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Topical application of beta-blockers accelerates epithelialization to mesh skin grafted full-thickness burn in sheep

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Abstract

Aim: Beta-adrenergic receptor blockers are conventionally used for the treatment of hypertension, tachycardia, and glaucoma. Research has shown that beta-blockers can accelerate wound epithelialization. In this study, we tested the efficacy of the beta-blocker timolol in an ovine model of grafted full-thickness burn wound healing, which closely mimics clinical scenarios.

Methods: Six full-thickness burn wounds were created on the sheep's posterior surface. Twenty-four hours later, eschars were excised and meshed skin was grafted (Day0). The wounds in the treatment group received topical application of timolol. Blood flow was measured using a blood perfusion imager. Cardiovascular hemodynamics and blood glucose levels were recorded daily. The epithelialization rate on Day 14 was determined by planimetric assay and analyzed by paired *t*-test. The days that the epithelialization rate exceeded 85%, 90%, and 95% were analyzed by survival analysis. To assess the potential influence of TGFβ, epithelial-mesenchymal transition (EMT), or myofibroblast activation (MFA) on wound healing, the RNA abundance of gene products related to these

pathways was measured by reverse transcription and quantitative polymerase chain reaction (RT-qPCR).

Results: The epithelialization rate on Day 14 was significantly higher in the treatment group. The days that the epithelialization rate exceeded 85%, 90%, and 95% were significantly shorter in the treatment group. There was no significant difference in wound blood flow or RNA abundance related to TGFβ, EMT, or MFA-related pathways among the groups at any time point.

Conclusion: The results demonstrate that the beta-blocker timolol accelerates epithelialization of mesh skin grafted full-thickness burn wounds through a mechanism other than improving wound blood flow.

Keywords: Beta-blocker, wound healing, burn, mesh skin graft

INTRODUCTION

Despite numerous studies and developments in burn treatment, limited donor skin and delayed wound healing are still major problems. Surgical treatment for burns generally involves the removal of eschar and skin graft for wound closure[1,2]. In large burns, donor skin is frequently limited, and often the skin is meshed to be grafted. The higher the mesh ratio, the greater the area that can be covered. However, this increases the area of mesh gaps, so epithelialization of the mesh gap takes longer[1,3]. Additionally, the closure of meshes is delayed due to complications such as infection, hematoma, or other factors, even if the wounds are covered with sufficient autologous skin. The longer epithelialization is delayed, the greater the risk for skin and soft tissue infection, the reduced quality of life for patients, and the increased cost of care[2]. Therefore, safer, more effective, readily available, and more cost-effective interventions that accelerate epithelialization of mesh gaps and raw surface areas due to graft loss are very valuable.

Wound healing includes hemostasis, inflammation, proliferation, and tissue remodeling phases[4]. Among these, keratinocyte migration during the proliferation phase is crucial for effective wound epithelialization. Keratinocytes, located in the epidermis, play an important role in skin barrier function. Adrenergic receptors expressed on keratinocytes are mainly beta-2 receptors[5-7], and several response systems triggered by stimulation of the receptors have been shown to reduce the migration ability of keratinocytes[6-8]. Endogenous adrenaline, which decreases the migration ability of keratinocytes, has been reported to cause delayed wound healing[7]. Conversely, beta-adrenergic receptor blockers increase the migration ability of keratinocytes.

Beta-adrenergic receptor blockers are drugs that act on beta receptors in the sympathetic nervous system and inhibit their activation. Beta-blockers are used orally or intravenously for hypertension and tachycardia, as eye drops for glaucoma, and recently for the treatment of infantile hemangiomas orally and topically. In severe burns, beta-blockers have been used as an intravenous therapy to reduce inflammation, resting energy expenditure, and hypermetabolism[9]. Timolol is a beta-adrenergic receptor antagonist and is used as an eye drop to treat glaucoma[10]. Previous studies have demonstrated that timolol can be safely applied topically to promote wound healing[8].

In recent years, some studies have demonstrated the efficacy of beta-blockers in wound healing. Previous studies have reported that beta-blocker infusion promotes epithelialization of flame burns in murine models[7], and that topical timolol improves wound healing of thermal burns in rat models[11]. Additionally, the topical application of timolol with dermal matrix, preconditioned human mesenchymal stem cells promotes wound healing of the total skin defect wound in porcine models[12], and the topical application of timolol promotes the epithelialization of donor wound of split-thickness skin graft in human[13]. However,

the efficacy of topical application of beta-blockers in a mesh skin grafted full-thickness burn wound has not been reported previously. Since endogenous adrenaline is elevated in burn patients and wound healing is delayed[7], topical application of beta-blockers could more effectively promote the epithelialization of burn graft wounds. Therefore, in the present study, we aimed to demonstrate the safety and efficacy of timolol in a clinically relevant large animal model that closely mimics a clinical scenario, i.e., eschar removal within 24 h followed by autologous meshed skin graft, adequate wound dressing, and proper daily wound care and assessment.

METHODS

Animal care

We followed the guidelines of the National Institutes of Health and the American Physiological Society regarding the care and use of laboratory animals, and the protocol of this study was approved by the University of Texas Medical Branch Animal Care and Use Committee (protocol #2003030). Adult Merino female sheep with body weights of 35 to 40 kg were purchased, and all sheep for this study were screened by a veterinarian and housed at the Animal Research Center, where they had free access to food and water.

