Effect of cryopreserved amniotic membrane on the mechanical properties of skeletal muscle after strabismus surgery in rabbits

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Abstract

PURPOSE: To study the functional recovery of the superior rectus muscle (SRM) after its partial resection in a rabbit model with and without cryopreserved amniotic membrane (AM).

MATERIAL AND METHODS: Resection of the right and left SRMs of 30 rabbits was performed. On the left eyes, a single sheet of equine cryopreserved AM was placed covering the muscle edge sutured. Active and passive mechanical properties of muscles operated with and without AM were monitored over time at 30 (n=10), 60 (n=10) and 90 (n=10) days after surgery. Muscle samples were extracted and electrically stimulated to register the force exerted by the samples, characterizing its active behavior. They were, then, subjected to stretching test to obtain its resistance to deformation, known as passive behavior. Moreover, right and left eyes of a control group (n=5) were equally subjected to active and passive tests to characterized the physiological behavior of SRM muscles.

RESULTS: On active function examination, statistically significant differences were documented between the following: control vs AM and no AM at 30 days (p=0.002 and p=0.04, respectively). All other comparisons were insignificant (p>0.05). On passive function analysis, significant differences were only found between control vs. no AM at 30 days (p=0.004) and between AM vs. no AM at 30 days (p=0.002). Indeed, muscle operated without AM did not recover a normal passive function until 60 days after surgery.

CONCLUSION: Cryopreserved AM is effective in accelerating recovery of SRM passive function in rabbits. Nevertheless, AM produced no significant effect on recovery of SRM active function.

Key words: rabbit; amniotic membrane; strabismus; extraocular muscle; mechanical behavior

1. INTRODUCTION

Strabismus is a misalignment of the visual axes due to an imbalance of the extraocular muscle (EOM) functions. This is a common ophthalmic pathology, affecting from 2% to 5% of general population (1–3). The surgical treatment of a high number of strabismus cases includes the recession to weaken an overacting muscle or the resection to strengthen an underacting muscle (4). As a consequence of surgical alterations, biomechanical changes could be induced leading on short and long-term effects on the EOM functions. Moreover, one of the major complications in strabismus surgery is the development of postoperative adhesions involving the EOM, Tenon's capsule, conjunctiva, orbital fat and sclera (5,6).

Among various techniques to reduce the formation of these postoperative adhesions and muscle fibrosis, the use of amniotic membrane (AM) has been widely described and numerous studies reported its positive effect as an adjunct in the strabismus surgery (7–14). AM acts as a temporary biological barrier between surfaces, preventing adhesion formation. Besides, its stromal matrix presents anti-inflammatory and anti-scarring actions and reduces neovascularization (15).

This tissue could be used fresh or preserved, through lyophilization or cryopreservation. Fresh AM is not recommended because of the risk of communicable diseases transmission (6). Lyophilized AM is incubated in EDTA to deprive it of epithelial cells, then air-dried, vacuum-packed and finally sterilized by gamma-irradiation (16). This method is safer than the fresh membrane and cheaper than the cryopreserved one. However, in their studies, Kassem et al. (17,18), revealed that lyophilized AM is not able to prevent adhesion formation or reduce EOM fibrosis. Moreover, Chun et al. (19) described a similar inflammation level between operated eyes with and without lyophilized membrane. Thus, cryopreserved AM, stored in glycogen and frozen at -80ºC, has been reported as the best method to avoid membrane damage and maintain its properties (20).

The efficacy of the cryopreserved AM for preventing adhesions and muscle fibrosis has been reported by Kirsch et al. (12), Kassem et al. (20) and Sheha et al. (8). However, all these investigations were based on histopathological studies or clinical case report, which do not give information about the functional recovery of the EOM. Since these muscles are the principal responsible of eye alignment, changes in motor tone or strength result in a modification of the rotational position of the globe (4).

Active behavior refers to the contractile capacity of the muscle to produce force, whereas passive behavior is the muscle resistance to deformation. Both functional characteristics of EOM contribute to the correct binocular vision (21) and they can be affected on a strabismus surgery (22,23). Therefore, it can be deduced the importance of EOM active and passive mechanical recovery after a strabismus surgery. However, to the

authors' knowledge no mechanical studies about EOM after surgery with cryopreserved AM have been published.

The aim of this work is to study the functional recovery of the superior rectus muscle (SRM) after its partial resection, together with cryopreserved AM transplantation in a rabbit model, by monitoring its active and passive mechanical properties over time.

