



Effects of Oral Propolis on Mucosal Wound Healing after Endoscopic Nasal Surgery in a Rabbit Model

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Original Investigation

Abstract

Objective: If the respiratory nasal mucosa is damaged and the mucosa does not heal properly during nasal or paranasal sinus surgery, a revision surgery may be required. The aim of this study is to investigate the effects of oral propolis application on mucosal wound healing following endoscopic nasal surgery in a rabbit model.

Methods: Twenty four New Zealand rabbits were randomly divided into three groups, namely the experimental group (EG), the control group (CG), and the negative control group (NCG). Mucosal resection was applied with 3-mm punch forceps in the bilateral ventral nasal concha in the experimental and control groups. 75 mg/kg/day propolis was added to the diet of the rabbits in the EG for 14 days. The CG continued with the standard diet postoperatively. In the NCG, no surgical intervention was made, and no dietary support was given. On postoperative day 14 all rabbits were sacrificed and left nasal specimens were

examined histopathologically, hydroxyproline levels were measured in right nasal specimens.

Results: There were no statistically significant differences in hydroxyproline level, epithelial and subepithelial thickness, ciliary and goblet cell count, subepithelial fibrosis and collagen density between EG and CG. Neutrophil count was statistically significantly lower in EG, compared to CG ($p=0.019$, Tamhane test).

Conclusion: Although there are many studies that show the positive effects of propolis on wound healing, such effect was not observed in this study. This study is deemed to constitute a unique experimental study that can be a resource for future similar studies to be performed with higher numbers of subjects and higher dosage of propolis.

Keywords: Endoscopic nasal surgery, hydroxyproline, propolis, wound healing

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Introduction

If the respiratory nasal mucosa is damaged and mucosa does not heal properly during a nasal surgical procedure, a revision surgery may be required. Wound healing is a highly complex, coordinated, and multi-stage system process, involving clot formation, inflammatory reaction, immune response, and tissue remodeling and maturation. Several endogenous and exogenous factors such as infection, nutrition, systemic factors, and surgical technique affect this process (1). Correctly applied postoperative care after nasal surgery shortens the recovery time of the patient and reduces the frequency of revision surgeries. No standard treatment protocol has yet been established in the literature on this subject (2).

The application of nasal saline irrigation and wound debridement provides cleaning of scabs and secretions and is, therefore, thought to prevent scar formation (3). Topical steroids are widely used after endonasal interventions owing to their local anti-inflammatory effects. Although the use of systemic steroids in the postoperative period provides a significantly improved appearance on endoscopic examination, their use is controversial, since they can slightly reduce the symptoms of the patient and have potential side-effects (2). That steroids are used following endonasal interventions for their anti-inflammatory effects has given rise to the idea that various anti-inflammatory agents can be used for this purpose. Propolis, a traditional medicinal, has been used for many years

in the treatment of burns and wounds and been shown to have antibacterial, anti-inflammatory and antioxidant effects (4).

There are many studies in the literature related to the therapeutic effects of the systemic or topical use of propolis in skin wounds, burns, ulcers, intra-abdominal adhesions and oral cavity lesions (5, 6). In the present study, we hypothesized that the systemic use of propolis could have a positive effect on wound healing by reducing inflammation in the nasal mucosa and accelerating epithelial closure. In our study, wound healing was examined histopathologically and by measuring the hydroxyproline level in the tissue. The tissue hydroxyproline level is considered a sign of the collagen metabolism during wound healing (7). Measurement of hydroxyproline levels in serum and homogenous tissues is a common practice. To the best of our knowledge, our study is the second study in the literature that measured hydroxyproline levels in a thin and non-homogenous tissue like the nasal mucosa. Although animals such as dogs, sheep, and pigs are also used, the rabbit model is the most common one used in nasal and paranasal sinus studies.