Surgical procedure and treatment protocol

For surgical procedures, sheep housed in individual cages were transferred to the Translational Intensive Care Unit after more than 14 days of quarantine. Sheep were sedated with an intramuscular injection of 500 mg of ketamine, followed by an intravenous injection (300 mg). They were then intubated under mask inhalation anesthesia with 2%-5% isoflurane. Then, anesthesia was further maintained by 2%-5% isoflurane inhalation via the endotracheal tube. The catheters were placed through skin incision in the groin into the femoral artery and vein for continuous monitoring of systemic arterial blood pressure, heart rate, and resuscitation and intermittent blood sampling, respectively. The analgesia was provided with a subcutaneous injection of 0.1 mg/kg long-acting (72 h) buprenorphine. After catheter placement, three 25 cm^2 full-thickness flame burn sites were made on each side of the dorsum (a total of six sites) [Figure 1], with 5 cm of space between the sites and 3 cm from the spine. A burn-resistant cloth with a 25 cm^2 open space in the middle was installed to prevent burns to the adjacent tissues. A metal frame enclosing an area of 25 cm^2 was put on the open space of the cloth. The burn was induced by flame until skin shrinkage stopped. This method was previously established in our laboratory to create full-thickness skin burns that can be confirmed through histological analysis[14]. No dressing was applied to the burn wounds during the first 24 h after burn creation. After wound creation, anesthesia was discontinued and the sheep were awakened and monitored for 24 h. For pain management, long-acting buprenorphine (72 h) was injected subcutaneously and additional short-acting buprenorphine was injected intravenously per need.

Twenty-four hours after burn creation, sheep were again anesthetized with ketamine and intubated with endotracheal tube for the anesthesia with inhaled isoflurane. The eschar was excised, under analgesia and continued anesthesia, down to the superficial fascia. Split-thickness skin grafts, measuring 0.03 inches in thickness, were harvested from both sides of the buttocks remote from the wounds. The harvested skins were meshed at a ratio of 4:1 and grafted to the wounds [Figure 2]. The day of skin graft surgery was set as Day 0. Wounds were randomly assigned to as either treatment or control. The groups were assigned to allow comparison of contralateral sites as pairs in order to avoid anatomic location-dependent wound healing variations. Wounds in the treatment group received topical application of timolol (0.02 mL/cm^2 of 0.5%), and wounds in the control group received saline in a similar manner. After the application, the wounds were covered with non-adherent strips (Curity, COVIDIEN, MA), and the non-adhesive hydrocellular foam dressing (ALLEVYN, Smith&Nephew, England) was placed on top and secured with skin staples. To apply proper pressure to the grafted skin, sterile gauze was placed on top of the hydrocellular foam, followed by a second layer of large hydrocellular foam that was secured with skin

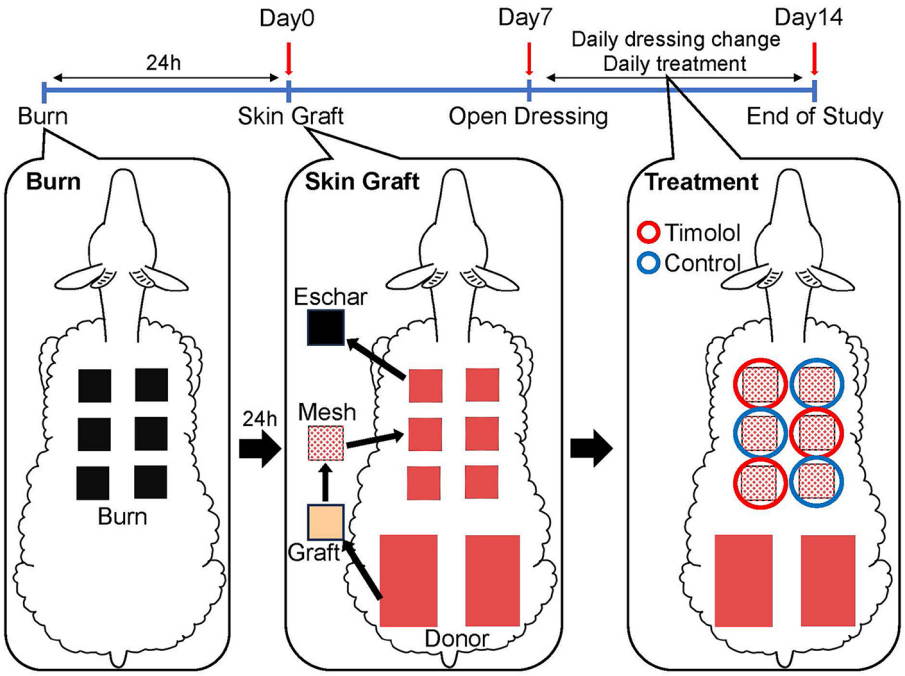

Figure 1. Study design of grafted full-thickness burn wound in ovine model that closely mimics clinical scenario. The full-thickness burn was induced by fire flame (below left). Split-thickness skin grafts were harvested to be meshed and grafted (below center). Wounds in the treatment group received topical application of timolol (0.02 ml/cm^2 of 0.5%), and wounds in the control group received saline in a similar manner. The groups were assigned to allow comparison of contralateral sites as pairs in order to avoid anatomic location-dependent wound healing variations (below right).

