The Effect of Collagen-Chitosan-Sodium Hyaluronates Intrastromal Implantation on Corneal Clarity and Transforming Growth Factor (TGF-ß) (Experimental Study On New Zealand Rabbit)

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Abstract

Background: Stromal haziness due to various diseases could results in permanent blindness. Currently, many biomaterials are developed to help aid the healing, so, haziness does not occur. This study aims to examine the effect of the collagen-chitosan-sodium hyaluronic (Col-Chi-NaHa) as an alternative to healing the corneal stroma so that corneal haziness does not occur.

Method: A total of 30 New Zealand male rabbit eyes were divided into three groups. The first group was not treated at all. The second group performed intrastromal injuries by making a stromal pocket with the aid of a crescent knife. The third group carried out implantation of the Col-Chi-NaHa biomaterial which was inserted into the stromal pocket. On day 14, the corneal haziness level was examined using an eye microscope and *handheld slit lamp*. Immunohistochemical staining of the cornea was carried out using anti-TGF-ß antibodies.

Result: After 14 days post-treatment showed that there was more significant haziness between the Col-Chi-NaHa composite implantation group and the control group ($p = 0.00$, $\alpha > 0.05$). There were also lower TGF-β levels between the Col-Chi-NaHa implantation group and the control group ($p = 0.00$, $\alpha > 0.05$).

Conclusion: Corneal clarity in the implanted group was lower than that of the control group but this was due to the short observation period which causing biomaterial not completely degraded. The TGF-β level in the implanted group was lower than control.

Keyword: Corneal clarity, stroma, TGF-β, biomaterial, Collagen, Chitosan, Sodium hyaluronic

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Introduction

Corneal damage is the second leading cause of blindness worldwide. According to WHO, there are 36 million people who suffer from blindness worldwide, and 217 suffer from vision damage, 2.4% of the total blindness is caused by corneal disorders[1]. The cornea is a transparent avascular tissue that functions protect the eye from infection and trauma. Due to its transparent

nature, the cornea has a refractive role in absorbing light and accounts for 2/3 of the eye's total refractive function[2]. Corneal damage to the stromal layer due to corneal surgery, refractive surgery, trauma, or infection will cause corneal haziness due to extracellular matrix and imperfect collagen fibers arrangement. Haziness in the stroma will reduce the cornea's clarity so that its refractive function will decrease, resulting in blindness[3].

Until now, corneal transplantation is the only way to correct blindness due to corneal loss of transparency, but there is an imbalance between the donor and the need for a corneal transplant. There are currently only 40,000 people who receive corneal donor annually in the United States, with conditions that may worsen in developing countries. Due to this imbalance, many artificial corneas and biomaterials are developed to help heal the cornea properly so that corneal scar that reduces the clarity does not occur. Biomaterials are currently widely studied to treat corneal defects by reducing mechanical stress and reducing pro-inflammatory cytokines. Biomaterials that are widely studied for use in aiding corneal healing include chitosan, hyaluronic acid, silk fibers, and polyarginins[4]. Biomaterials to be implanted on the cornea must be non-toxic, non-immunogenic, transparent, flexible to adjust during implant insertion surgery, and must be able to support the growth of corneal cells. One of the biomaterials being developed is a combination of collagen-chitosan-sodium hyaluronate (Col-Chi-NaHa) discovered by Chen in 2005 fulfils the biomaterial criteria for the cornea because these three materials are similar to the extracellular matrix of the cornea[5].

