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Experimental Study

Treatment of Corneal Alkali Burn with Chestnut Honey, Royal Jelly, and Chestnut Honey-Royal Jelly Mixture

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Abstract

Objectives: The alkaline burn of the cornea usually results in a decreased vision with opacification. In this study, we investigated the potential role of endemic chestnut honey (CH) and royal jelly on corneal healing after an alkaline burn.

Methods: We created an alkaline burn on the center of the corneas of four groups of Wistar rats by applying round filter paper soaked with 1 N NaOH for 30 seconds. The animals were treated with RJ, CH, RJ-CH combination and Na-Hyaluronate (Na-HA) eye drops. We performed a serial evaluation with anterior segment photography on the first, 7th, and 14th days of the experiment. Pathologic examination conducted with hematoxylin and eosin stains and immunostaining for SMA and α4β1 integrin. We evaluated the corneal healing process with a scoring system, which was estimating the degree of corneal edema, the size of the corneal ulcer, and limbal hyperemia.

Results: There was no statistically significant difference between groups on the first, 7th, and 14th days concerning the healing scores (p=0.88, p=0.06, p=0.80, respectively). However, there were significantly better scores in the repeated measures of CH (p=0.012) and RJ-CH (p=0.00) groups. The RJ group and Na-HA group did not show a significant difference in repeated measures (p=0.19 and p=0.10, respectively). The α4β1 integrin levels on immunostaining showed a significant difference among groups on the 14th day (p=0.002).

Conclusion: We found better corneal healing after treatment with the RJ-CH containing eye drops concerning corneal healing sore and α4β1 integrin staining.

Keywords: Cornea, corneal alkaline burn, chestnut honey, royal jelly, smooth muscle antigen, α4β1 integrin.

Introduction

The corneal alkaline burn is still a significant cause of visual disruption. The proper healing of cornea after an alkaline injury is vital for the restoration of vision and ocular comfort (1). An inflammatory reaction begins shortly after an alkaline insult to the cornea. The release of tissue remodeling enzymes, such as matrix metalloproteinases (MMPs), and pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), were observed in the early periods of inflammation (2, 3). The function and structure of epithelial and stromal cells change so that they secrete several intracytoplasmic and cell membrane proteins, including smooth muscle antigen (SMA) and integrins, to interact with the environment, especially the basement membrane (BM) (4). The balance in tissue response against alkaline damage results in an excellent transparent three-dimensional integrity.

Several treatment strategies, including early irrigation with buffered solutions and vigorous artificial tear drop in-

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stallation, used to limit the tissue degradation with some degree of success (5, 6). The artificial tear drops containing sodium hyaluronate (Na-HA) help corneal reepithelization with the restoration of pH stability of the ocular surface and improve patient comfort (7, 8). Despite shown beneficial effects of the Na-HA on corneal healing, better treatment options are required to restore the corneal structure.

The ophthalmologic usage of different types of honey and royal jelly (RJ) was tried in the treatment of several experimental animal models of ocular surface diseases, including corneal burns, with variable degrees of improvement (9–14). The chestnut honey (CH) is endemic in Turkey and is used widely as complementary medicine for several conditions. However, a gap exists in current knowledge about the potential effects of RJ, CH, and RJ-CH combination on the corneal healing process after an alkaline insult. In this study, we investigated the potential role of topical RJ, CH, and RJ-CH combination drops for the treatment of experimentally induced rat model of corneal alkaline burn.