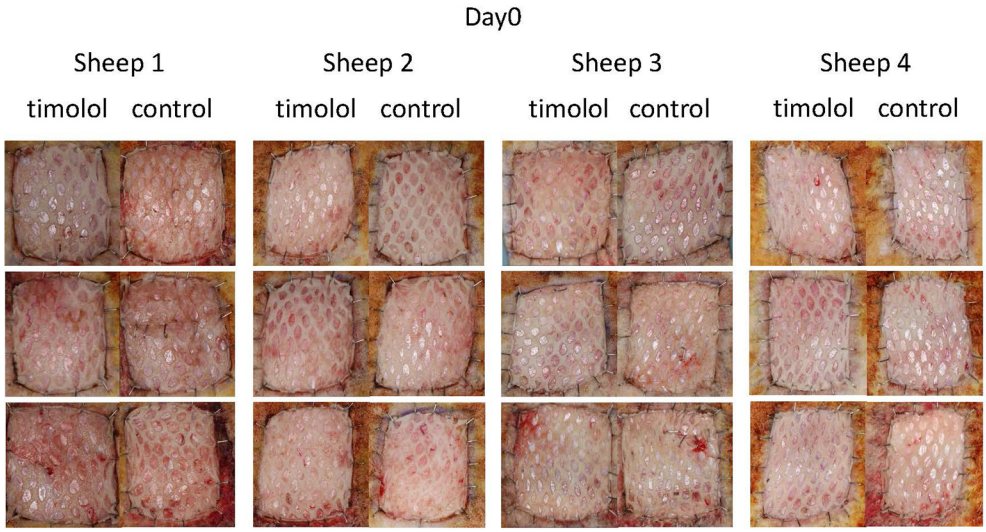

Figure 2. Digital photographs of the wounds of each sheep in the treatment and control groups at the time of mesh skin graft (Day 0).

staples. These dressings were removed on Day 7 [Figure 3]. Wounds were washed with saline and cleaned gently with a sharp spoon. After the application of either timolol or the saline vehicle, the wounds were covered with petroleum jelly and non-adherent pads (Telfa, COVIDIEN, MA). These dressing changes were performed once a day thereafter up to Day 14. On Day 14, animals were euthanized under deep anesthesia with intravenous injections of ketamine (40 mg/kg), xylazine (3.0 mg/kg), and analgesia [buprenorphine

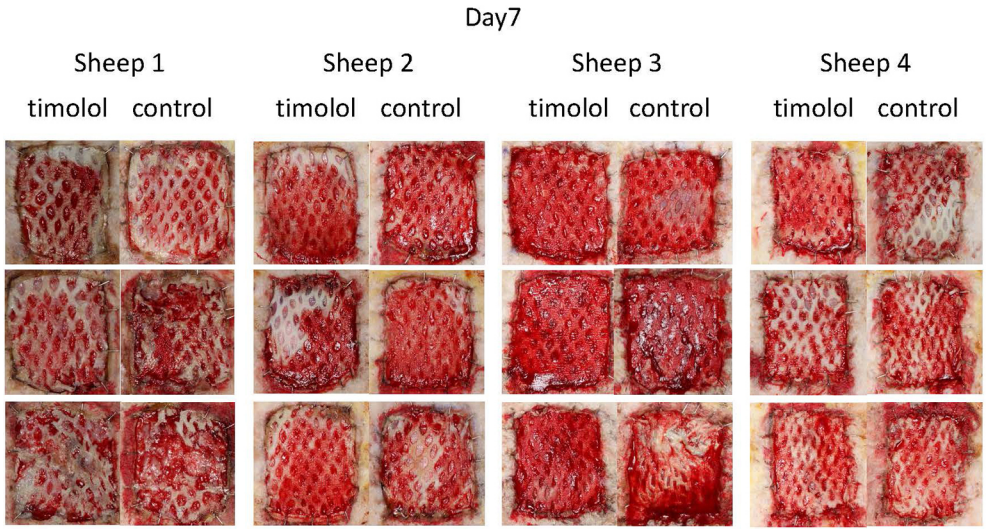

Figure 3. Digital photographs of the wounds of each sheep in the treatment and control groups on Day 7.

(0.01 mg/kg)] following IACUC-approved protocol and the American Veterinary Medical Association Guidelines for Euthanasia.

Measurements and samples

Digital photographs of each wound were taken at the time of skin graft and at each dressing change. The total wound area and epithelialized area were measured from the digital photographs using ImageJ software version 1.53 (National Institutes of Health, Bethesda, MD), and the epithelialization rate was calculated on different days. The number of days for the epithelialization to reach 85%, 90% and 95% were determined. The blood flow in the wounds was measured immediately after excision, after skin grafting, on Day 7, Day 10, Day 12, and Day 14 using the blood perfusion imager PeriCam (Perimed, Stockholm, Sweden). Cardiovascular hemodynamics such as arterial blood pressure, heart rate, and blood glucose levels were measured once a day from Day 0 to Day 14 to reveal any possible systemic effects of timolol. On Day 14, the skin tissues from the wounds were harvested and preserved either by formalin fixation or snap freezing.

Histology

Formalin-fixed skin samples that were harvested on Day 14 were stained with hematoxylin and eosin (HE) according to standard histologic procedures. Images were acquired with an Olympus CKX41 microscope at a low magnification (4 × 10). The thickness of the epithelial layer in each group was measured with ImageJ software. Images were taken for all wound sites on each pathology slide. Eight measurements per image were taken at evenly spaced locations. The numbers of neutrophils, macrophages, and blood vessels per field (total ten fields per wound) of view were measured at a magnification of 400 by an independent masked pathologist.