2. MATERIAL AND METHODS

2.1. ANIMALS AND ETHICS REQUIREMENTS

Thirty-five male New Zealand white rabbits, two-month aged (2150 ± 100 g) obtained from the Animal Experimentation Service of the University of Zaragoza were used. All animals were healthy and free of clinically observable ocular diseases. This research was conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All procedures were performed under Project License 27/16 approved by the in-house Ethics Committee for Animal Experiments of the University of Zaragoza. The care and use of the animals were performed in accordance with the Spanish Policy for Animal Protection RD53/2013, which meets the European Union Directive 2010/63 on the protection of animals used for experimental and other scientific purposes.

2.2. GROUPS

SRM from rabbit eyes were divided into three groups as explained:

- **Control group (n=10, right and left eyes)**: this group was used to determine active and passive physiological behavior of SRM, without being subjected to surgery.
- **AM group (n=30, left eyes)**: these SRM were resected and wrapped with cryopreserved amniotic membrane.
- **No AM group (n=30, right eyes)**: these SRM were resected without an amniotic membrane wrap.

The AM and No AM groups were each divided in three subgroups (n=10 each), in order to determine the functional recovery of SRM over time at 30, 60 and 90 days after surgery, respectively.

2.3. Surgical procedure

Animals were intramuscularly anesthetized with medetomidine (0.14 mg/kg, Medeson®; Uranovet, Barcelona, Spain), buprenorphine (0.02 mg/kg, Buprex®; Fort Dodge, Girona, Spain) and ketamine (20 mg/kg, Imalgene 1000®; Merial, Barcelona, Spain), and topically with tetracaine hydrochloride 0.1% and oxybuprocaine 0.4% eye drops (Colircusi anestésico doble®; Alcon Cusi, Barcelona, Spain). A superior rectus muscle resection described as strabismus treatment was performed in both eyes (24) (Figure 1). Firstly, the muscle was isolated through a conjunctival incision parallel to the limbus followed by dissection (Fig. 1A, 1B and 1C). The muscle was secured placing two sutures (temporal and nasal) at 3 mm of the scleral insertion of the muscle (Fig. 1D and 1E). Subsequently, 1 mm muscle segment anterior to the sutures was resected (Fig. 1E), and the muscle was sutured back to its original insertion site using the sutures previously fixed. An additional suture was placed in the center to ensure a correct apposition of the muscle edges (Fig. 1F). All sutures included the full-thickness of the muscle, and scleral or conjunctival passes were avoided. Finally, the conjunctival incision was closed by a continuous suture (Fig. 1H). In both procedures, 7/0 absorbable monofilament polyglycolic acid (Sinusorb®; Péters Surgical, Bobigny, France) was used. All surgeries were performed by the same surgeon (A.O.) to avoid inter individual variations.

The effect of cryopreserved equine AM on the recovery of the muscular mechanical behavior was assessed. Therefore, on the left eyes a single sheet of this biomaterial (5 mm wide) was placed following the technique described by Kassem et al. 2013 (20). The muscle edges sutured were covered with AM as a bandage without sutures, with the stromal side towards the muscle (Fig. 1G).

Immediately after the surgical procedure, to minimize pain and avoid infections, intramuscular buprenorphine (0.05 mg/kg twice a day, Buprex®; Fort Dodge, Girona, Spain) and enrofloxacine (10 mg/kg twice a day, Alsir 5%®; Esteve, Barcelona, Spain), subcutaneous meloxicam (0.2 mg/kg once a day, Loxicom®; Karizoo, Barcelona, Spain), and a topical broad-spectrum antibiotic (twice a day, Oftalmowell®; UCB Pharma, Madrid, Spain) were administered in all animals for five days.

2.4. Mechanical testing

2.4.1. *Muscle preparation*

All animals were humanely euthanized with intravenous sodium pentobarbital (150 mg/kg, Dolethal®; Vétoquinol, Madrid, Spain). After eye exenteration, conjunctiva was dissected to expose the EOM. A blunt dissection of the SRM was carried out, cutting off the muscle with a small section of sclera. Length, width and

thickness measurements of each sample were registered with a digital caliber (Mitutoyo Absolute Digimatic Series 227, Mitutoyo Inc., IL, USA). Finally, samples were oriented vertically in a methacrylate organ bath (20x20x20 cm) and installed in an electromechanical Instron Microtester 5248 (Illinois Tool Works Inc., Glenview, IL, USA) with a 5 N full scale load cell (see Fig. 2). This electromechanical test machine allows us to register active and passive forces developed by the muscles.

The scleral end was fixed inside the bath and the upper end (distal tendon) to the machine actuator by means of a grip, based on a method for the muscle sample positioning inside the bath previously published (25). Temperature of the bath solution (Ringer's solution) was maintained at 27ºC and saturated with carbogen gas. A pair of platinum plate electrodes was used to field stimulate the muscles, and they were connected to a CIBERTEC CS-20 electrical signal generator (25).