Methods

General Information

The Animal Experiments Local Ethics Committee of Bagcilar Teaching and Research Hospital (HADYEK project number: 2017-10) approved this experiment. This study was conducted between 11-03-2017 and 11-17-2017 by the principles of the Declaration of Helsinki and carried out by considering animal rights. Our study included four groups of Wistar rats. Each group consisted of six animals. We created an alkaline burn on the center of the corneas by applying a 3.5 mm in diameter round filter paper soaked with 1 N NaOH for 30 seconds. We irrigated the ocular surface of each eye with 10 ml of sterile 0.09% NaCl as a first-line standard treatment. A gamma irradiation process with 25 kGy doses was done to sterilize RJ and CH. We arranged 1% eye drop solutions for the RJ and the CH groups. We prepared a solution containing 0.05% RJ and 0.05% CH for the RJ-CH treatment group. The animals were treated four times a day only with RJ, CH, RJ-CH combination, and 0.15% Na-HA (Eyestil ™, SIFI S.p.A.) eye drop.

Interpretation of Inflammation and Corneal Epithelium Healing

The description of the measurement of inflammation and corneal epithelium healing process is present in previous studies with detail (15, 16). We have used a slightly modified approach for the evaluation of inflammation and corneal healing. Serial slit lamp biomicroscopic photographs were taken at first, 7th and 14th days of examination. The direct images were gained at first, later for taking a second picture the cornea stained with one drop of 0.1% fluorescein sodium solution to measure the size of the epithelial defect. We calculated the corneal healing score with the use of three markers. The first marker was ciliary hyperemia (absent=0; present but less than 1 mm=1; present between 1 and 2 mm=2; present and more than 2 mm=3). The second marker was central corneal edema (absent=0; present with visible iris details=1; present without visible iris details=2; present without visible pupil=3); peripheral corneal edema (absent=0; present with visible iris details=1; present without visible iris details=2; present with no visible iris=3). The third marker was the corneal epithelial staining (no staining=0; slight punctate staining=0.5; diffuse punctate staining=1; diffuse staining covering less than one-third of the cornea=2; diffuse staining covering more than one-third of the cornea=3; and staining covering more than two-thirds of the cornea=4). A total score for each examination date was used for the overall physiologic healing response.

Evaluation of Histological Sections

A single pathologist (AC) handled the histopathologic examination of specimens. After enucleation and removal of corneas, the samples fixed in a 10% formaldehyde solution of 20 ml for 24 hours, samples from each group were macroscopically taken into cassettes and then dehydrated in a tissue tracking device by being passed through alcohol, acetone, xylene, and paraffin phases. The paraffin blocking was conducted thereafter. The hematoxylin and eosin (HE) staining was done to each block after taking cuts of 4μm. The covering solution was dripped on the painted lames, and they were closed with lamellas. After the necessary examination with HE, an immunohistochemical examination for SMA and α4β1 integrin were applied to each block with 4 nm sections. In the evaluation of the SMA, the cells that stained from 100 cells in the stroma were counted at X400 magnification. The value is given in percentage. The cells that were painted from 100 cells in the stroma at X400 magnification were counted in the evaluation of α4β1 integrin. The amount is given in percentage. We used a light microscope (BX51TF ™, Olympus, Tokyo, Japan) to evaluate tissue sections.

Statistical Methods

We used open-source software, namely PSPP (a GNU project), for statistics. We expressed all continuous data as a mean±standard deviation where applicable. Categorical variables were analyzed with the chi-square test (X2). Repeated measures were analyzed with the Friedman test. The Mann-Whitney U test was used for comparing two groups. The Kruskal-Wallis test was used to test more than two groups. The values of less than 0.05 were considered statistically significant for the measured P-values.

Results

Corneal Healing Scores

We measured the corneal healing scores on the first, 7th, and 14th days after the corneal alkaline burn induction. Figure 1 shows the fluorescein staining patterns of groups. We observed a decrease in total healing scores in all groups emphasizing a stable healing activity. Table 1 shows the corneal healing scores of all groups. There was no statistically significant difference between groups on the first, 7th, and 14th days concerning the corneal healing scores (p=0.88, p=0.06, p=0.80, respectively). However, there were significantly better scores in repeated measures of CH (p=0.012) and RJ-CH (p=0.000). RJ and Na-HA did not show a significant difference in repeated measures (p=0.19 and p=0.10, respectively).

