THE EFFECT OF COLLAGEN-CHITOSAN-NATRIUM HYALURONATE COMPOSITE ON NEOVASCULARIZATION AS ANGIOGENESIS REACTION IN RABBIT CORNEAL STROMA WOUND
(Experimental Study on *Oryctolagus cuniculus*)

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Introduction

The cornea is an essential refractory tissue in the eye that supports two-thirds of the eye's total refractive power. Corneal tissue is avascular and has angiogenic privilege, which is the cornea's ability to maintain its avascular condition by preventing neovascularization that grows from the surrounding tissue. However, the inflammatory process in corneal wound healing can affect this condition, causing cloudiness and blood vessels to appear in the corneal tissue that can lead to visual disturbances to blindness. Keratoplasty is the primary therapeutic modality to replace pathological corneal tissue. Due to limited donor keratoplasty, many alternative therapies developed to restore corneal regeneration, biomaterials in prosthetic devices with composite materials. An excellent composite composition can reduce the accumulation and replacement of extracellular matrix components in tissue injury, mediate the immune response, prevent neovascularization and reduce fibroblast deposition, thereby reducing scar tissue formation. Several recent studies have developed compositions to achieve these criteria, including collagen-chitosan-sodium hyaluronic composites [9].

Collagen-chitosan-sodium hyaluronic composite is a natural biopolymer considered to have good biocompatibility, biodegradable, and non-toxic properties. Collagen is the most abundant protein in mammalian tissues and a significant component of the biological extracellular matrix. Chitosan is a polysaccharide chitin derivative compound, has a similar structure to glycosaminoglycans, has properties like human body tissues, and can be broken down by enzymes in humans. Hyaluronic acid is the simplest glycosaminoglycan and is found in almost every mammalian tissue. It is the extracellular matrix's main polysaccharide, located both on the surface and inside the cell. So that from the three compositions, advantages can be obtained; in addition to being easy to get and produce, the composite is safe and can increase corneal tissue regeneration.

In [12], researching artificial corneas based on collagen - chitosan - NaHa showed no changes in chemical interactions, indicating that the three ingredients were successfully made. The artificial cornea is hydrophilic because the degree of contact is below 90°, then the artificial cornea also has a water absorption rate of 90%. These properties support cellular interactions and provide hydration to maintain eye clarity and permeability. Therefore, collagen-chitosan-sodium hyaluronic composites can be an excellent choice for making engineered corneas. The collagen-chitosan-sodium hyaluronic biomaterial composite also has advantages over the amniotic membrane, that it is easier to fabricate, so it does not depend on donor availability.

Materials and methods

Methods

It is a type of experimental laboratory research with a randomized post-test-only control group design. The test was carried out on three groups of experimental animals in New Zealand rabbits without injury, which was injured by the corneal stroma, with and without implanted collagen-chitosan-sodium hyaluronic composite biomaterial for two weeks on the corneal stroma of rabbit eyes in vivo. Clinical evaluation was carried out, that is, the level of neovascularization.

Animal

This study sample was an adult male New Zealand rabbit species *Oryctolagus cuniculus* who had inclusion criteria rabbits weighing between 2.5 - 3 kg and declared healthy by a veterinarian. The exclusion criteria are rabbits with eye disorders and diseases. Drop out criteria rabbits died and experienced pain in both their eyes and other organs during the clinical trial evaluation.

Rabbits are placed in a cage with 90 cm x 60 cm x 60 cm; each rabbit occupies one cage, room temperature, and safe environment, adequate ventilation and good nutrition.

Ethical eligibility was obtained from the Research Ethics Commission of the Faculty of Veterinary Medicine, Animal Care and Use Committee (ACUC), Airlangga University, Surabaya.