After preparation, muscle samples were subjected to *in vitro* electrical stimulation to register its contractile properties, considered its active behavior. Then, the samples were stretched to determine its resistance to stretch, equivalent to its passive behavior

2.4.2. *Active behavior*

Each SRM from Control, AM and No AM groups, was subjected to electrical pulses (1 ms and 60 V), applied for the development of isometric twitch forces, varying muscle length. The length at which the muscle force production was maximum, was considered as the optimal length of each muscle sample.

After optimal length determination, stimulation protocols differed between control and operated muscles. Control muscles were subjected to electrical voltage and frequency sweeps in order to determine the optimal stimulation parameters (length, voltage and frequency values at which the force production was maximum) of the SRM. Thereby, electrical pulses from 40 to 100 V were applied to the muscle for 1 ms each one. Two minutes were respected between stimuli. At the end, the sample rested for five minutes. Then, trains of pulses at increasing frequencies (from 20 to 100 Hz) were applied. Finally, 90 Hz, 100 V and 500 ms were chosen as the optimal parameters of SRM stimulation.

Operated muscles were tested 30, 60 and 90 days after surgery. To register their active forces, all samples (with and without AM) were stimulated with 3 trains of pulses at 90 Hz, 100 V and 500 ms. These parameters were established and fixed according to the control sample results that provided maximum force. This active test allowed us to monitor the active behavior, or capacity to produce force, of the SRM over the time.

In order to compare the active behavior of all the samples, the active force produced by each one was normalized. Therefore, stress was calculated as active force (F) divided by muscle cross sectional area (Width x Thickness) of the control and operated SRM over time.

2.4.3. *Passive behavior*

After force characterization, the resistance to stretching, or passive behavior, of all the samples was measured. Thus, muscles were subjected to stretching through uniaxial tension tests at $0.1\% \cdot s^{-1}$ deformation rate. The experiment finished when the testing machine achieved the load or stretch limits, or the muscle sample broke. This passive tests allowed us to obtain stress (σ) – stretch (λ) curves of each sample, i.e. passive force exerted per unit area at different stretch levels (stretch is defined here as the final length divided by the initial length).

To compare the resistance to stretching of the muscles operated with and without AM over time, the slope of lineal regions of stress (σ) – stretch (λ) curves obtained after passive tests, was also calculated. This slope is called the maximum tangent modulus (E_{max}) and it mesaures the tissue stiffness, or resistance to stretching (Fig. 3).

After carrying out the mechanical tests, samples were weighted and their volumes determined.

STATISTICAL ANALYSIS

Central tendency and dispersion of the quantitative variables were calculated and expressed as mean ± standard deviation. The statistical analysis was based on the multifactorial analysis of variance (ANOVA), with a standard threshold of 5%. Scheffe's correction for multiple comparison groups was used. Before the ANOVA test, normal distribution of sample population was verified by a Kolmogorov-Smirnov test, also with a standard threshold of 5%.

3. RESULTS

Characteristic parameters of the samples analyzed are shown in Table 1 for the Control, AM and No AM groups. Thickness values showed statistically significant differences between control and 30, 60 and 90 groups ($p=0.004$, $p=0.001$ and $p=0.001$, respectively) in muscles operated with AM and between control and 60 ($p=0.012$) and 90 ($p=0.001$) groups in muscles operated without AM (Table 1). However, comparing the results at each time separately, there were not statistically significant differences between operated muscles with and

without AM. No correlation was found between thickness and functional muscle behavior, suggesting that thickness increasing of extraocular muscles didn't have consequences on functional recovery.

3.1. Active behavior

Figure 4 shows the % maximum stress values, corresponding to normalized muscle active behavior. As explained before, stress was calculated as active force divided by muscle cross sectional area. Control stress values were compared to AM and No AM stress.

At 30 days, muscles operated with ($p=0.002$) and without AM ($p=0.04$) showed a statistically significant drop of stress production ($35.04 \pm 22.13\%$ and $57.88 \pm 13.53\%$ of the control stress, respectively). At that time, AM and No AM force productions per sectional area were lower than control muscle force production. After 60 days, no statistically significant differences were found between maximum control stress and SRM with ($p=0.369$) or without AM ($p=0.953$): $65.33 \pm 11.07\%$ and $77.53 \pm 9.58\%$ of maximum initial stress, respectively. No statistically significant differences were observed neither at 90 days after surgery between control and operated muscles (AM group ($p=0.720$) produced $75.36 \pm 21.31\%$ of maximum initial stress and No AM group ($p=0.753$), $72.68 \pm 21.22\%$). Moreover, a comparison between groups with and without AM at each time was carried out. There were no statistically significant differences, showing an insignificant effect of the AM use in the muscle force production at 30, 60 and 90 days ($p=0.114$, $p=0.354$ and $p=725$, respectively). Thus, after 60 days SRM would recover its normal capacity to produce force indistinctly of AM use.

