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Comparative Analysis of the Use of Domestic Bioresorbable Collagen Membranes at the Closure of Postoperative Defects of the Oral Mucosa in an Experiment *In vivo*

Natalia Blagushina [1], Ekateina Diachkova [1,*], Maria Volkova [1] Svetlana Pankush [1], Svetlana Tarasenko [1,]

[1] Department of Oral Surgery of Borovsky Institute of Dentistry, Sechenov University, Russia; blagushina.natalia@yandex.ru (N.B.), secu2003@mail.ru (E.D.), yablokoland@yahoo.com (M.V.), tetra_ivi@mail.ru (S.P.), prof_tarasenko@rambler.ru (S.T.);
* Correspondence: secu2003@mail.ru;

Scopus Author ID 57200189811

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Abstract: The success of using soft tissue transplants is enough for their spreading in the clinic, but the need to cover the surface, where the transplant was taken from, can be forgotten. It can lead to long-term discomfort of the patient in real life, in some cases to complications alike bleeding; We performed the analysis of the use of different new xenogenic resorbable membranes created within our University in compare with the natural healing of oral mucosa defects in the experiment *in vivo* on 36 rabbits after performing of the surgical wound on the palatine side (5x5 mm). All animals were separated (divided) for 3 groups: #1 group of control and main groups #2 and #3, where we used pericardium and collagen film for covering mucous defects. We assessed the edema, hyperemia in the operation side, the pain according to animal behavior, the histological picture after animals completion of the experiment (on 3rd, 6th and 10th days); The decrease of clinical signs of inflammation in groups of collagen and pericardium films use ($p<0.05$) was statistically confirmed. Analysis of histologic investigation of biopsy specimens has shown the faster and massive growth of soft tissue in the donor site after application of pericardium and collagen films ($p<0.05$). An analysis of the experiment results allows recommending their possible use for closing the donor site after taking a free gingival graft or in the zone of postoperative wound defect in the oral mucosa in clinical oral surgery after specific clinical trials.

Keywords: membrane; collagen; pericardium; mucosa defect; oral surgery.

1. Introduction

In recent decades, the problem of mucous membrane wound closure of a donor site, mostly in the palate, stays actual in oral surgery. Due to the need of providing some specific procedures for increasing of the attached gum width in case of its deficiency, to treat local and multiple gingival recessions (the prevalence is up to 100% in patients over 64 years old), as well as performed vestibuloplasty (the frequency of the low alveolar ridge height is 62.6% according to WHO), dental implant surgery and removing of the oral mucous areas affected by premalignancy [1- 15]. Despite the effectiveness of the technique at the recipient site, one of its drawbacks is the presence of an open wound at the donor zone. The healing period is quite painful and uncomfortable for patients [18]. There is a high probability of mechanical trauma

to the open wound surface with solid food or a food bolus, which may cause pain, bleeding, suppuration, and damage to the growing granulation tissue [1-15]. Treatment of open wounds in the oral cavity is a challenge since it is impossible to completely protect the surface from adverse mechanical, chemical, and physical factors, as well as from the penetration of microorganisms. Due to the continuous rinsing of the wound with saliva, it is difficult to use pharmacological agents that provide analgesic, antibacterial, anti-inflammatory, and reparative effects [1,16,17]. The literature sources contain quite diverse data on the methods for closing a donor site. Some authors placed guiding sutures with chromic catgut or silk thread to ensure hemostasis [15]. Later, the microfibrous hemostatic collagen can be in the form of powder or a non-woven membrane. Other authors recommend the use of periodontal and resorbable cellulose dressings, cyanoacrylate medical adhesives [15, 18, 19]. To ensure the maximum postoperative comfort at the donor site, a preformed dental splint was used. However, closure of the wound surface at the donor site with an iodoform swab, hemostatic sponge, and guiding sutures turned out to be a temporary measure, while the use of thermoplastic dental splints requires additional cost for the patient and significantly increases the price of treatment. Therefore, the development and introduction of materials and improvement of treatment methods that contribute to the protection and regeneration of the oral mucosa in the wound area are relevant tasks for surgical treatment of patients with dental diseases [16, 18]. The positive experience of using collagen membranes, including acellular xenopericardium, as a substrate for closing mucous membranes in plastic surgery of the neovagina, orbit, and urethra was described in the literature. These materials have several advantages, such as elasticity, sufficient porosity, excellent biocompatibility with tissues of the recipient site [19, 20]; however, we have not found in the literature data about their use in dental practice. In this regard, it is interesting to conduct a comparative analysis of the use of bioresorbable collagen membranes in combination with levomicitin and methyl uracil to close wounds of the oral mucosa in the experiment *in vivo*, followed by the introduction in clinic practice. The aim was an increase in the efficiency of the surgical treatment of dental patients with the use of bioresorbable collagen membranes to close postoperative wound defects in the oral mucous membrane.