Collagen is the largest component of the extracellular matrix of the cornea. The extracellular matrix allows organogenesis and reconstruction during the wound healing process, so collagen is good for corneal repair and regeneration. Collagen is biodegradable, has low antigenicity, and has good biocompatibility due to its low toxicity and immune reactions. Collagen is widely used in ophthalmology for thread materials, bandage contact lenses, punctual plugs, and viscoelastic fluids during surgery. Collagen is also used for corneal reconstruction with good results because it has been shown to facilitate corneal cell and nerve regeneration in vitro. When implanted into the cornea, collagen has a brittle consistency, so it should be mixed with other ingredients to improve its strengths[6]. Chitosan is a linear polysaccharide consisting of glucosamine and N-acetylglucosamine and is a deacetylated derivative of chitin. Chitosan is biocompatible, biodegradable, inert and non-toxic[7]. Chitosan also have anti-fungal, anti-bacterial, and hemostatic properties so it is suitable for use as a material for tissue engineering[29]. Sodium hyaluronate is a glycosaminoglycan in the extracellular matrix, which has a vital role in wound healing and inflammation. In the eye plane, sodium hyaluronate has viscoelastic properties, so it is often used to protect the corneal endothelium and maintain the anterior chamber's depth during cataract surgery. Animal studies have also shown that sodium hyaluronate supports corneal epithelial wound healing by stimulating corneal epithelial migration, adhesion, and proliferation[8].The addition of chitosan to collagen can improve its stability and structural integrity so that it can be implanted in the eye[9] and the addition of sodium hyaluronate improves light transmission[28,29]. This study attempted to evaluate the differences in Collagen-Chitosan- sodium hyaluronates (Col-Chi-NaHa) intrastromal implantation to the corneal haziness level TGF-ß expression in stromal injury on New Zealand rabbits eye.

Methods

Animal

Thirty New Zealand male albino rabbits weighing 2–3 kg were used. Ethical approval was given by the animal care and Use Committee of the Faculty of Veterinary Airlangga University (Surabaya, Indonesia). Animals were given at least one week for acclimatization. The rabbits then divided into three groups, with each group consists of 10 rabbits. The first group are the treatment group which was given a circular complex of Col-Chi-NaHa biomaterial intrastromal implantation. The second group is the positive control group who was given injuries by making the stromal pocket in the cornea. The

third group is a negative control group where rabbit eyes were not treated at all

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

Collagen-Chitosan-Sodium Hyaluronic (Col-Chi-NaHa) membrane was made at the biomaterial laboratory at the Institute of Tropical Disease (ITD) Airlangga university. Collagen 20% solution was mixed with 0.1 M acetic acid using a magnetic stirrer for 60 minutes. Then the solution was cross-linked with hydroxypropyl methylcellulose (HPMC). 10% w / v DD 95% chitosan is then dissolved in 13.4 M acetic acid. Collagen and chitosan, which had been wholly dissolved were mixed using a magnetic stirrer for 1 hour. Sodium hyaluronates with a concentration variation of 0.6% were then added to Col + Chi solution and stirred for 30 minutes. The homogeneous Col + Chi + NaHA solution was cast on a Perspex plate and heated using an incubator for 24 hours at 35^0C until the membrane dries. After that, the membrane was immersed in PBS until the pH became neutral. The biomaterial membrane was then cut with a diameter of 3 mm and a thickness of 0.2 mm. Sterilization is carried out by rinsing thoroughly in distilled water, then rinsing again with 75% ethanol. The final stage was sterilization with UV for 30 minutes and immersed in sterile PBS buffer liquid, and the biomaterial was ready to be applied intrastromal to the rabbit's cornea.

Intrastromal implantation

The corneal stromal pocket was created at 2 mm from the superior limbus with a diameter of 4 mm and a thickness of 0.2-0.3 mm using crescent knife. The first group was given a circular complex of Col-Chi-NaHa biomaterial intrastromal implantation of 3 mm and a thickness of 0.2 mm, and the stromal pocket was closed without suturing. The second group is the positive control group who was given injuries by making the stromal pocket in the same way as stated above. The third group is a negative control group where rabbit eyes were not treated at all. Each group consists of 10 rabbits with a total population of 30 rabbits. Levofloxacin 0.5% and fluorometholone 0.1% eyedrops were applied six times daily for 2 weeks. The rabbits were examined 2 weeks post op using eye surgery microscope and handheld slit lamp for corneal clarity grading. All the animals were euthanized, the eye were enucleated, and the samples were fixed in 10% NBF (BBC chemicals, Mount vernon, USA) and processed for light microscopy.