3.2. Passive behavior

As explained in Methods' section, muscle stiffness, measured in N/mm^2, was calculated as the slope of the linear zones of the curves obtained from passive tests. Then, muscle stiffness comparisons were carried out between Control group and AM and No AM groups (Fig. 5).

Thus, control stiffness was 2.06 ± 0.44 N/mm^2 whereas the operated muscles with AM presented a stiffness of 1.21 ± 0.81 N/mm^2, 1.95 ± 0.95 N/mm^2, and 1.83 ± 1.25 N/mm^2 after 30, 60 and 90 days, respectively. No statistically significant differences were found between groups ($p=0.402$, $p=0.998$ and $p=0.975$). Regarding the operated muscles without AM, the stiffness was 3.98 ± 1.15 N/mm^2, 2.14 ± 0.82 N/mm^2, and 1.65 ± 0.52 N/mm^2 after 30, 60 and 90 days, respectively. Statistically significant differences were found between control and 30 days ($p=0.004$). At 60 and 90 days, statistically insignificant differences ($p=0.998$ and $p=0.839$,

respectively) were determined. Comparing at 30 days, muscle stiffness from SRM operated without AM was significantly different of muscle stiffness from SRM operated with AM (p=0.002). Hence, AM would have a positive effect on passive behavior recovery of SRM.

4. DISCUSSION

Since strabismus is caused by an imbalance between EOM, it is important to know their functional characteristics after surgery, as well as the effect of complementary treatments on muscle active and passive behavior. The *in situ* models have been described as the suitable models to study absolute power production of extraocular muscles. However, its high complexity is an important limitation. By contrast, *ex vivo* models, presenting lower complexity, are accepted to carry out comparative studies, where relative active and passive tension are calculated (26,27). Thus, the *ex vivo* rabbit model of strabismus described here is considered as a valid model to assess the recovery of contractile properties and resistance to stretching in extraocular muscles.

The use of cryopreserved amniotic membrane after strabismus surgery has been deeply studied in the literature (9,10,12,17). Among their main effects, AM reduces the tissue inflammation, neovascularization and scar formation of the muscle resected or recessed (15). However, despite the importance of EOM active and passive response, no mechanical studies have been published to compared its functional recovery after using AM. The *ex vivo* model presented in this study allowed to study the benefits of using cryopreserved AM after a strabismus surgery and its effects on the mechanical recovery of the SRM.

The active stress data through time observed in muscles operated with and without AM, did not showed statistically significant differences. At 60 days, both groups (AM and No AM) recovered its functional capacity to produce force. These data suggested that cryopreserved AM did not improve the recovery of the active muscle properties. The significant muscle stress reduction showed at 30 days AM could be related to the increase of inflammation provoked by the membrane, previously described by different authors (12,20,28,29). This phenomenon, related to the xenogenic nature of the equine AM, would reverse the anti-inflammation effect of the membrane, slowing down the muscle active properties recovery. Further studies are needed to investigate allogeneic use of AM and its effects on muscle functional recovery.

Regarding the passive behavior, several studies have demonstrated the cryopreserved membrane effectiveness for reducing postoperative adhesions and scar formation (8,12,13,20). However, no mechanical characterizations of *ex vivo* extraocular muscle passive properties have been described; although their histological analysis showed a fibrosis reduction when AM was used. Whereas Kirsch et al. (12), reported this

after 30 days, Sheha et al. (8), demonstrated a positive effect of the AM in the eye alignment six weeks after surgery. Nevertheless, Kassem et al. (20), did not observe differences in muscle fibrosis production regardless the use of AM after 42 days. Therefore, these results would be coherent with the data presented in this study. Thus, muscles without membrane showed a significantly higher stiffness than muscles with membrane at 30 days after surgery. Together with the histological studies previously published, the results suggest that cryopreserved AM prevents muscle fibrosis production, improving an earlier recovery of the EOM resistance to stretching after a strabismus surgery. The initial tension of EOMs plays an important role in keeping the eye suspended, and it comprises passive and active contributions of the muscle (30). Considering the eye alignment as the principal goal of strabismus surgery, it is clear the importance of passive and active properties recovery after the operation. Thus, accelerating passive behavior recovery, cryopreserved AM would improve global recovery after surgery.

In conclusion, cryopreserved equine AM has not showed a positive effect on recovery of rabbit SRM active properties. However, muscles operated with AM showed a significantly faster recovery of passive properties. Since eye alignment depends on passive and active behavior of EOM, cryopreserved AM could improve the global recovery after strabismus surgery. Moreover, the *ex vivo* rabbit model of strabismus described here has been described as a valid model to study the recovery of the active and passive properties in extraocular muscles. Thereby, in the future this model could be applied to test the effect of strabismus alternative methods (botulinum toxin, etc.), in the muscle mechanical behavior.