2. Materials and Methods

An experimental model of a pilot study was developed using 36 male chinchilla rabbits weighing 4000 g at the Central Vivarium of the Federal State Autonomous Educational Institution of Higher Education I.M.Sechenov First Moscow State Medical University under the Ministry of Health of Russia (Sechenov University). The experiment, as well as the maintenance of animals, was carried out in accordance with the Directive № 63 of 22.09.2010 of the Presidium and Parliament of Europe and the Order of the Ministry of Health of the Russian Federation № 267 of 19.06.2003 (approved by Local Committee of Ethics, protocol № 05-19 from 10/04/2019)

The number of animals we count with sample size formula for finite population, where incidence was equal 50%, α=0.05, power was 0.8:

$$N_1 = \left\{ z_{1-\alpha/2} * \sqrt{\bar{p} * \bar{q} * \left(1 + \frac{1}{k}\right)} + z_{1-\beta} * \sqrt{p_1 * q_1 + \left(\frac{p_2 * q_2}{k}\right)} \right\}^2 / \Delta^2$$

$$q_1 = 1 - p_1$$

$$q_2 = 1 - p_2$$

$$\bar{p} = \frac{p_1 + kp_2}{1 + K}$$

$$\bar{q} = 1 - \bar{p}$$

$$N_1 = \left\{1{,}96 * \sqrt{0 * 1 * \left(1 + \frac{1}{1}\right)} + 0.84 * \sqrt{0 * 1 + \left(\frac{0.5 * 0}{1}\right)}\right\}^2 / 0.5^2$$

$$N_1 = 11$$

$$N_2 = K * N_1 = 11$$

Where:

p_1, p_2 = proportion (incidence) of groups #1 and #2

$\Delta = |p_2 - p_1|$ = absolute difference between two proportions

N_1 = sample size for group #1

N_2 = sample size for group #2

$\alpha = 0.05$

$\beta = 0.2$

z = critical Z value for certain mean of α or β

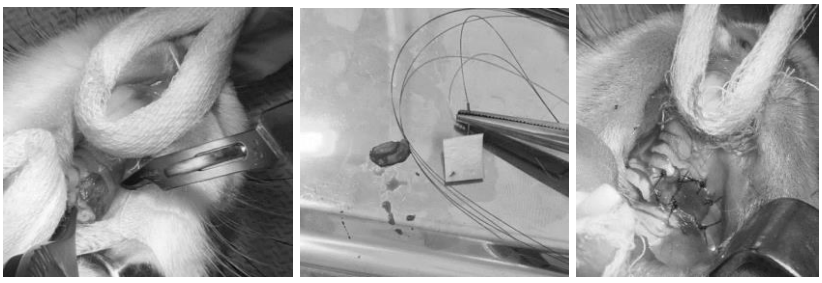
K = ratio of sample size for group #2 to group #1

Sample size was equal 11.

According to the need for assessment of drop out of research, the number of animals in one group was equal to 12.

2.1. The operation technique.

A veterinary certificate of animal health will be obtained and, once purchased, animals were kept in quarantine for two weeks. Animals were kept in cages with feed grooves and latticework made of steel, sawdust made of hardwood as bedding. The temperature in the vivarium was 18-22ºC, the relative humidity was 50-65%, the lighting cycle was twelve hours, and the air volume of the cage was changed tenfold per hour. Standard vivarium ration: the animals were fed with combined feed for laboratory animals (microbiological status of the water corresponds to GOST standard 51849-2001), as well as filtered tap water ad libitum in standard drinking bottles (microbiological status of the water corresponds to Sanitary Regulations and Norms 2.1.4.1074-01). The entire experiment, as well as the maintenance of animals, was carried out in accordance with the Directive No. 63 of 22.09.2010 of the Presidium and Parliament of Europe "On the protection of animals used for scientific research" and Order of the Ministry of Health of the Russian Federation No. 267 of 19.06.2003 about good laboratory practice.



(a) (b) (c)

Figure 1. Operative technique in animals: (a) Creation of defect of palatine mucosa; (b) View of collagen membrane; (c) Covering the mucous defect with the domestic membrane.