The aim of this study is to investigate the effects of oral propolis application on mucosal wound healing following endoscopic nasal surgery in a rabbit model. To the best of our knowledge, apart from our study, there is only one study in the literature that has examined the effects of systemic propolis on nasal mucosal wound healing (8).

Methods

The approval for all the experimental procedures in the study was granted by the Ondokuz Mayıs University Ethics Committee for Animal Studies (2013-HADYEK-62). Institutional guidelines on animal experimentation were followed. The study included a total of 24 adult male New Zealand rabbits weighing 2500 to 4000 g and was conducted between March 2014 and March 2015. The power analysis of the study was performed with Minitab 15 statistical software (Minitab, Inc., State College, Pennsylvania, USA). According to the result of the statistical study, the sample size was found to be 8 for each group with 99% power based on a mean tissue hydroxyproline level of 2.105 µg/g difference and 656 µg/g deviation in a 95% confidence interval (CI).

Two rabbits were kept in each of the special cages in sun-shaded rooms with suitable ventilation conditions. During the seven days before the start of the work, the rabbits were housed in a natural light/dark period for 12 hours at room temperature ($22\pm2^{\circ}\text{C}$). The standard diet on the market and drinking water were provided as needed for feeding. Their water was changed every day and cages were cleaned throughout the day. No dietary restrictions were applied before and during the study.

The 24 New Zealand white rabbits were randomized to three groups as the experimental group (EG) (n=8), the control group (CG) (n=8), and the negative control group (NCG) (n=8). Each animal in EG and CG was anesthetized with an intramuscular injection of ketamine 10% (50 mg/kg; Richter Pharma AG,

Wels, Austria) and xylazine 2% (5 mg/kg; Bayer AG, Leverkusen, Germany). Bilateral mucosal resection of the concha nasalis ventralis was performed with 3-mm punch forceps on all EG and CG animals. Surgery was performed by the same surgeon without the knowledge of the animal's group. Following endoscopic mucosal resection, the rabbits in EG were administered 75 mg/kg/day ethanolic propolis extract (EPE) by oral gavage for 14 days in addition to the normal diet. Euthanasia was applied on day 14 postoperatively. The rabbits in CG continued to be fed with a normal diet for 14 days postoperatively and were not given any supportive dietary product. Euthanasia was applied on day 14 postoperatively. The rabbits in NCG did not undergo any surgical intervention and were not given any supportive dietary product. Euthanasia was also applied to this group on day 14.

Preparation of the Propolis Extract: The propolis, which was collected by honeybees in Samsun region of Turkey, was obtained from Ondokuz Mayıs University Agricultural Faculty in 2013. The EPE to be used in the study was prepared according to the method defined by Krell (9). Raw propolis of 100 g was mixed with 1.900 mL of 70% ethanol in a bottle wrapped in aluminum foil to protect the mixture from light. At the end of one week, the EPE was filtered through Whatman filter paper. Then, in a vacuum evaporator, the ethanol in the mixture was evaporated and eventually alcohol-free EPE was obtained.

Sampling: After sacrificing, the skin on the rabbit maxilla was peeled and the maxilla was resected at the inferior edge of the eyes, and the specimen was separated on a vertical plane, with a cut through the septal cartilage. Left nasal specimens, including the septum, were kept in 10% formaldehyde for histopathological examination. The damaged mucosal area in the right nasal wall was shaved with a scalpel over the bone and placed in Eppendorf tubes and stored at -80°C until hydroxyproline level analysis. The specimens were numbered without group information and were sent to the pathologist and the pharmacologist.