Histopathologic Examination Results

The HE staining of all groups shows complete reepithelization without any sign of inflammation. Anti-SMA immunohistochemical staining at 14th day showed no staining with SMA antigen in any of the groups (0%). However, there was a significant difference between the groups for the α4β1 integrin staining of stromal cells on the 14th day (p=0.002) (Table 2), which was due to the significantly different α4β1 integrin staining levels between the RJ and the CH groups (p=0.019) and the RJ and the RJ-CH groups (p=0.003). When the RJ and the Na-HA groups compared for the α4β1 integrin staining, no significant difference was analyzed (p=0.335). The staining for the α4β1 integrin was significantly different between the CH and the RJ-CH groups (p=0.027). When we compared the CH and the Na-HA groups, we found no significant difference for the α4β1 integrin staining (p=0.18).

Table 1. Corneal healing score

	RJ*	CH*	RJ-CH*	NaHA*	P1*
CHS 1st day	5.16±0.40	5.33±0.51	5.66±1.03	5.25±0.95	0.88
CHS 7th day	3.66±2.02	2.50±0.63	3.58±0.73	2.50±0.0	0.06
CHS 14th day	2.12±1.10	2.50±0.61	2.66±0.68	2.50±0.70	0.80
P2*	0.19	0.012	0.00	0.11	

*P1 Stands for Kruskal-Wallis test significance level; P2 stands for the Friedman test significance level; CHS stands for the corneal healing score; RJ: Royal Jelly; CH: Chestnut Honey; RJ-CH: Royal Jelly-Chestnut Honey; NaHA: Sodium Hyaluronate.

The RJ-CH and the Na-HA groups were significantly different in staining for the α4β1 integrin (p=0.008). Figure 2 displays some of the pathologic sections of the NaHA group and RJ-CH group for different staining techniques.

Discussion

A transparent cornea, which is necessary for a good vision, can be harmed with an alkaline burn. This type of injury has been tried to treat with several medications with limited success. Honey and RJ are two remedies successfully used for some ocular diseases. Here, we investigated the potential role of CH, RJ and CH-RJ combination for the treatment of alkaline burn of the cornea and found better treatment results in CH-RJ treated group concerning improved corneal healings scores in repeated measures and α4β1 integrin staining characteristics.

Corneal epithelial healing is a complex process with the propagation of corneal epithelial cell in three-dimensional x, y, and z-axis. The failure of this healing process for any reason results in an ulcerated corneal surface. The inflam-