Collagen-chitosan-sodium hyaluronates (Col-Chi-NaHa) membrane preparation

Chitosan (Molecular Weight: 500 000-600 000) is made from chitin through a deacetylation process (85-90% deacetylation). Collagen and chitosan were dissolved in 0.001 N hydrochloric acid separately. Then, the collagen and chitosan solutions were poured into a beaker and stirred with a homogenizer for 30 minutes. Sodium hyaluronate solution with a purity of 95% is dropped into the mixture and mixed for 30 minutes. HPMC was added to the collagen solution as

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a crosslinker in this study. The ratio of collagen and HMPC used was 1: 1 by weight. So that the overall concentration of collagen and chitosan does not change, HPMC is added without increasing the volume of the solvent. The collagen that has been added by HPMC is then stirred using a magnetic stirrer for 12 hours and the temperature is maintained at 40ºC. After the collagen solution was cross linked, the next procedure was the addition of 0.6% w / v chitosan and NaHA solutions. The homogeneous solution was placed on a Perspex plate and heated for 24 hours at 35ºC to obtain a dry membrane. The dry membrane was then immersed in PBS. Before being used in in vivo research, the dry membrane was thoroughly rinsed in PBS, prepared by 75% ethanol for 30 minutes to be sterilized and rinsed in a sterile PBS buffer solution [11,12].

Intrastromal implantation
Rabbits were placed on a sterile operating table, anesthetized with 50 mg/kg ketamine hydrochloride and 5 mg/kg xylazine given intramuscularly. The eye to be tested was given 0.5% pantocaine topical anesthetic. The intrastromal bag was made through a lamellar incision using a cataract surgery knife, then a composite with a thickness of 0.2 mm and a diameter of 3 mm was inserted into the bag [8].

Corneal neovascularization grading
Examination of the level of corneal neovascularization in this study was carried out on the 14th day after treatment, using slit lamp biomicroscopy with diffuse illumination technique. The results of the examination are then classified as 0 if there is no neovascular in the entire area, 1 if there is peripheral neovascular <2 mm, 2 if there is peripheral neovascular> 2 mm but has not reached the center of the cornea, 3 if there is neovascular reaching the center of the cornea, 4 if there is neovascular reaching the center of the cornea is accompanied by fibrosis.

Statistics
Comparison of dependent variables, the ordinal scale will be analyzed using the Kruskal-Wallis test.

Results and discussion

The Effect Of Col-Chi-NaHa Intrastromal Implantation On Corneal Neovascularization
The study results showed significant differences in the level of physiological neovascularization between the negative control group, the positive control group, and the composite implantation group ($p = 0.012$, $\alpha < 0.05$). Forty percent of rabbits receiving the collagen-chitosan-sodium hyaluronic composite resulted in significantly higher physiologic neovascularization levels than stromal injuries that were not given the biomaterial composite. New blood vessels were used to initiate the wound healing process with healing factors in the lesions. Likewise, the proportion of VEGF expression in the untreated group, the stromal injury group, and the hyaluronic collagen-chitosan-sodium composite implant group had differences found ($p = 0.000$, $\alpha < 0.05$).

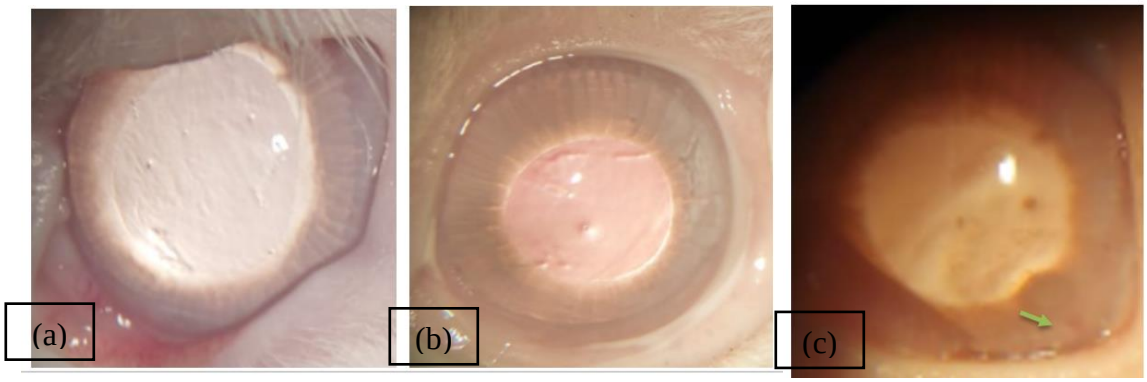

Fig 1. Neovascular images in the group (a) control negative (b) control positive (c) composite implantation treatment.