Histopathological Examination: Tissue samples were fixed in 10% buffered neutral formaldehyde solution before examination, and then, decalcified with an acid solution containing acetic acid and formic acid. After decalcification, the tissues were embedded in paraffin blocks. Sections 5 µm in thickness were cut from the paraffin blocks, stained with Hematoxylin and eosin (HE) and Masson's trichrome (MT), and examined under a light microscope. Modifications were made to the studies of Khalmuratova et al. (10) and Garcia et al. (11) for the HE staining evaluation of inflammatory cell count, ciliary cell count, epithelial thickness, and subepithelial thickness. The MT evaluation examined subepithelial collagen thickness, goblet cell count, and subepithelial fibrosis. Epithelial and subepithelial thickness examinations were made morphometrically. While ciliary cell count, goblet cell count, and inflammatory cell count were evaluated quantitatively, subepithelial fibrosis and collagen density were evaluated semi-quantitatively (Table 1). All specimens were examined by the same veterinary pathologist without knowledge of the specimen's group.

Table 1. Method of morphometric, quantitative and semi-quantitative histopathological measurements. (Evaluation criteria were based on the modification of the study by Khalmuratova et al. (10) and Garcia et al. (11))

Variable	Instrument	Method	
Epithelial thickness	HE X20	The mean value was taken of the measurements of epithelial thickness in 5 different areas	
Subepithelial thickness	HE X20	The mean value was taken of the measurements of subepithelial thickness in 5 different areas	
Inflammatory cell count	HE X40	After calculation of the total neutrophil count in 5 different areas, the group average was calculated	
Ciliary cell count	HE X40	After calculation of the total ciliary cell count in 5 different areas, the group average was calculated	
Goblet cell count	MT X40	After calculation of the total goblet cell count in 5 different areas, the group average was calculated	
Collagen density	MT X20	Collagen density was determined for each specimen, then the group average was calculated Normal collagen density:0 Slight increase:1 Moderate increase:2 Severe increase:3	
Subepithelial density	MT X20	Subepithelial fibrosis density was determined for each specimen, then the group average was calculated No fibrosis: 0 Slight increase:1 Moderate increase:2 Severe increase:3	

Table 2. Epithelial and subepithelial thickness

		Study Group	Control Group	Negative Control Group	p
Epithelial Thickness	AM±SD	41.30±8.21	45.60±5.97	19.00±5.34	SG-CG p=0.2
	Med (Min-Max)	40.68 (30.4-54.4)	45.38 (37.9-55.0)	17.77 (13.4-30.5)	SG-NCG p<0.05
Subepithelial thickness	AM±SD	159.49±64.28	162.74±72.87	94.09±18.78	SG-CG p=0.92
	Med (Min-Max)	159.65 (80.07-229.6)	135.63 (89.83-270.15)	94.02 (66.84-128.46)	SG-NCG p<0.05

AM: Arithmetic Mean; SD: Standard Deviation; Med: Median; Min: Minimum; Max: Maximum

Hydroxyproline Measurement: Hydroxyproline levels in wet tissues (mg/g) were measured using the method described by Hutson et al (7) with some modifications. Hydroxyproline, sarcosine, iodoacetamide, and 9-fluorenylmethyl-chloroformate (FMOC) were purchased from Sigma-Aldrich (St Louis, Missouri, USA). Sodium acetate, sodium hydroxide o-phthalaldehyde (OPA), 2-mercaptoethanol, acetonitrile, glacial acetic acid, boric acid, and ethyl ether were obtained from Merck (Darmstadt, Germany). The mobile phase consisted of 3% glacial acetic acid buffered with sodium acetate to pH 4.3 (650 mL) with acetonitrile (350 mL). The high-performance liquid chromatography (HPLC) with a fluorescence detector system was supplied by Shimadzu (LC-20A Prominence; Shimadzu, Kyoto, Japan). Separation was obtained using a LiChrospher 100 RP18, 5 mm, 4x250 mm (Teknokroma, Barcelona, Spain). The mobile phase was pumped at a constant rate of 1 mL/min. Samples were homogenized in 1 mL 6M HCl with a motorized homogenizer (WiseTis HG-15D; Daihan Scientific Co, Seoul, Korea). Then, 200 mL of each homogenate was placed in a glass test tube with an additional 3.8 mL 6M HCl and 2 mM sarcosine added and the tubes were placed on a heating block for 18 hours at 110°C. Aliquots of the 900-mL homogenate supernatant were removed for the derivatization process conducted using borate buffer, OPA solution, iodoacetamide, and FMOC reagent. The remaining aqueous phase was injected into the HPLC system. Standard calibration curve for seven concentrations between 25