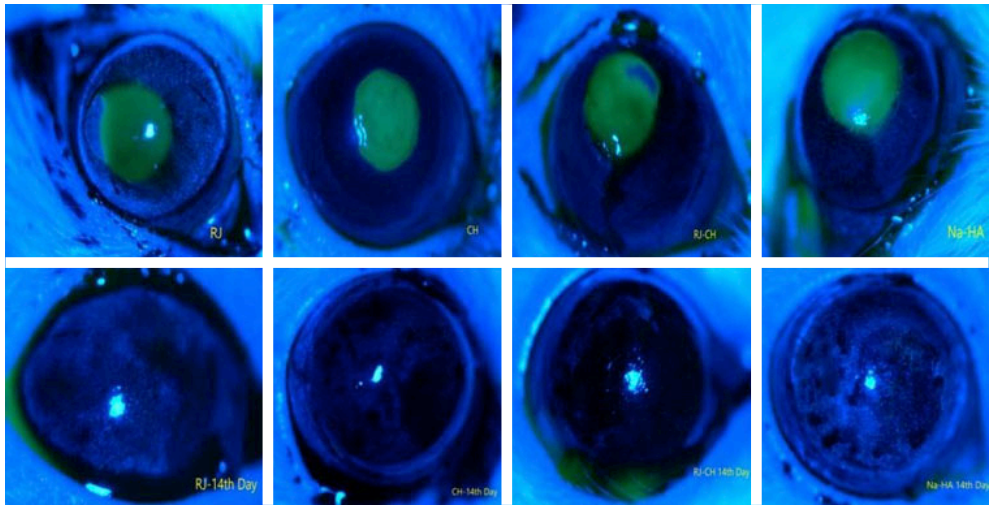

Figure 1. The frames on the upper part of the figure show the fluorescein staining of each group on the first day. The lower part of the figure contains the pictures taken on the 14th day (RJ=Royal Jelly, CH= Chestnut Honey, RJ-CH= Royal Jelly-Chestnut Honey, NaHA= Sodium Hyaluronate).

Table 2. The number of α4β1 staining cells

	Groups				P
	RJ	CH	RJ-CH	Na-HA	
Number of α4β1 staining cells	96.66±5.16	73.33±16.02	55.00±8.36	88.75±14.36	0.002

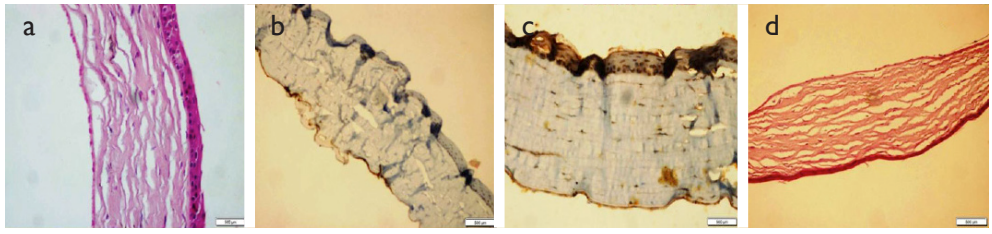

Figure 2. Some of the pathologic sections of the Sodium Hyaluronate (NaHA) group and Royal Jelly (RJ-CH) group for different staining techniques. **(a)** NaHA group, full epithelialization in the section, moderate edema is observed, and no active and chronic inflammatory cell infiltration is observed, HE, X400; **(b)** RJ-CH group, SMA immunoreactive stromal cell in cross-section was not observed, SMA, X200; **(c)** RJ-CH group, Half of the stromal cells in the section are immunoreactive with α4β1 integrin 4, X400; **(d)** RJ-CH group, full epithelization in the section, moderate edema is observed, and no active and chronic inflammatory cell infiltration is observed, HE, X200.

mation of the cornea is one of the critical causes of corneal ulceration (17). Also, the inflammatory cells in the wound area increase paracrine secretion of oxidizing agents, inflammatory cytokines, and tissue remodeling enzymes (18). There are many studies on the protective effects of different honey varieties and RJ against excessive inflammatory responses and oxidative stress (9, 19-21). In an experiment that used the rabbit corneal alkali burn model, Tualang honey displayed reduced conjunctival hyperemia, reduced corneal edema and reduced neutrophilic infiltration, which was not significantly different from the treatment with prednisolone acetate one percent, ciprofloxacin 0.3 percent and oral ascorbic acid (20). In another experiment, Uwaydat et al. showed a faster epithelisation and decreased corneal expression of vascular endothelial growth factor (VEGF), transforming growth factor-beta (TGF β), interferon-gamma, interleukin 12, and tumour necrosis factor-alpha (TNF-α) in a rat model of corneal abrasion and P. aeruginosa toxin immune-mediated keratitis treated with soybean/wildflower honey (14). The keratocytes and polymorphonuclear cells secrete MMPs during the early phases of wound healing (2, 22, 23). The inhibition of MMPs shows some beneficial effects on the healing of corneal ulceration (24, 25). Paterson et al. reported that the tissue inhibitors of MMPs were equal to the synthetic inhibitor of MMPs at ameliorating corneal ulcers in a rabbit model of corneal alkali burn. Majtan et al. examined the major royal jelly protein one (MRJP1) that is an essential active compound of RJ and acacia honey on the expression of MMP mRNA from human keratinocytes and found an increased MMP mRNA expression in acacia honey group but no induction in production of mRNA of MMP in (MRJP1) group (26). In our study, corneal ulceration improved on the 14th day of treatment in all groups, suggesting that agents used in all groups were active. In our research, there are clinical findings that support the improvement of cellular propagation in the mixture of CH-RJ and CH on epithelial healing.