FUNDING

Spanish Ministry of Economy and Competitiveness (Project DPI2014-54981-R).

Department of Industry and Innovation (Government of Aragón) and European Social Fund 2014-2020 (FSE-DGA T88).

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge Carlos III Health Institute (ISCIII) through the CIBER initiative and the ICTS NANBIOSIS, specifically by the Tissue & Scaffold Characterization Unit (U13) of CIBER-BBN. The authors also thank the Government of Aragón and the Spanish Ministry of Education, Culture and Sports for the financial supports to M. Sierra and A. Ortillés, respectively, and the use of Service General Research Support (SAI), University of Zaragoza. The authors are grateful to AJL Ophthalmic S.A. (Victoria-Gasteiz, Spain) for providing sutures (Sinusorb®).

REFERENCES

1. Donnelly UM, Stewart NM, Hollinger M. Prevalence and outcomes of childhood visual disorders. Ophthalmic Epidemiol. 2005;12:243–250.

2. Friedman DS, Repka MX, Katz J, Giordano L, Ibironke J, Hawes P, et al. Prevalence of decreased visual acuity among preschool-aged children in an American urban population: the Baltimore Pediatric Eye Disease Study, methods, and results. Ophthalmology. 2008;115:1786–95, 1795-1804.

3. Stidwill D. Epidemiology of strabismus. Ophtalmic Physiol Opt. 1997;17:536-539.

4. McLoon LK, Anderson BC, Christiansen SP. Increasing Muscle Strength as a Treatment for Strabismus: Sustained Release of Insulin-like Growth Factor-1 in Rabbit Extraocular Muscle. J AAPOS. 2006;10:424–429.

5. Mora JS, Sprunger DT, Helveston EM, Evan AP. Intraoperative sponge 5- fluorouracil to reduce postoperative scarring in strabismus surgery. J AAPOS. 1997;1:92–97.

6. Ozkan SB, Kir E, Culhaci N, Dayanir V. The effect of Seprafilm on adhesions in strabismus surgery, an experimental study. J AAPOS. 2004;8:46–49.

7. Yamada M, Shinoda K, Hatakeyama A, Nishina S, Mashima Y. Fat adherence syndrome after retinal surgery treated with amniotic membrane transplantation. Am J Ophthalmol. 2001;132:280–282.

8. Sheha H, Casas V, Hayashida Y. The use of amniotic membrane in reducing adhesions after strabismus surgery. J AAPOS. 2009;13:99–101.

9. Mehendale RA, Dagi LR. Amniotic membrane implantation to reduce extraocular muscle adhesions to a titanium implant. J AAPOS. 2011;15:404–406.

10. Strube YNJ, Conte F, Faria C, Yiu S, Wright KW. Amniotic Membrane Transplantation for Restrictive Strabismus. Ophthalmology. 2011;118:1175–1179.

11. Tugcu B, Helvacioglu F, Yuzbasioglu E, Gurez C, Yigit U. Amniotic membrane in the management of strabismus reoperations. Jpn J Ophthalmol. 2012;57:239–244.

12. Kirsch D, Lowen MS, Cronemberger MF, Sato EH. Amniotic Membrane for Reducing the Formation of Adhesions in Strabismus Surgery: Experimental Study in Rabbits. J Pediatr Ophthalmol Strabismus. 2014;51:341–347.

13. Frangouli O, Adams GGW. The use of amniotic membrane for the management of fibrosis in complex strabismus surgery. Strabismus. 2013;21:13–22.

14. Kassem RR, Kamal AM, El-Mofty RMA-M, Elhilali HM. A controlled study of the role of cryopreserved amniotic membrane transplant during strabismus reoperations. J AAPOS. 2017;21:97–102.

15. Tseng SCG, Espana EM, Kawakita T, Di Pascuale MA, Li W, He H, et al. How does amniotic membrane work? Ocul Surf. 2004;2:177–187.

16. Gomes JAP, Romano A, Santos MS, Dua HS. Amniotic membrane use in ophthalmology. Curr Opin Ophthalmol. 2005;16:233–240.

17. Kassem RR, Gawdat GI, Zedan RH. Severe fibrosis of extraocular muscles after the use of lyophilized amniotic membrane in strabismus surgery. J AAPOS. 2010;14:548–549.

18. Kassem RR, Abdel-Hamid MA, Khodeir MM. Effect of lyophilized amniotic membrane on the development of adhesions and fibrosis after extraocular muscle surgery in rabbits. Curr Eye Res. 2011;36:1020–1027.

19. Chun BY, Kim HK, Shin JP. Dried human amniotic membrane does not alleviate inflammation and fibrosis in experimental strabismus surgery. J Ophthalmol. 2013;2013:369126.