and 1000 mM hydroxyproline was obtained under the HPLC condition. Linear regression was R²=0.9999.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS) version 15.0 software (SPSS Inc., Chicago, IL, USA). Descriptive data were expressed in arithmetic mean and standard deviation (SD), and median (min-max) values. Numerically obtained data were expressed in percentages (%). Conformity to normal distribution was evaluated using the Shapiro-Wilk test. Normally distributed data among the three groups were analyzed using the one-way variance of analysis (ANOVA), while the Tamhane Test was used for the comparison of two groups which did not meet the homogeneity hypothesis. Abnormally distributed data among the three groups were analyzed using the Kruskal-Wallis variance analysis, and the paired groups were evaluated with the Bonferroni-corrected Mann-Whitney U test. A *p* value of <0.05 was considered statistically significant.

Results

Histopathological Results: In one subject of EG and three subjects of CG, ulcerative areas were seen in the wound site where the epithelium had fallen due to widespread granulation tissue, and erosion of scattered bone trabeculae was observed in these areas.

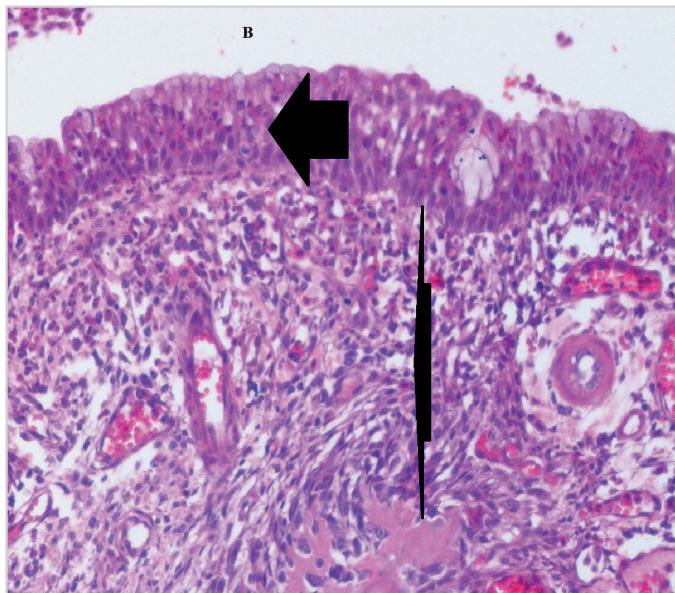


Figure 1. Epithelial and subepithelial hypertrophy in experimental group (HE X20; thick arrows: epithelial, thin arrows: subepithelial area)

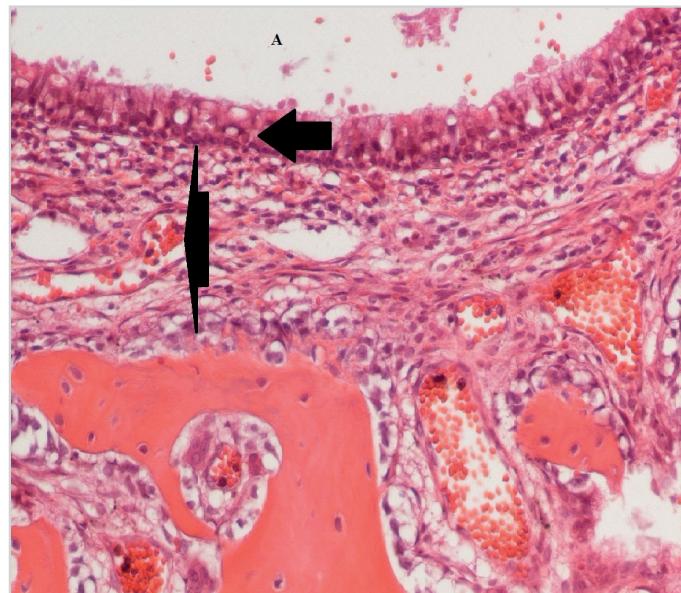


Figure 2. Epithelial and subepithelial hypertrophy in control group (HE X20; thick arrows: epithelial, thin arrows: subepithelial area)

