM as a result of blinking. This hypothetical movement may have stimulated inflammation, mechanically. Although it is demonstrated that AM does not cause immune rejection at the first implantation [41], a xenograft reaction can be postulated as another possible reason.

In conclusion, the stronger fibrovascular reaction that occurred in our histopathological examinations indicates that cryopreserved free human AM without suturing is not useful to decrease subconjunctival fibrovascular reaction at the first postoperative month in rabbit eyes and postulates that it wouldn’t be useful in a clinical basis. However, the morphologic effects of tightly-sutured subconjunctival amniotic membrane to episclera have not been studied and needs to be evaluated histopathologically.

Disclosure of interest
The authors declare that they have no conflict of interest concerning this article.

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