RNA exaction and reverse transcription and quantitative polymerase chain reaction (RT-qPCR) analysis

At the terminal endpoint, snap-frozen tissue was collected, and ~ 100 mg was used for RNA extraction by homogenizing the tissue, followed by RNA purification using a Trizol-based phase separation protocol. The aqueous phase was loaded onto a Promega (Catalog # Z6012) column and further RNA purification was carried out, including DNase treatment, following the protocol previously described by Fagg *et al.*[15]. The specificity of each amplification was confirmed through melt curve analysis for each set of primers used to measure the RNA abundance of KRT1, KRT10, PCNA, TGFb1, TGFbR1, TGFbR2, VIM, FN1, COL1a1,

COL3a1, ACTA2, and MMP1, with the abundance of each target normalized to EEF1A1 [Table 1]. The data shown for each target are the ΔΔCt values. In each wound, the relative expression of each gene was determined with EEF1A1 as the housekeeping geneΔ.

Statistical analysis

Values are expressed as means ± standard error of the mean (SEM). All analyses were performed with GraphPad Prism 10 (GraphPad Software, La Jolla, CA) software. Statistical significance was set at $P < 0.05$.

The differences in epithelialization rate on Day 14 between groups were compared using a paired *t*-test.

The time from the skin graft to when the epithelialization rate exceeded a certain percentage (85%, 90%, and 95%) was compared using survival time analysis with the log-rank test.

The blood flow data from the PeriCam at each time point were compared using two-way ANOVA with repeated measures, followed by Bonferroni test.

The mean values for epidermal thickness and the numbers of neutrophils, macrophages, and blood vessels were calculated for each wound. Then, these values were compared using paired *t*-test.

The relative gene expression levels in each wound were compared by paired *t*-test.

RESULTS

Wounds epithelialization results

All wounds had no complications such as infection or hematoma [Figure 4]. The epithelialization rate on Day 14 in the beta-blocker group (84.46% ± 5.893%) was significantly higher than that in the control group (74.30% ± 5.861%) ($P = 0.0158$) [Figure 5]. The days when the epithelialization rate exceeded 85%, 90%, and 95% in the beta-blocker group were significantly shorter than those in the control group ($P = 0.0354$, $P = 0.0104$, and $P = 0.0313$, respectively) [Figure 6]. There were no significant differences in the wound blood flow between the groups at any measured time point [Figure 7]. There were no hypoglycemia [Table 2] and hemodynamic changes, i.e., hypotension [Table 3] and bradycardia [Table 4]. These results indicate that topical application of timolol significantly promotes epithelialization of mesh skin grafted full-thickness burn wounds through mechanism(s) other than improving wound blood flow.

Histological results

The thickness of the epithelial layer in the treatment group was significantly thicker than those in the control group (213.1 ± 15.64 μm in the treatment group *vs.* 183.5 ± 16.25 μm in the control group, $P = 0.0203$) [Figure 8]. This result supports the finding that the epithelialization rate on Day 14 in the treatment group was significantly higher. There were no significant differences in the numbers of neutrophils, macrophages, and blood vessels per field between the treatment and control groups [Figure 9]. The number of neutrophils was 28.88 ± 9.864 in the treatment group and 40.61 ± 10.95 in the control group ($P = 0.2433$). The number of macrophages was 1.492 ± 0.269 in the treatment group and 1.733 ± 0.4517 in the control group ($P = 0.5763$). The number of blood vessels was 2.817 ± 0.4156 in the treatment group and 3.475 ± 0.6433 in the control group ($P = 0.2688$). These results indicate that timolol's positive effects on wound epithelialization are not linked to the severity of inflammation and angiogenesis in the wounds.

RNA abundance measurements

There were no significant differences in the levels of RNA abundance relative to EEF1A1 for several

Table 1. Primer sequences used for RT-qPCR assays

	Used pair 1
shEEF1A1-qF	GGCACGTAGATTCAGGGAAG
shEEF1A1-qR	CCCAGGCATATTTGAAGGAG
shKRT1-qF	CTTCTGCAACCCCTCAATGT
shKRT1-qR	GTTCTGCTGCTCCAGGAATC
shKRT10-qF	GGTAATTCAAGCCAGCGAGA
shKRT10-qR	CAGCCTGGCATTATCAACCT
shPCNA-qF	CTTGGTGCAGCTAACCCTTC
shPCNA-qR	ATGTCTTCATTGCCAGCACA
shTGFb1-qF	GGGTACCACGCCAATTTCT
shTGFb1-qR	GGTTGTGCTGGTTGTACAGG
shTGFBR1-qF	CAACCAGGACCACTGCAATA
shTGFBR1-qR	AAGCAGACTGGTCCAGCAAT
shTGFBR2-qF	CCCTGTCGGTAGATGACCTG
shTGFBR2-qR	CAGGGCCATGGAGTAGACAT
shVim-qF	CTTCAGGAGGCTGAGGAATG
shVim-qR	GTTGTTGCGGTTAGCAGCTT
shFN1-qF	CTCGAAGAGCAGGAGACAGG
shFN1-qR	CGCTCCCACTGTTGGTTTAT
shCol1a1-qF	CAGGAAGAAGGCCAAGAAGA
shCol1a1-qR	CACACGTCTCGGTCATGGTA
shActa2-qF	AGCTATGAGCTGCCTGATGG
shActa2-qR	GTACGTGGTCTCATGGATGC
shCol3a1-qF	GGTGGACAGATTCTGGTGCT
shCol3a1-qR	GGACATCTTCGGGAAGTTCA
shMMP1-qF	AAATCCTCGTTGGGAGAACA
shMMP1-qR	TTGGTCCACATCTGCTCTTG

RT-qPCR: Reverse transcription and quantitative polymerase chain reaction.

healing-associated target transcripts between the treatment and control groups [Figure 10]. PCNA was used as a biomarker to measure any differences in cell proliferation. KRT1, KRT10, COL1A1, COL3A1, ACTA2, and MMP1 were used as biomarkers for myofibroblast activation (MFA). VIM and FN1 were used as biomarkers for epithelial-mesenchymal transition (EMT). TGF1, TGFR1, and TGFR2 were used for TGF-associated pathways. These results indicate that timolol does not affect cell proliferation, at least at the transcriptional level.