Table 3. Ciliary cell, goblet cell, neutrophil cell count

		Study Group	Control Group	Negative Control Group	p
Ciliary Cell Count	AM±SD	8.00±3.46	9.75±13.43	60.00±29.92	SG-CG p=0.72
	Med (Min-Max)	7.00 (5-16)	4.50 (1-40)	66.00 (12-103)	SG-NCG p<0.05
Goblet Cell Count	AM±SD	65.62±12.92	77.25±20.95	55.38±12.43	SG-CG p=0.38
	Med (Min-Max)	66.50 (39-83)	70.50 (48-108)	53.50 (43-84)	SG-NCG p<0.05
Neutrophil Cell Count	AM±SD	52.63±17.32	96.38±32.19	22.88±13.41	SG-CG p=0.019
	Med (Min-Max)	50.0 (25-85)	99.5 (50-140)	21.50 (5-44)	SG-NCG p<0.05

AM: Arithmetic Mean; SD: Standard Deviation; Med: Median; Min: Minimum; Max: Maximum

Table 4. Subepithelial fibrosis and collagen density

		Study Group	Control Group	Negative Control Group	p
Subepithelial Fibrosis	AM±SD	1.88±0.83	1.75±1.03	0.38±0.51	SG-CG p=0.72
	Med (Min-Max)	2 (1-3)	1 (1-3)	0(0-1)	SG-NCG p<0.05
Collagen Density	AM±SD	2.0±0.53	2.0±0.92	0.38±0.51	SG-CG p=1
	Med (Min-Max)	2 (1-3)	2 (1-3)	0 (0-1)	SG-NCG p<0.05

AM: Arithmetic Mean; SD: Standard Deviation; Med: Median; Min: Minimum; Max: Maximum

No significant difference was observed with respect to the epithelial and subepithelial thickness between EG and CG ($p=0.2$, Mann Mann-Whitney U test, $p=0.92$, Tamhane test) (Table 2). The epithelial and subepithelial thickness measurements of specimens from EG and CG are shown in Figure 1 and 2.

Decrease in the ciliary cell count and increase in the goblet cell count were seen in both EG and CG, compared to NCG. There

was no statistically significant difference between EG and CG ($p=0.72$, $p=0.38$, Mann-Whitney U test). A significant increase was seen in the neutrophil count in both EG and CG, compared to NCG, with a higher increase in CG. The increase in neutrophil count was less significant in EG, compared to CG. Comparison of the groups showed that the increase in the neutrophil count was less significant in EG with reduced inflammation ($p=0.019$, Tamhane test) (Table 3).

Table 5. Hydroxyproline level

	Study Group	Control Group	Negative Control Group	p
AM±SD	41.1488±11.14563	40.4057±23.92726	52.6029±21.84858	SG-CG p>0.05
Med (Min-Max)	46.19 (22.56- 54.38)	34.45 (24- 93.08)	46.1300 (35.12- 94.27)	SG-NCG p>0.05 CG-NCG p>0.05

AM: Arithmetic Mean; SD: Standard Deviation; Med: Median; Min: Minimum; Max: Maximum