The Effect Of Col-Chi-NaHa Intrastromal Implantation On Corneal Neovascularization
Research by Widiyanti et al. in 2019 [12] made a composite Collagen - Chitosan - Sodium Hyaluronic with a collagen ratio of 20% w / v and chitosan 10% w / v, then the two solutions were dissolved in 0.1 M and 0.6 M acetic acid separately and added NaHA 0, 6% indicates hydrophilic ability with a percentage of more than 90% and the contact angle is reduced with the addition of NaHA. Engineered corneas made from Col-Chi-NaHA have hydrophilic properties to support cellular interactions and provide hydration to maintain eye clarity and permeability. In [10] was shown, state that the collagen-chitosan-sodium hyaluronic composite has good biocompatibility with a percentage of living cells above 85%, a pore size that matches pore standards for keratoprosthesis, and has strong antibacterial properties. In [11] shown, stated that the degradation test for the Collagen-Chitosan-Glycerol-HPMC composite was 0.6861%, and the Collagen-Chitosan-Glycerol composite was 0.9469%. Widiyanti, and Siswanto, 2020 [10], stated that sodium hyaluronate can increase

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artificial corneal transmittance, degradability, and surface morphology that still need to be improved further research. Widiyanti et al., 2020 [10], showed good cytotoxicity results where the biomaterial showed a percentage of living cells above 70%, which indicated that the biomaterial was not toxic. After eight months of implantation, it showed the same corneal stromal thickness as a normal cornea. The study results showed good biocompatibility in vivo. That is, there was no inflammation and exudation in the anterior chamber and transparent cornea.

The cornea maintains a balance between proangiogenic and anti-angiogenic factors to keep it avascular and transparent. This unique angiogenic ability occurs through passive mechanisms such as the cornea's anatomical structure and active molecular mechanisms. The angiogenic balance can be disrupted by pathological conditions such as injury, infection, or activation of an immune response that proceeds to an inflammatory process. It triggers the process of corneal neovascularization, which causes scarring, lipid deposition, and corneal edema. Also, pathological blood vessels' presence worsens the prognosis for corneal transplantation because it increases the rejection reaction, which is mediated by the immune response to foreign transplanted tissue. All of these changes can lead to decreased vision and blindness [4]

Tang et al. [8] made a neovascularized stromal pouch model in mice by way of anesthetized mice. They then performed a 1.2–1.4 mm lamellar incision from the corneal limbus using a cataract surgery knife. A sac is created under the cornea's epithelial layer by inserting a knife horizontally into the center of the cornea and extending it to a size sufficient to insert a membrane containing angiogenesis factors. The membrane is inserted using tweezers into the pocket slowly and ensures that the membrane is not folded. Wound healing occurred within the first 48 hours postoperatively then, seven days after membrane implantation, the mice were systemically and topically anesthetized, and blood vessels were observed in the cornea. The results showed that the corneas treated with the poly-HEMA membrane did not show any blood vessel growth, while in the corneas treated with the bFGF membrane, new blood vessel growth occurred.

This study used a composite implantation method in the intrastromal bag such as Tang et al., 2011 [8], clinical observations of corneal blood vessels were carried out on day 14 using slit-lamp biomicroscopy and assessed the level of neovascularization in the cornea with a scoring technique.