With general anesthesia (intramuscular injection) (Zoletil (Virbac, France) (5mg/kg animal weight) and Xila (0.2ml/kg animal weight)) and additional infiltration anesthesia Sol. Ultracaini DS 1:200000 with vasoconstrictor (adrenaline) for local narrowing of the lumen of the vessel and stop the bleeding was measured in the alleged shape and size of the defect and followed by the withdrawal of free gum autograft. Mucosal defects were formed on a hard palate of all animals using a 15C scalpel (figure 1a,b).

During the operation, thorough hemostasis of the donor site was performed. All experimental animals were divided into 3 groups. In 12 animals from the control group, wound regeneration was with secondary healing. In 12 animals from the main group 1, wound healing occurred under the xenopericardium collagen membrane (figure 1c); in 12 animals from main group 2, the wound healed under a collagen film. For the remaining 2 groups, the edges of the sterile membrane packaging were cut off after 70% alcohol irrigation; collagen membranes were removed from the packaging using sterile tweezers (each group had its own collagen membrane). The implant was cut out with scissors of the required size and shape, which corresponded to the size of the resulting defect. Then the membrane was placed on the defect in the area of hard sky in such a way that it covered the obtained wound surface with 3 mm overlap of the wound edges and was fixed with the suture material "Prolene 6.0" on an atraumatic needle with 3/8 knot seams. The 3 mm overlap was the most favorable as it prevented additional damage. To prevent injury to the resulting defect in the postoperative period, feeding the rabbits were carried out with softened feed.

Animals completed their participation in the experiment from the experiment on day 3, 6, 10 after surgery. The samples of biomaterial were fixed in 10% formalin solution, embedded in paraffin, 4-5 μm thick microtome sections were stained with hematoxylin-eosin, examined under a Leica DM4000 B LED universal microscope, photographed with a Leica DFS 7000T camera, phase-contrast, and polarized light microscopy were performed.

Morphometric analysis of the square of granulation and connective tissues in samples was provided with the application of the planimetric grid in the program Adobe Photoshop.

Statistical analysis was provided according to comparing all three groups for approval of the null hypothesis, where ɱ0 told about the absence of differences between groups and was not equal ɱ1 hypothesis. We checked the presence of normal distribution with the help of the Shapiro-Wilx test, where the null hypothesis approved the normal distribution presence of the test and found that the control group, group 2 (first main group), and group 3 (second main group) were not normally distributed (p-value = 0.0004971; 8.317e-08; 1.857e-06) that is why we used criteria of non-parametric statistics for compared results in different groups (Kruskal-Wallis rank-sum test) and coefficients of Pearson and Spearman for assessment of correlation and its density between different signs in statistics program R.

3. Results and Discussion

After the assessment of results, we found that clinically it was observed a significant decrease of edema in dynamics in both the main group in comparison with the control group ($p<0,05$).

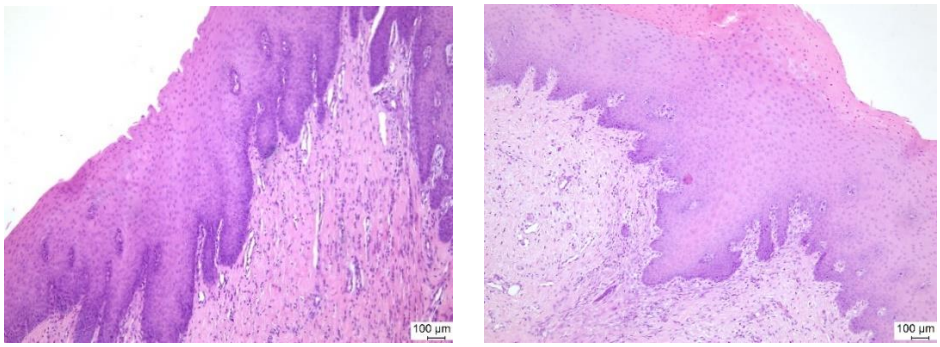
Hyperemia in the recipient site was approximately equal in control, and both main groups ($p>0.05$) in common assessment and all control monitoring days.

We evaluate pain according to the analyses of animals' behavior as significantly weaker in both main groups in comparison with the control group during all days of research ($p<0.05$) (table 1).

An experimental histological study has shown the following results. The animals from the control group had no epithelization of the wound surface on day 3, inflammatory infiltrates, microvascular disorganization, and the absence of granulation tissue was noted. By day 6, the surface of the wound was not epithelized, although the proliferation of epithelial cells was noted around the defect. This area was filled with immature granulation tissue, inflammation signs improved. After 10 days, the surface of the defect was covered with epithelium, under which fibrous granulation tissue was observed (figure 2).