20. Kassem RR, Khodeir MM, Salem M, Abdel-Hamid MA, El-Mofty RMA-M, Kamal AM, et al. Effect of cryopreserved amniotic membrane on the development of adhesions and fibrosis after extraocular muscle surgery in rabbits. Acta Ophthalmol (Copenh). 2013;91:e140–48.

21. Robinson DA, O'Meara DM, Scott AB, Collins CC. Mechanical components of human eye movements. J Appl Physiol. 1969;26:548–553.

22. Christiansen SP, Soulsby ME, Seifen EE. Effect of antagonist weakening on developed tension in cat extraocular muscle. Invest Ophthalmol Vis Sci. 1995;36:2547–2550.

23. Rosenbaum AL, Egbert JE, Keogan T, Wheeler N, Wang C, Buzard K. Length-tension properties of extraocular muscles in patients with esotropia and intermittent exotropia. Am J Ophthalmol. 1994;117:791–799.

24. Merriam SW. Surgical Management of Strabismus. Am Orthopt J. 2006;56:202–203.

25. Grasa J, Sierra M, Lauzeral N, Muñoz MJ, Miana-Mena FJ, Calvo B. Active behavior of abdominal wall muscles: Experimental results and numerical model formulation. J Mech Behav Biomed Mater. 2016;61:444–454.

26. Croes SA, Baryshnikova LM, Kaluskar SS, Bartheld CS von. Acute and long-term effects of botulinum neurotoxin on the function and structure of developing extraocular muscles. Neurobiol Dis. 2007;25:649–664.

27. Frueh BR, Gregorevic P, Williams DA, Lynch GS. Specific force of the rat extraocular muscles, levator and superior rectus, measured in situ. J Neurophysiol. 2001;85:1027–1032.

28. Barton K, Budenz DL, Khaw PT, Tseng SC. Glaucoma filtration surgery using amniotic membrane transplantation. Invest Ophthalmol Vis Sci. 2001;42:1762–1768.

29. Demirel S, Atilla H, Okcu Heper A, Erkam N. Effects of amniotic membrane on wound healing and adhesions in experimental strabismus surgery. Eur J Ophthalmol. 2009;19:899–904.

30. Gao Z, Guo H, Chen W. Initial tension of the human extraocular muscles in the primary eye position. J Theor Biol. 2014;353:78–83.

FIGURE LEGENDS

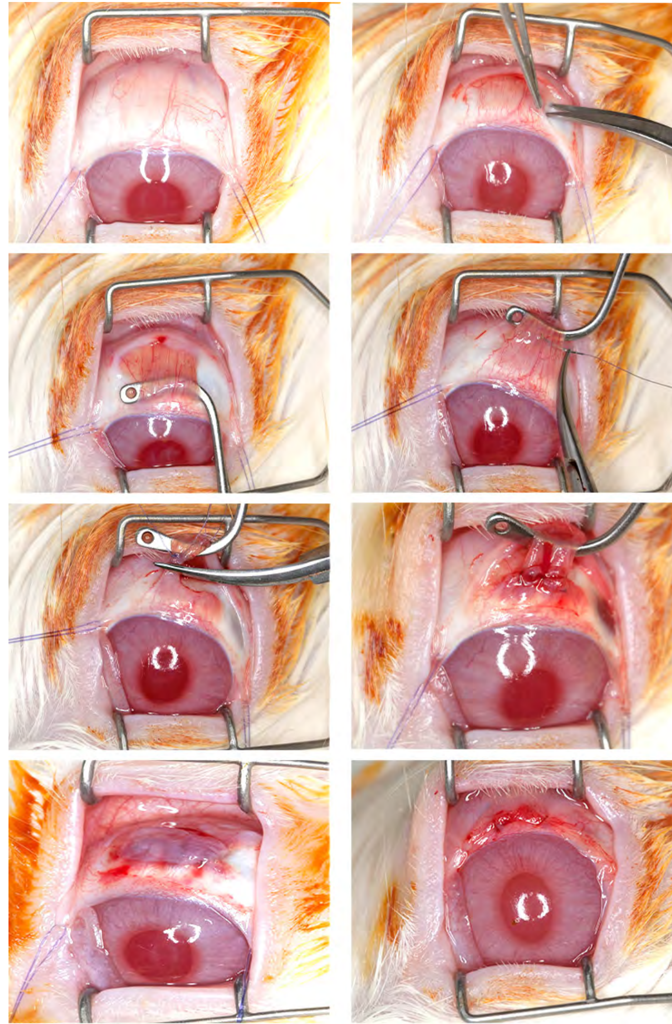
Figure 1. Surgical protocol of superior rectus muscle resection (A-F) and cryopreserved amniotic membrane transplant (G).