The results of the evaluation of the level of neovascularization obtained were that there were significant differences in the composite treatment group compared to other groups. This is following the corneal angiogenesis process related to blood vessels' activity in the wound healing process. The formation of new blood vessels aims to infiltrate the leukocytes into the corneal lesions. Clinically, vascularization in the corneal wound healing process can be classified as young active phase, old active phase, mature phase, partial regression, and complete regression. The active young phase characterized by newly formed blood vessels, appearing bright red filled with blood, covered with minimal fibrous tissue, and growing actively on the cornea with a visible capillary network. The corneal stroma around young active blood vessels shows signs of extravasation and edema. Active old-phase blood vessels appear less bright and have rapid circulation; blood vessels have reached and surrounded or covered the corneal lesion. Mature vessels appear with little capillary remain in the scar tissue or the corneal stroma after the corneal lesions have healed. These vessels contain blood and maintain circulation to the area of the lesion. Vascular partial regression when the corneal lesions had reduced due to therapy or due to the healing factors of the corneal vessels. In this phase, circulation in the vascular complex appears relatively slow, the vessels are not enlarged as much, and some parts of the vessels appear less visible. The ghost vessels appear as fine white lines and do not have active circulation accompanied by thinning of the cornea's lesions [6].

New blood vessels in the corneal wound healing process differ from blood vessels in rejection reactions characterized by diffuse edematous corneal lesions from the first postoperative day, persistent corneal opacities, new blood vessels are immature and brittle, and high permeability causes corneal lesions to become edematous in a long time. New blood vessels are immature and fragile, which is a lousy marker leading to the rejection process, in contrast to physiological new vessels, which are mature and do not break easily. Corneal lesions in rejection reactions appear to be edema that does not decrease over time, in contrast to corneal lesions in the wound healing process. They become smaller and gradually clear up as the wound healing time goes on [3].

Limitation

1. This study has the limitation of making a stromal pocket not carried out with the guidance of optical coherence tomography, which has the advantage of increasing the accuracy of the pocket's location confined to the corneal stroma.
2. The study did not carry out serial observations. Suggestions for further research are necessary for serial observations (3, 7, 21, and 28 days) following the corneal wound healing timeline with a more extended study time. Observations in the timeline included the histology of leukocyte infiltration on day 3, fibroblast density on day 7, vascular endothelium on day 21, and collagen density on day 28.
3. Clinical examination of neovascularization of the cornea should use in vivo confocal microscopy to observe the emergence of new blood vessels earlier.

Conclusion

Research on the effect of the collagen-chitosan-sodium hyaluronic composite on the level of

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neovascularization as an angiogenesis reaction in rabbit corneal stromal injuries can be concluded that the rate of physiological neovascularization in the corneas of rabbits treated with collagen-chitosan-sodium hyaluronic composite implants was higher than in the negative and positive control groups.

The effect of collagen-chitosan-natrium hyaluronate composite on neovascularization as angiogenesis reaction in rabbit corneal stroma wound (Experimental Study on *Oryctolagus cuniculus*)

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Background: The inflammatory process in the healing of corneal wounds can lead to cicatricial tissue resulting in cloudiness, and blood vessels in the corneal tissue cause visual disturbances to blindness. Keratoplasty is the primary therapeutic modality to replace pathological corneal tissue. Due to the limited availability of donors, many alternative therapies have been developed to restore corneal regeneration, one of which is the use of biomaterials in prosthetic devices with composite materials. Collagen-chitosan-sodium hyaluronic composite is a natural biopolymer that is considered to have good biocompatibility, biodegradable, and non-toxic properties so that it is hoped that we can obtain advantages. Besides being easy to get and produce, the composite is safe and can increase corneal tissue regeneration**. Materials and Methods.** Twenty adults New Zealand male white rabbits (30 eyes) were divided into three groups. The first group is control negative, the left eye without any treatment. The second group is control positive gro, which was the right eye with the corneal stromal injury. The third group was the right eye with corneal stromal injury and implantation of the collagen-chitosan-sodium hyaluronic composite. On day 14, the sedation was performed, and the degree of neovascularization was assessed clinically. **Results & Discussion.** The study results 14 days after treatment showed a significant difference in the level of neovascularization in the stromal injury group with the collagen-chitosan-sodium hyaluronic composite implantation group ($p = 0.012$, $\alpha > 0.05$). The type of neovascularization indicating that the wound healing process is ongoing. **Conclusion.** There was a significant difference in the level of neovascularization between the stromal injury group and the collagen-chitosan-sodium hyaluronic composite implantation group.

Keywords: cornea, biomaterials, composites, collagen, chitosan, sodium hyaluronic, neovascular.

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