Myofibroblasts play an essential role in the closure of the wound by podophilic movements through actin microfilaments that they have during wound healing (27). Corneal haze may arise from myofibroblasts that occur during corneal wound healing because they do not turn into keratocytes, which are specialized fibroblasts (27, 28). The emergence of inflammatory responses in tissue supports the activation of myofibroblasts (29). At the end of the normal healing process, myofibroblasts stop the production of SMA (30). They may become scar keratocytes or fade with apoptosis (30). In our study, no SMA positive cells were detected in the stroma after SMA staining to detect the presence of myofibroblasts in tissue. An inflammatory cell response typically seen in the early stages of wound healing is not shown in HE staining of all groups, and the absence of SMA positive cells suggests that the treatments given in all groups effectively control inflammation.

Integrins that are also known as very late activation (VLA) antigen are a group of heterodimeric integral membrane proteins (31). They have several cells to cell and cell to ECM interactions. The corneal keratocytes express α4β1 integrin after an inflammatory response (32, 33). The α4β1 integrin binds to fibronectin, which is a critical ECM component. The sources of the fibronectin (Fn) are tissue fi-

broblasts or hepatocytes. The tissue fibroblasts secrete a subtype of fibronectin called EDA Fn after an injury. Recent reports show a mutual relation between α4β1 integrin and EDA Fn secretion during the healing process (34). Besides, α4β1 integrin is a receptor for soluble vascular cell adhesion molecule-1 (VCAM-1) which is induced after secretion of pro-inflammatory cytokines, such as tumor necrosis factor α (TNF-α) (35). Inhibition of the interaction between α4β1 integrin and soluble VCAM-1 blocks the neovascularization (3, 36). Both disorganized ECM and neovascularization impair the corneal clarity. The honey exerts different effects in wound healing employing inflammatory cytokine production. That is, some studies relate a pro-inflammatory action, while some studies do not connect with pro-inflammation (14, 37, 38). On the other, RJ decreases inflammatory response through reduced production of cytokines (12, 39). In our study, in RJ (96.66±5.16) and Na-HA (88.75±14.36) groups, stromal fibroblast α4β1 integrin staining rates were higher than CH (77.33±16.02) and RJ-CH groups (55.00±8.36). We observed a significant difference in the α4β1 integrin staining levels between the groups, and this difference was due to the low level of α4β1 integrin staining of RJ-CH and CH groups. Also, in the comparison of the CH group and RJ-CH group, a low level of staining was detected in the RJ-CH group. According to our study, there was no clinical difference in corneal healing scores in RJ, CH, and RJ-CH groups compared to the NaHA group. However, concerning the amount of α4β1 integrin staining at the cellular level, better corneal healing seems to be present after installation of the RJ-CH containing eye drops, which may be due to the synergistic action of both of these materials on immunomodulation and fibroblast interaction with ECM.

Conclusion

In conclusion, we find better corneal healing after treatment with the RJ-CH containing eye drops concerning corneal healing sore and α4β1 integrin staining in an experimental animal model of corneal alkaline injury.

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Disclosures

Ethics Committee Approval: The Animal Experiments Local Ethics Committee of Bagcilar Teaching and Research Hospital (HADYEK project number: 2017-10).

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Conflict of Interest: None declared.

Authorship Contributions: Involved in design and conduct of the study (KSC, KA, AK, AKC); preparation and review of the study (KA); data collection (KA, KSC, AK); and statistical analysis (KA).

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