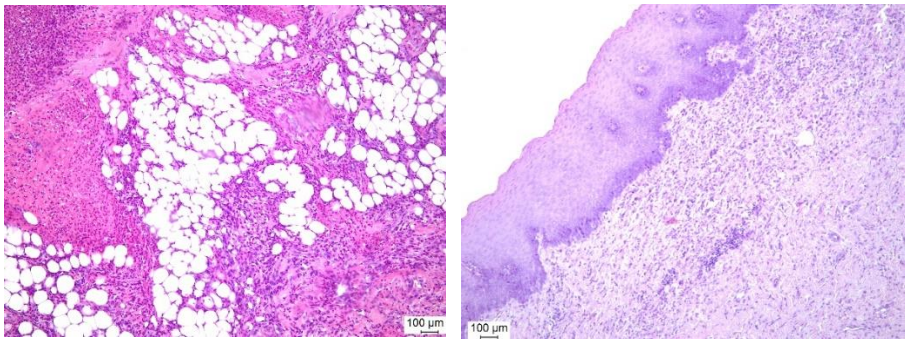
Table 1. The clinical differences in dynamics *in vivo* after the operation.

Symptom	Group 1 Mean Min-Max	Group 2 Mean Min-Max	Group 3 Mean Min-Max	p-value
Edema				
3rd day	2.5±0.67	1.25±0.45	1.33±0.49	**0.0006**
6th day	2±0.76	1±0	1.25±0.46	**0.02705**
common	2.3±0.73 (1-3)	1.15±0.37 (1-2)	1.3±0.47 (1-2)	**0.00002**
Hyperemia				
3rd day	2±0.74	1.42±0.51	1.58±0.67	0.17054
6th day	1.75±0.71	1.25±0.46	1.375±0.74	0.35059
common	1.9±0.72 (1-3)	1.35±0.49 (1-2)	1.5±0.69 (1-3)	0.064
Pain				
3rd day	2.42±0.74	1.58±0.51	1.67±0.67	**0.00857**
6th day	1.875±0.46	1±0	1±0	**0.0133**
common	2.2±0.62 (1-3)	1.35±0.49 (1-2)	1.4±0.5 (1-2)	**0.00033**



(a) (b)

Figure 2. Results of histological investigation in control group: (**a**) 3rd day (stained with hematoxilin-eosin); (**b**) 10th day (stained with hematoxilin-eosin).



(a) **(b)**

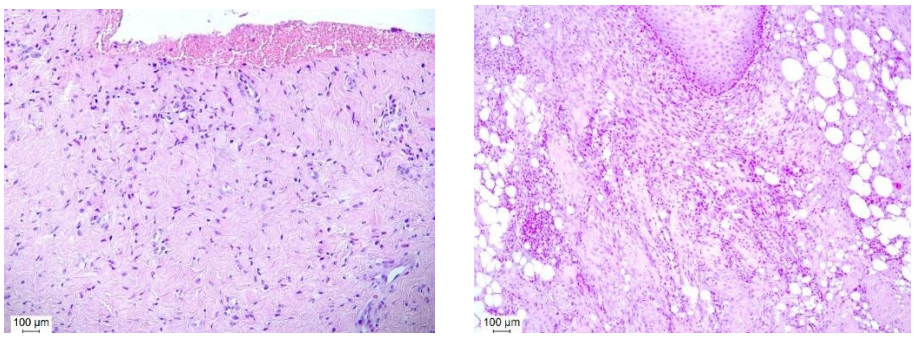
Figure 3. Results of histological investigation in main group No 1: (**a**) 3rd day (stained with hematoxylin-eosin); (**b**) 10th day (stained with hematoxylin-eosin).

The histological picture of biopsy specimens of animals from the main study groups differed from that in the control group. After 3 days, epithelization was absent in animals from

both study groups, but epithelial hyperplasia was noted at the edges of the defect. Inflammatory infiltration was less pronounced. In the study group with pericardium (main group No 1), a part of the defect was already filled with immature granulation tissue (figure 3).

After 6 days, complete epithelialization of the wound was noted; a mature granulation tissue was formed under the epithelium, turning into the scar tissue. Inflammatory infiltration was less in comparison to the control group. The maximum activation of wound repair was noted in the area closed with the pericardium.