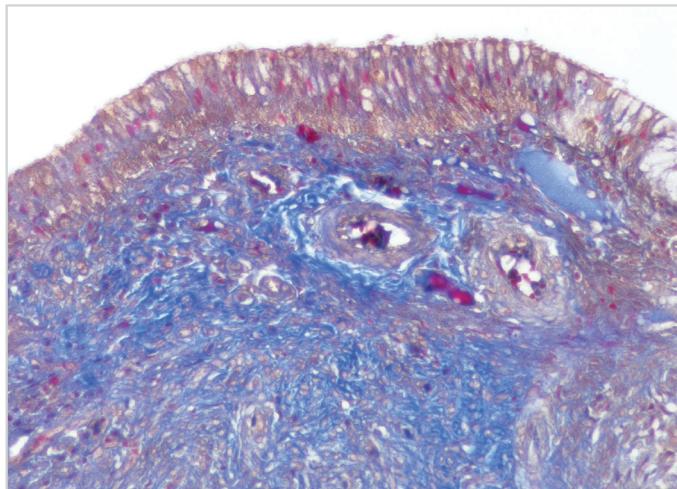


Figure 3. Severe fibrosis, severe increase in collagen density in control group (MT X20)

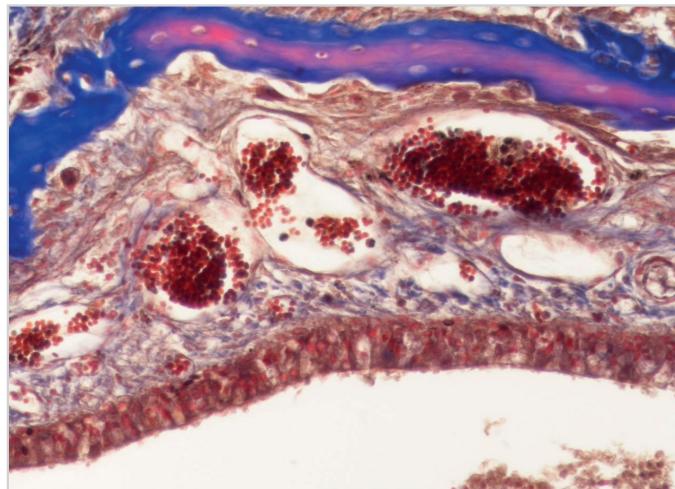


Figure 5. Mild fibrosis, slight increase in collagen density in experimental group (MT X20)

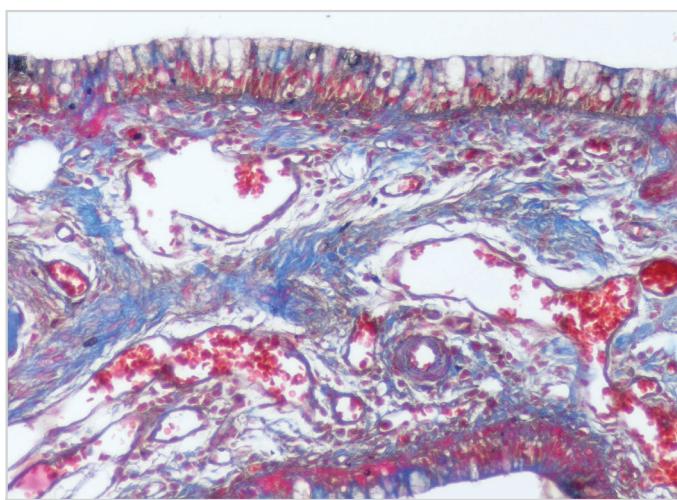


Figure 4. Moderate fibrosis, moderate increase in collagen density in control group (MT X20)

Severe fibrosis was found in two subjects in EG and in three subjects in CG. A severe increase in collagen density was observed in one subject in EG and in three subjects in CG. There was no significant difference in fibrosis and collagen density between EG and CG ($p=0.72$, Mann-Whitney U test, $p=1$, Mann-Whitney U test) (Table 4). Different fibrosis and collagen densities of subjects in EG and CG are shown in Figure 3, 4 and 5.

Evaluation of Hydroxyproline Levels: The hydroxyproline levels in the wet tissue of both EG and CG were lower compared to NCG, however, the differences among the groups were not

statistically significant ($p>0.05$, Mann-Whitney U test) (Table 5).