DISCUSSION

This study demonstrates that the topical application of timolol accelerates the epithelialization of mesh skin grafted full-thickness burn wounds in sheep. Furthermore, the histology results showed that epidermal thickness was significantly higher in the treated group. Our results also indicate that the treatment with timolol did not affect the wound blood flow, suggesting that the beneficial effects of beta-blockers are not attributable to wound blood flow improvement. In addition, the histology result showed that the number of blood vessels in the wound was comparable. Although the exact reason is unknown, we speculate that beta-blockers may not promote neovascularization. If the hypothesis is correct, this suggests that combined use

Table 2. Blood glucose level (mg/dL) for each sheep from Day 0 to Day 14

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sheep1	70	59	59	57	60	55	55	68	68	69	58	57	75	56	61
Sheep2	73	70	63	59	60	58	77	86	77	68	62	49	69	77	73
Sheep3	63	66	71	81	39	67	63	56	77	61	65	66	63	62	70
Sheep4	68	64	70	66	72	66	57	52	66	62	60	63	64	54	57

Table 3. Mean arterial blood pressure (mmHg) for each sheep from Day 0 to Day 14

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sheep1	94	93	91	99	106	97	102	97	101	111	94	101	96	101	93
Sheep2	105	102	115	104	115	115	116	114	104	111	111	120	119	112	112
Sheep3	81	90	101	94	87	96	78	77	95	93	86	91	91	86	95
Sheep4	95	100	107	104	107	90	90	100	90	100	105	94	99	93	95

Table 4. Heart rate (min) for each sheep from Day 0 to Day 14

Day	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Sheep1	65	71	73	80	73	86	69	92	95	77	82	87	81	79	61
Sheep2	55	60	63	74	70	72	56	74	56	65	72	62	86	57	58
Sheep3	68	69	100	110	92	100	73	63	101	91	96	99	95	63	99
Sheep4	76	88	89	88	85	88	105	102	103	100	102	72	97	98	95

of beta-blockers with angiogenetic agents could further accelerate the wound epithelialization rate.

It has previously been shown that beta-blockers suppress inflammation[16]. Therefore, beta-blockers could offset the increased blood flow by suppressing inflammation, even if blood flow was increased by neovascularization. However, in the present study, the numbers of neutrophils and macrophages in the wounds were comparable, suggesting that the beta-blocker timolol failed to modulate inflammation. Future studies should correlate the number of neovascular vessels to the severity of local inflammation to clarify the hypothesis.

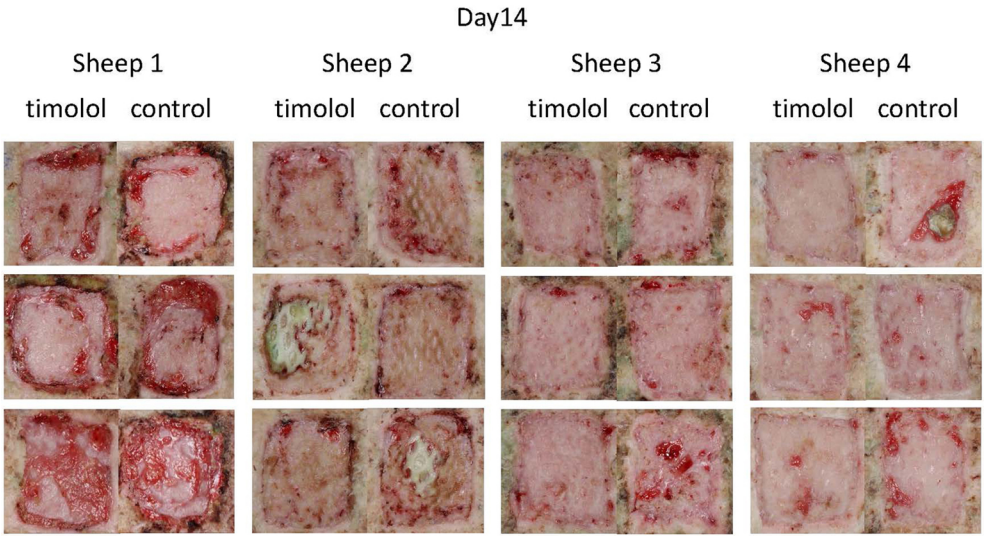

Figure 4. Digital photographs of the wounds of each sheep in the treatment and control groups on Day 14. Wounds that were adjacent to each other were analyzed as a pair.