By day 10, the wound was epithelialized; the fibrous tissue with minimal inflammatory infiltration was located under the epithelium. In the study group with the pericardium, the tissue was more mature in comparison to the study group with collagen film (figure 4).



(a) **(b)**

Figure 4. Results of histological investigation in main group No 2: (**a**) 3rd day (stained with hematoxylin-eosin); (**b**) 10th day (stained with hematoxylin-eosin).

According to morphometric analysis, a different square of granulation and connective tissues in samples on the 6th and 10th days in all 3 groups were investigated ($p<0.05$). On the 3rd day, we found the absence of cells of these tissues in all groups. The dynamics of changing granulation tissue on connective tissue were more significant in both the main groups in comparison with the control group. Also, we found a faster decrease of the neutrophil amount in tissues, although the probability was not significant ($p>0.05$) what can relate to insufficient sample size (Table 2). We did not count for neutrophils on the 10th day because of their absence in samples.

Table 2. The differences in histologic dynamics *in vivo* after the operation.

Criterium	**Group 1** **Mean** **Min-Max**	**Group 2** **Mean** **Min-Max**	**Group 3** **Mean** **Min-Max**	**p-value**
Granulation square, % 3rd day 6th day 10th day	 0 20±4.32 12.25±3.86 (0-26)	 0 45±12.25 20±6.73 (0-60)	 0 40±5.78 25±4.1 (0-45)	**0.00842**
Connective tissue square, % 3rd day 6th day 10th day	 0 15±2.16 25±4.1 (0-30)	 0 30±7.7 60±10.9 (0-70)	 0 27±6.27 40±8.12 (0-50)	**0.00316**
Neutrophil amount 3rd day 6th day 10th day	 31.5±1.29 12.5±1.29 0 (0-33)	 13.25±1.7 0 0 (0-15)	 16.5±1.29 0 0 (0-18)	0.05506

We assessed the correlation between the changing square of connective and granulation tissue in samples and found a strong and significant correlation between changing in dynamics

square of granulation and connective tissue in the control group ($r=0.59$, $p<0.05$) and the 2nd main group ($r=0.67$, $p<0.05$), but with a mild density of correlation and insufficient statistical probability (r_s,= 0.44 and r_s,= 0.46, $p>0.05$, respectively).

Rocchietta *et al*. (2012), in the experimental model on animals, studied the response of the body to the introduction of the collagen membrane. In the first case, the collagen membrane was placed on the periosteum after the apical displacement of the flap and left open. The study confirmed optimal integration of the collagen matrix with surrounding tissues without any adverse reactions during the whole period of observation. In the group where the collagen matrix remained open in the oral cavity for 7 days, no signs of membrane presence were found on the wound surface [22].

F. Wehrhan *et al*. (2010) also conducted an experimental study in piglets in which they placed the same collagen matrix on an artificially created ear defect. The authors observed the membrane presence between 1 and 7 days and its degradation after 7 days. By the 14th day, there were no differences in tissue architecture, fiber orientation between the recipient area and the augmented zone [23].

According to many researchers, the use of collagen matrixes of xenogenic origin demonstrates good healing of soft tissues and growth of keratinized epithelium, and even for bone regeneration [24-32]. It is assumed that the use of such membranes causes a greater increase in keratinized tissue compared to dermal matrices, due to the more porous structure that allows retention of the blood clot.

Most of the tissue-equivalent characteristics important for surgeons, in addition to cellular material, determine the type of cell carrier (substrate). There is currently no ideal material capable of serving as the basis for any tissue-equivalent [33].

The substrate is selected individually for each equivalent, considering the characteristics of the cells as well as the characteristics necessary for functioning in each specific area of reconstruction. The question of selecting a tissue equivalent substrate material for mucous membrane reconstruction remains relevant since the materials proposed in the world literature have a number of drawbacks, including poor biodegradation indices, insufficient strength, lack of elasticity, insufficient porosity, difficult transportation and storage, inconvenience in operation, and high cost [34].

4. Conclusions

The absence of optimal material for the creation of tissue tissue-equivalent of mucous membranes, absence of generally accepted surgical criteria for selection of substrate materials in the available literature, as well as an absence of an experimental model for investigation of properties of such materials, were the grounds for this study.

Thus, the results of a histological study of biopsy specimens of oral mucosa in experimental animals showed that the closing of wound defect of the hard mucous palate with pericardium and collagen film had a noticeable stimulation and decreased time of epithelisation and cicatrization of the wound were observed, especially in the group with the pericardium.

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Conflicts of Interest

The authors declare the absence of conflict of interest.

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