Discussion

Endoscopic nasal surgery is applied primarily for chronic rhinosinusitis, nasal polyposis, paranasal region tumors, septal deviation, turbinate surgery, hypophysis tumors, cerebrospinal fluid rhinorrhea, and encephalocele treatment. Correctly applied postoperative care, shortens the recovery time of the patient and reduces the frequency of revision surgery (2). Nasal saline irrigation, local wound debridement, systemic and topical steroids, and antibiotics can be used for early postoperative care. That steroids are used following endonasal interventions owing to their anti-inflammatory effects, has given rise to the idea that various anti-inflammatory agents can be used for this purpose.

Propolis, which exerts antibacterial, anti-inflammatory and antioxidant effects, has been used for many years in the treatment of burns and wounds (4). In our study, we, therefore, investigated whether propolis had a positive effect on wound healing by reducing inflammation in the nasal mucosa and accelerating epithelial closure. Propolis, a resin-like substance, is collected by honeybees and modified by bee enzymes (12). It has been suggested that propolis is used by honeybees for many purposes, such as mending the cracks and tears in the hive, preventing the putrefaction of foreign insects that die in the hive, maintaining the inner warmth of the hive and preventing contamination (12). Previous studies have demonstrated the positive effects of using propolis in the treatment of inflammation-induced arthritis, peritonitis, and pleurisy, and in the healing process of frac-

tures, surgical anastomotic lines, and skin and mucosal injuries (5, 13, 14).

In a study by Hu et al. (14) in which rats were experimentally induced with edema in the paw, pleurisy and arthritis, the use of EPE and water-soluble derivative (WSD) propolis extract showed an anti-inflammatory effect similar to steroids. These findings support the idea of our study to use systemic propolis following nasal surgery as an alternative to steroids, owing to its anti-inflammatory effects.

Propolis can be used systemically and locally. Kilicoglu et al. (15) evaluated the effects of systemic propolis on healing in the anastomotic area following colon resection. In addition to its anti-inflammatory effects, propolis was also shown to have provided an early start to angiogenesis, increased and regular collagen production, accelerated epithelial regeneration, thereby significantly accelerating wound healing in the surgical site. In an experimental rat model, Iyyam Pillai et al. (5) induced skin wounds and reported that propolis had a positive effect on wound healing, similar to that of nitrofurazone which is often used in local skin wound care. In a study by Temiz et al. (13) in which healing was evaluated in the anastomosis region after resection, local and systemic propolis was used. The use of both systemic and local propolis showed a positive effect on wound healing in the anastomosis region. Based on the previous findings, we used the oral route in our study, as the easier application. In a similar study which El-Anwar et al. (8) induced nasal mucosal damage in rats and fed them with oral propolis for 15 days, they reported that propolis had an anti-inflammatory effect; however this effect wasn't observed in our study.

As there are variations in the chemical structure of propolis and no standardization has yet been achieved, the therapeutic or toxic doses for humans and animals are not fully known (16). Previous reports have demonstrated that doses between 100 mg/kg/day and 600 mg/kg/day can be safely used in rats (13, 17). In a study by Nassar et al. (18) 50 mg/kg/day propolis showed an immunostimulant effect and did not cause any side-effects in rabbits. Also Nader et al. (19) reported that the development of the inflammatory process of atherosclerosis was reduced in rabbits fed with oral propolis 75 mg/kg/day. Considering the propolis dose used in the mentioned study, we decided to administer propolis at a dose of 75 mg/kg/day to EG.