Figure 2. Scheme of experimental set up. One muscle end is fixed in a methacrylate organ bath. Then, the bath is placed in a universal electromechanical testing machine (Instron Microtester 5248) where the muscle is fixed and stimulated by means of two platinum plates.

Figure 3. Example of maximum tangent modulus (Emax) or maximum resistance to stretching determination from control SRM tension-deformation curves.

Figure 4. Active behavior: percentage of maximum stress and standard deviation. Statistically significant differences were only found between Control and SRM operated with and without AM at 30 days (*p=0.002 and *p=0.04, respectively).

Figure 5. Passive behavior: Mean Emax (stiffness) SD of operated SRM. Statistically significant differences were determined between Control and SRM operated without AM at 30 days (*p=0.004). Comparing the stiffness results of operated muscles with and without AM, statistically significant differences were also found at 30 days (*p=0.002).



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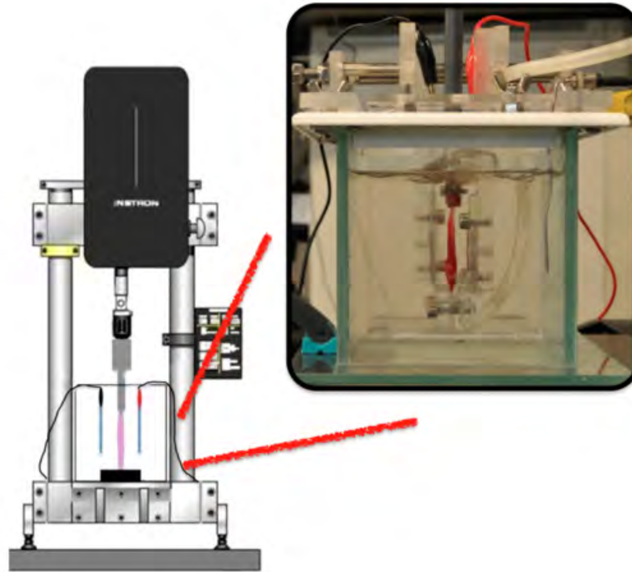

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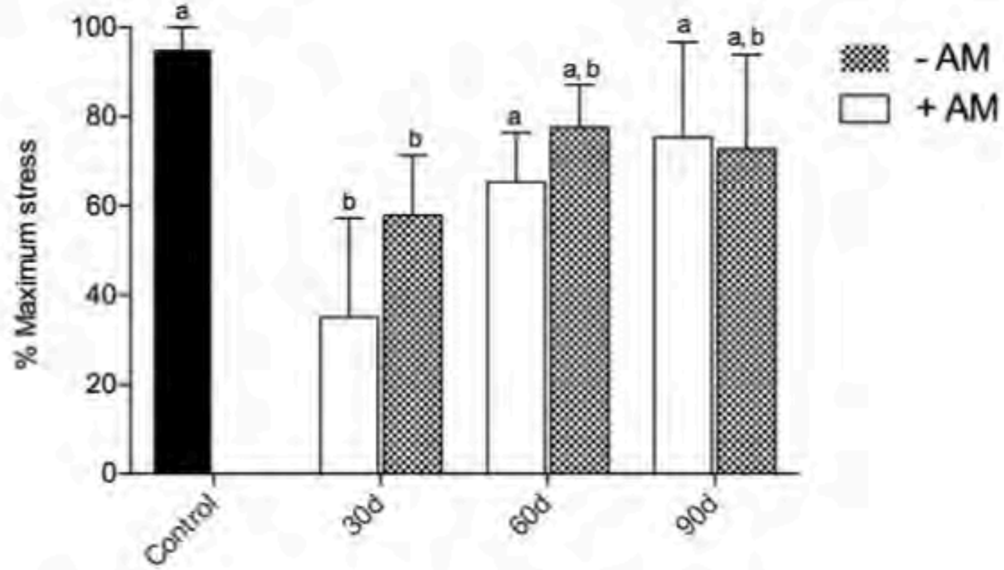
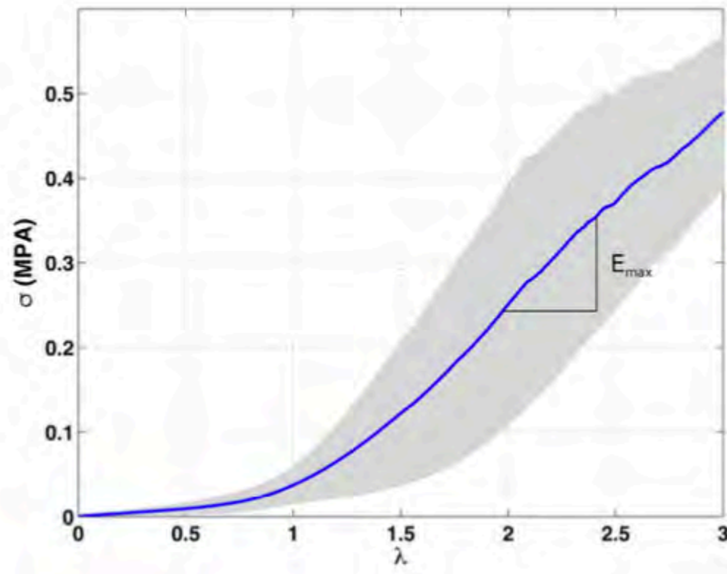