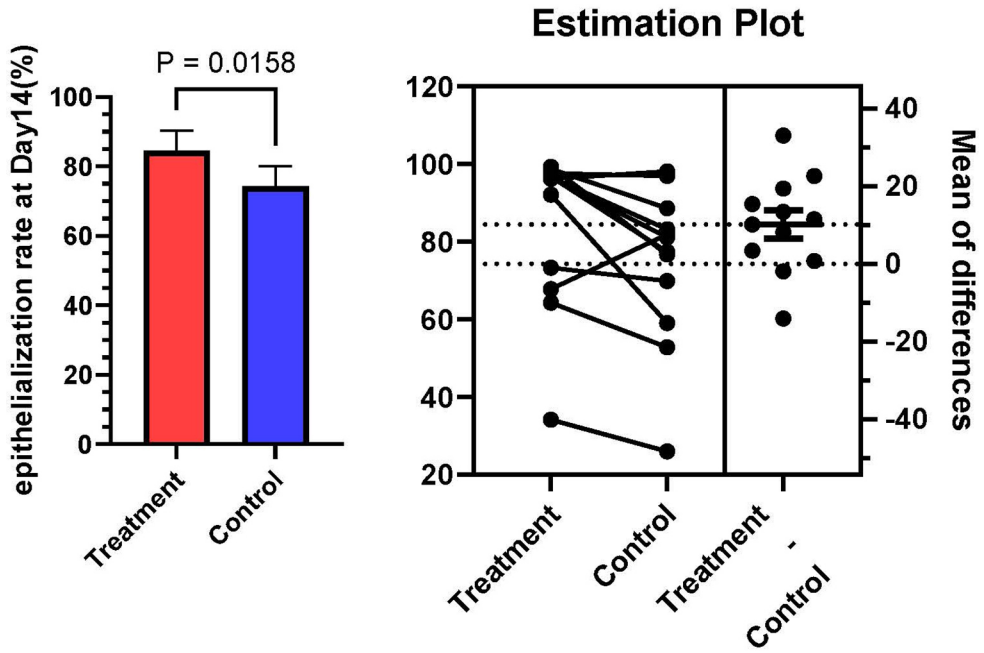

Figure 5. The graph shows the epithelialization rate (%) of the mesh skin graft wounds on Day 14 in the treatment and control groups. The red bar represents the treatment group and the blue bar represents the control group. Estimation plots show the difference between pairs. Error bars indicate SEM. SEM: Standard error of the mean.

In the present study, we report that the epidermal thickness (histological analysis) on Day 14 was significantly thicker in the treatment group than in the control group. This confirms the significantly higher epithelialization rate in the treatment group on Day 14. Since topical application of timolol accelerates epithelialization of mesh skin grafted full-thickness burn wounds, timolol may have the potential to improve cosmetic appearance and reduce scar contracture. Future studies are needed to compare the effects of beta-blockers on cosmetic aspects and scar contracture.

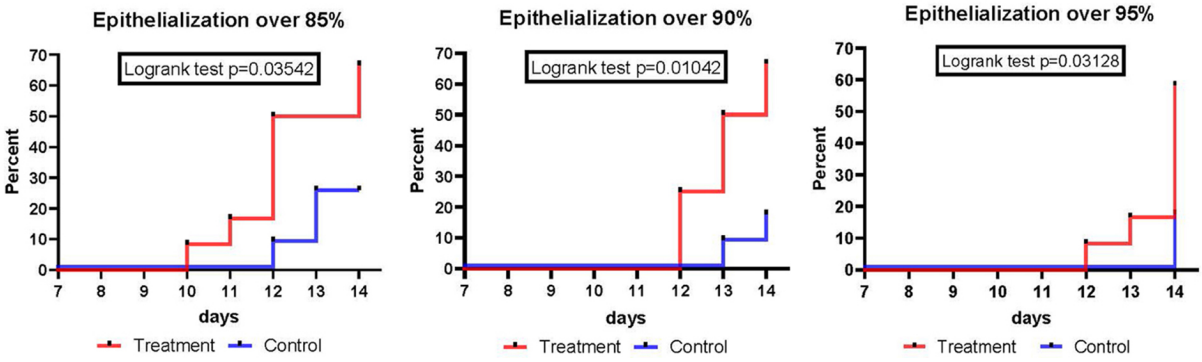

Figure 6. These graphs are survival curves comparing the treatment and control groups for the number of days that the epithelialization rate exceeded 85%, 90%, and 95%, respectively. The red line represents the treatment group and the blue line represents the control group.

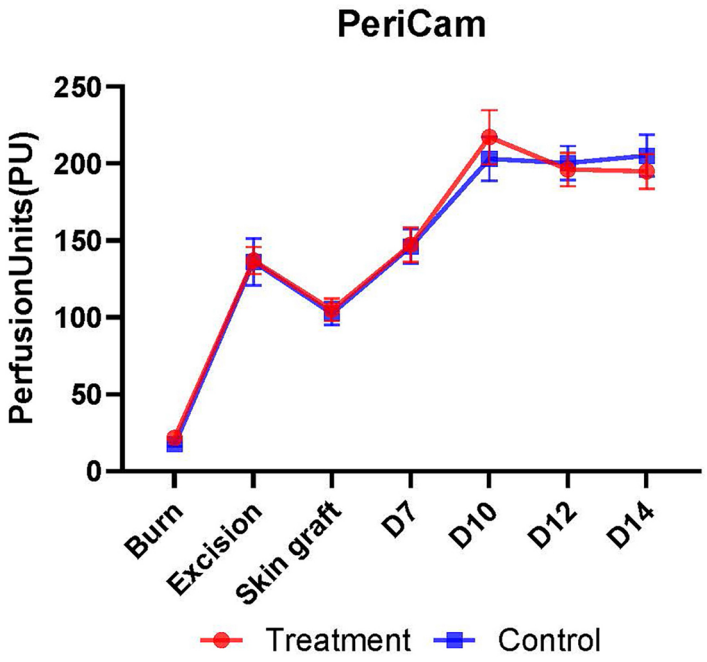

Figure 7. The graph shows the blood flow in the wounds measured immediately after excision, after skin grafting, on Day 7, Day 10, Day 12, and Day 14 using the blood perfusion imager PeriCam. Red line and red circles indicate the treatment group; blue line and blue squares indicate the control group. Error bars indicate SEM. SEM: Standard error of the mean.