Despite the use of animals such as dogs, sheep, and pigs, the rabbit maxillary sinus model is the most frequently used one (20, 21). Sun et al. (20) created an injury in the rabbit maxillary sinus medial wall and applied no treatment. It was reported that on day four, there was an opening to a significant degree in the maxillary sinus medial wall and on day 14, this was completely closed. In addition, collagen deposition gradually increased in the regenerated mucosa and was seen to peak on day 14. In another study, Forsgren et al. (22) reported that re-epithelialization of the rabbit maxillary sinus was completed in 14 days. Proctor et al. (21) examined the effects of hyaluronan on wound healing on days 14 and 21 following damage in the rabbit max-

illary sinus medial wall and found that the wound size and histopathological appearance were similar in both time periods. In the current study, we examined epithelial cells and epithelial and subepithelial thicknesses, and, in the light of the previous findings, we terminated the study on day 14 when the epithelial closure was completed.

Apart from the studies that use the rabbit maxillary sinus in experimental paranasal sinus diseases, there are several studies where nasal septal or conchal mucosa were used (10, 23). In a study by Bayraktar et al., (23) endoscopic mucosal damage was created with punch forceps in the bilateral ventral nasal concha of all rabbits. The surgical protocol applied by Bayraktar et al. (23) was utilized in the current study. Also basing on the study of Khalmuratova et al. (10) as a reference study, where the authors evaluated the effects of systemic dexamethasone on wound healing in septal mucosal damage created in a rat model, the epithelial and subepithelial thicknesses were measured and goblet, ciliary, and inflammatory cells were counted in our study. Since we considered that the mucosal damage with punch forceps on the concha, given the folds could cause inflammation in the adjacent tissues, the newly developed epithelial and subepithelial thicknesses and cell counts were evaluated and the groups were compared to baseline values.

During wound healing, over production and deposition of collagen occurs to restore the damage. Hydroxyproline occurs by enzymatic hydroxylation of proline amino acid. The level of hydroxyproline is accepted as an indicator for assessing collagen production or metabolic degradation (7). The level of hydroxyproline can be measured in different tissues such as plasma and urine in order to determine normal and pathological states of collagen metabolism (7). Several previous studies have been conducted to examine the relationship between hydroxyproline levels and wound healing in tissues such as the colon, the skin, and the lungs (5, 7, 13). Our study is the second study in the literature that measures hydroxyproline levels in a thin and nonhomogeneous tissue like nasal mucosa. Consistent with the study of Bayraktar et al. (23), the hydroxyproline level was measured with the HPLC method in wet tissue as a marker of collagen metabolism in our study.

Conclusion

Our study results showed that the use of propolis significantly reduced the neutrophil cell count in endoscopic mucosal damage-induced rabbits, although there was no significant difference in epithelial and subepithelial thicknesses, goblet cell count, ciliary cell count, subepithelial fibrosis, collagen density, and hydroxyproline level. Although there are many studies showing the positive effects of propolis on wound healing, we didn't observe this effect in our study. Nonetheless, there are some limitations of this study. Our sample size was relatively small. Also, we had difficulty in choosing the propolis dosage as due to limited studies there has not been a standardized dosage in the wound healing process. This study can be used as a source for future studies that will be performed on more subjects and with a higher propolis dosage.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ondokuz Mayıs University Local Ethics Committee for Animal Studies (2013-HADYEK-62).

Informed Consent: N/A.

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Author Contributions: Concept - E.K., S.Ç.K., D.G., M.Y., A.A.; Design - E.K., S.Ç.K., D.G., M.Y., A.A.; Supervision - E.K., S.Ç.K., D.G., M.Y., A.A.; Resource - E.K., S.Ç.K., D.G., M.Y., A.A.; Materials - E.K., S.Ç.K., D.G., M.Y., A.A.; Data Collection and/or Processing - E.K., S.Ç.K., D.G., M.Y., A.A.; Analysis and/or Interpretation - E.K., S.Ç.K., D.G., M.Y., A.A.; Literature Search - E.K., S.Ç.K., D.G., M.Y., A.A.; Writing - E.K., S.Ç.K., D.G., M.Y., A.A.; Critical Reviews - E.K., S.Ç.K., D.G., M.Y., A.A.

Conflict of Interest: The authors have no conflicts of interest to declare.

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