Figure 3. Percentage of maximum stress and standard deviation. In SRM operated with AM, significant differences were detected between control (a) and 30 days (b), 30 (b) and 60 days (a), and 30 (b) and 90 days (a). In SRM operated without AM, significant differences were only detected between control (a) and 30 days (b).



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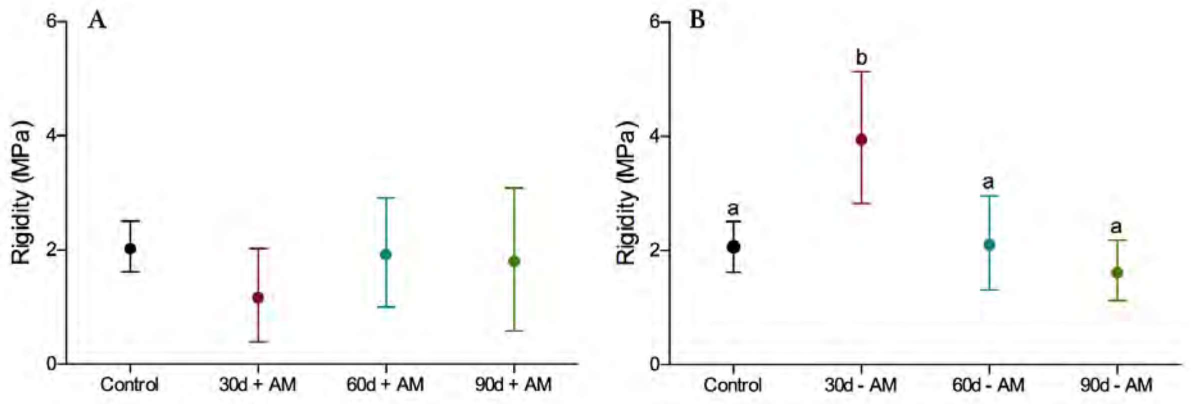

Figure 5. Mean Emax (stiffness) SD of operated SRM. A) With AM no significant differences were found between groups. B) Without AM significant differences were observed between control (a) and 30 days (b), 30 (b) and 60 days (a), and 30 (b) and 90 days (a).

Group	**30 days**	**60 days**	**90 days**
Control (n=10)	-	-	-
With AM	n=10	n=10	n=10
Without AM	n=10	n=10	n=10

Table 1. Distribution of control, with AM and without AM groups.

With AM	Weight (g)	Length (mm)	Width (mm)	Thickness (mm)
Control	0.083 ± 0.03	11.38 ± 3.36	4.05 ± 0.53	0.57 ± 0.17[a]
30 days	0.130 ± 0.03	14.00 ± 2.91	4.34 ± 0.59	0.97 ± 0.20[b]
60 days	0.120 ± 0.03	13.95 ± 2.73	4.21 ± 0.75	1.17 ± 0.26[b]
90 days	0.110 ± 0.04	14.70 ± 3.44	4.09 ± 0.59	1.12 ± 0.23[b]

Table 2. Dimensions of SRM operated with AM (mean SD) for the four groups defined (control, 30, 60 and 90 days). Significant differences were observed between control (a) and 30, 60 and 90 days (b) thickness (*p<0.05, **p<0.01).

Without AM	Weight (g)	Length (mm)	Width (mm)	Thickness (mm)
Control	0.083 ± 0.03	11.38 ± 3.36	4.05 ± 0.53	0.57 ± 0.17^{a}
30 days	0.110 ± 0.06	13.32 ± 2.16	3.91 ± 0.80	$0.69 \pm 0.17^{a,b}$
60 days	0.120 ± 0.04	14.45 ± 2.64	5.53 ± 0.76	0.96 ± 0.23^{b}
90 days	0.090 ± 0.02	15.15 ± 2.74	4.19 ± 0.53	1.07 ± 0.32^{b}

Table 3. Dimensions of SRM operated without AM (mean SD) for the four groups defined (control, 30, 60 and 90 days). Significant differences were observed between control (a), and 60 and 90 days (b) thickness (*p<0.05).