Furthermore, the present study showed no significant differences in the levels of RNA abundance relative to EEF1A1 for any of the markers of proliferation measured between the treatment and control groups. Specifically, the treatment with timolol did not affect RNA expression of MFA, EMT, and TGF-associated pathways in the wound tissue. The RNA abundances relative to EEF1A1 for biomarkers of cell proliferation and MFA and EMT were compared to assess whether the topical application of beta-blockers has any proliferative effects beyond enhancing keratinocyte migratory ability. However, there is not any significant difference, and this suggests that combining a drug with a proliferative effect on keratinocytes with beta-blockers may have an additional therapeutic effect. In addition, although our approach suggests there is no significant difference between the RNA levels of the targets we measure, we cannot rule out the possibility that these targets and pathways might be affected through other means, such as differential post-

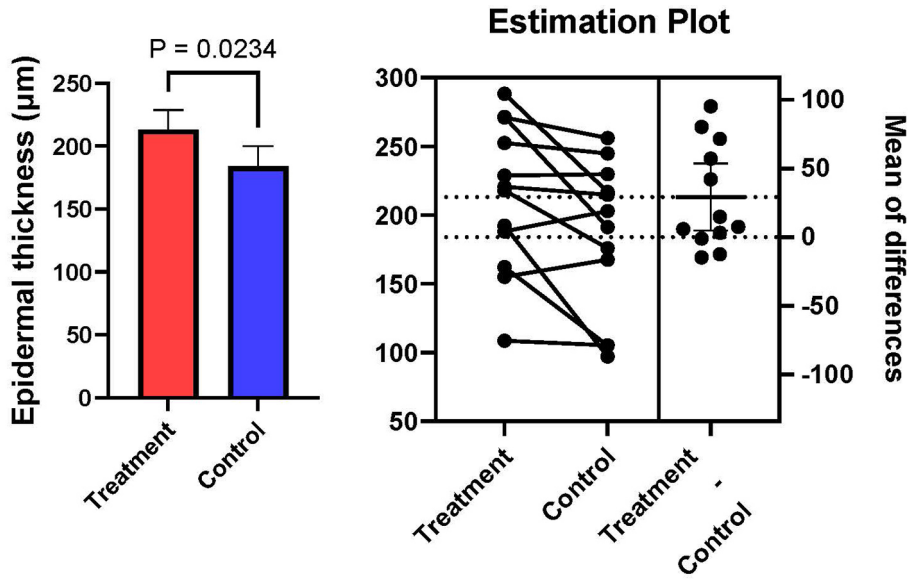

Figure 8. The graph shows epidermal thickness (μm) in the treatment and control groups. The red bar represents the treatment group and the blue bar represents the control group. Estimation plots show the difference between pairs. Error bars indicate SEM. SEM: Standard error of the mean.

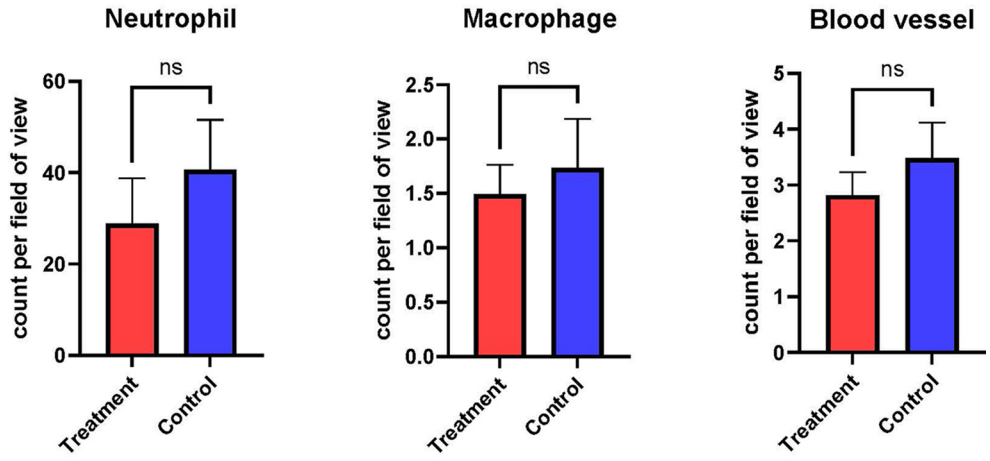

Figure 9. These graphs show the numbers of neutrophils, macrophages and blood vessels per field in the treatment and control groups. Red bars represent the treatment group and blue bars represent the control group. Error bars indicate SEM. SEM: Standard error of the mean.

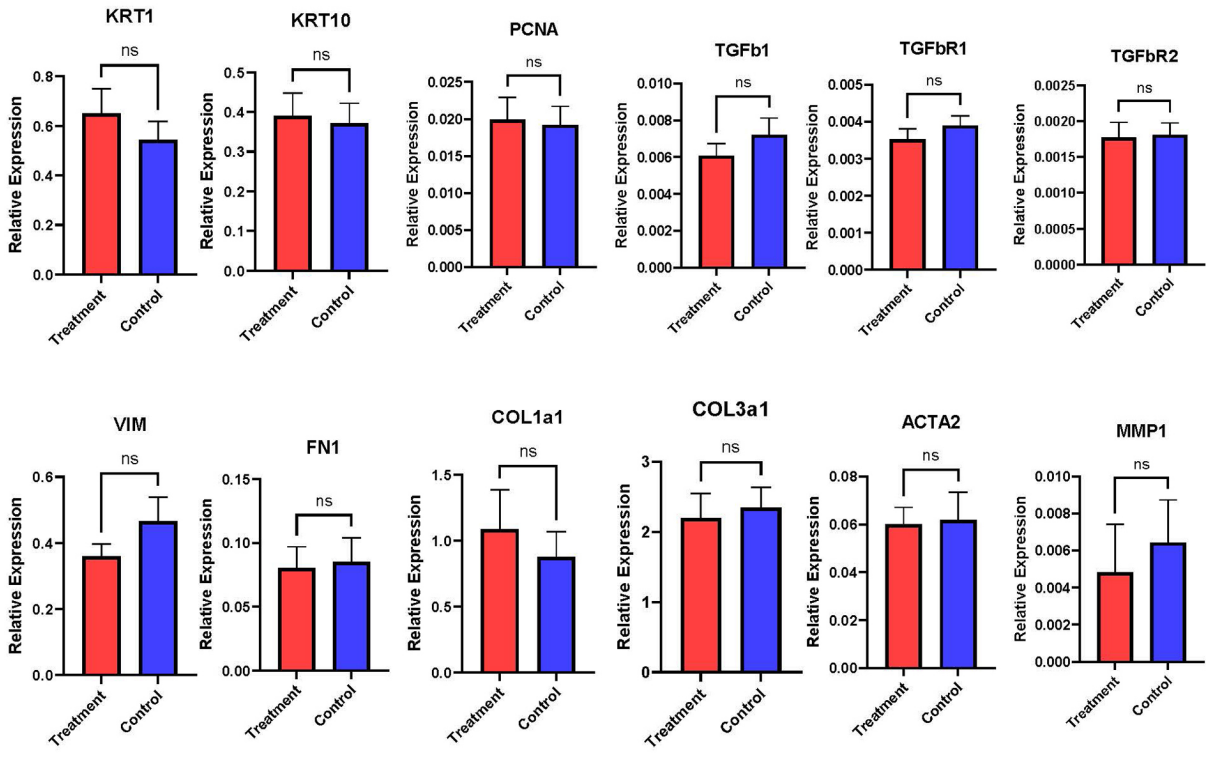

Figure 10. These graphs show the relative expression levels of each gene in the treatment and control groups. Red bars represent the treatment group and blue bars represent the control group. Error bars indicate SEM. SEM: Standard error of the mean.

translational modifications or abundance.

We have monitored possible systemic effects of topically applied timolol throughout the study period, because beta-blockers can generally cause bradycardia, hypotension, and hypoglycemia. The frequency of systemic effects of beta-blockers varies depending on the dose and method of administration. Previous research has demonstrated that treating infantile hemangiomas with beta-blockers is less likely to result in systemic effects when administered topically compared to oral administration[17]. Conventionally, beta-blockers have been used intravenously in the treatment of burn patients to control hypermetabolic responses[18]. The systemic effects of intravenously administered beta-blockers include lowering blood pressure, heart rate, and resting energy expenditure. This study showed that topical application of timolol did not cause any systemic effects. Therefore, the topical application of beta-blockers is particularly noteworthy, as it provides maximum local effects without amplifying the systemic effects associated with intravenous administration.

While various topically applied agents for wound care have been developed, many of them are associated with significant drawbacks, such as high costs and complex manufacturing and availability. Beta-blockers are relatively inexpensive, broadly accessible, and Food and Drug Administration (FDA)-approved. Previous studies have indicated that these readily accessible agents could promote wound healing. However, to date, studies have yet to report on their effects on skin grafted full-thickness burn wounds. Both conservative and surgical treatments for burns can be painful and require ongoing care until complete epithelialization occurs. Additionally, longer healing times increase the risk of skin and soft tissue infections. This study is important because it demonstrates that timolol can promote wound healing in mesh skin grafted full-thickness burn wounds.

The present study has several limitations. First, it did not explore possible mechanisms by which the beta-blocker accelerated the epithelialization in grafted wounds. Second, the study lasted only 14 days, which limited the evaluation of cosmetic outcomes and scar contracture. Third, a dose-dependency study for timolol was not conducted to determine the optimal concentration. Fourth, the study did not assess the protein levels (post-transcriptional level) of the measured markers. Finally, RNA levels of these markers were only measured on Day 14, leaving their potential changes at early time points unexamined. Therefore, studies are needed to investigate the mechanistic aspects of the beneficial effects of beta-blockers in wound healing.

DECLARATIONS

Authors' contributions

Developed the concept and planned the experiments: Nakamoto K, Enkhbaatar P
Carried out the experiments: Nakamoto K, Batsaikhan TA, Kakizaki R, Heathman T
Contributed to RT-qPCR analysis: Liu N, Fagg WS
Provided critical feedback and helped analyze data and prepare the manuscript: Nakamoto K, Batsaikhan TA, Liu N, Fagg WS, Kakizaki R, Heathman T, Enkhbaatar P

Availability of data and materials

The data that support the findings of this study are available from the corresponding author, Nakamoto K, upon reasonable request.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

This study followed the guidelines of the National Institutes of Health and the American Physiological Society regarding the care and use of laboratory animals, and the protocol of this study was approved by the University of Texas Medical Branch Animal Care and Use Committee (protocol #2003030).

Consent for publication

Not applicable.

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