Histopathology

Immunohistochemical staining was performed on the corneal surface of the enucleated eye tissue in the second week after corneal stromal injury treatment was given. Paraffin-blocked tissue was cut with a thickness of 3µm and placed on a glass polylysine object, then incubated at 45^0C for one night. The preparations were paraffinized and washed with flowing distilled water, then incubated with H_2O_2 for 3 minutes and washed with flowing distilled water. The preparations were incubated with citrate buffer PH 6 at 95^0 C for 45 minutes, then cooled for 30 minutes and washed with PBS 2x for 3-5 minutes. The preparations were then incubated with a blocking serum for 15 minutes and drained. Subsequently, immersion was carried out in alcohol with levels of 70%, 96%, 100%, and xylol. The tissue is then cooled for 15 minutes at room temperature and rinsed in phosphate-buffered saline (PBS) for at least 5 minutes. Tissues to be incubated in anti-TGF-β were given 0.5% casein in PBS for 10 min to block non-specific binding sites.The primary antibody incubation with rabbit polyclonal antibody TGF-β (1:50 Serotec MCA797, Oxford, UK). was carried out for 30 minutes. The tissue was rinsed in water, and 0.5% cobber sulfate in PBS was added to increase the staining intensity. The stained tissue was observed using a light microscope with a magnification of 400x.

Corneal haze grading

Corneal haze was graded using Sonoda and Streilen grading at two weeks post-op using ophtalmic surgery microscope and handheld slit lamp. The opacification observed and scored according to an established grading system as follows: 0, completely transparent cornea; 1, minimal corneal opacity, iris vessels easily visible; 2, moderate corneal opacity, iris vessels still visible; 3,

moderate corneal opacity, only pupil margin visible; 4, complete corneal opacity, pupil not visible.

TGF-β immunoreactivity grading

The level of TGF-β expression to assess the healing process of the corneal stroma was analyzed by semiquantitative histopathological examination using the Allred scoring method. Immunoreactive-score (IRS) can be assessed quantitatively by adding the results between proportion score (PS) and Intensity score (IS). Proportion score is the proportion of positive cells that are immunoreactive (scored on a scale of 0-5). Intensity score is staining intensity (scored on a scale of 0-3)from light brown to silver brown. The IRS scores range from 0-8, with the scores attached to table 1. Each sample's data is the average IRS scores observed at 5 Fields of View at 400x magnification.

Table 1 Guideline for Allred scoring method

Proportion Score (PS)	Intensity Score (IS)
score 0: no positive cell	score 0: no colour reaction
score 1: Positive cells 0-1%	score 1: weak intensity
score 2 : Positive cells >1-10%	score 2 : intrermediate intensity
score 3 : Positive cells >10-33.3%	score 3 : strong intensity
score 4 : Positive cells >33.3-66.6%	
score 5 : Positive cells >66,6-100%	
Total PS+IS score 0-8	

Statistics

The groups' distribution was analyzed using the Shapiro-Wilk normality test. The comparison of variables between groups was analyzed using Annova if the variables were normally distributed or Kruskal-Wallis if the variables were not normally distributed. All the statistical tests are carried out with the help of SPSS 23 software.

Results

The Effect Of Col-Chi-NaHa Intrastromal Implantation On Corneal Haze

In the negative control group, all samples had clear corneas on the 14th day after surgery. In the positive control group that was given intrastromal injury, the mean corneal haze level was 0.30 ± 0.949, with the lowest corneal haze on a scale of 0 and the highest corneal haze was on scale 3 which was found on one rabbit. In the treatment group given the Col-Chi-NaHa implant, the mean corneal haze level was 1.80 ± 1.033 with the with the lowest corneal haze scale of 0 in two rabbits, a scale of 3 in two rabbits and a scale of 2 in the remaining six rabbits. The Kruskal-Wallis statistical test showed that there were significant differences between groups ($P=0.00$, $P<0.01$).

Table 2. Corneal haziness grading

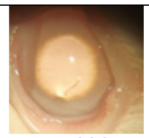
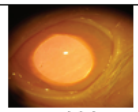
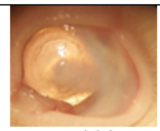
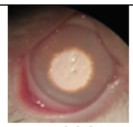
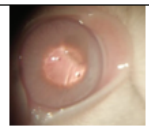
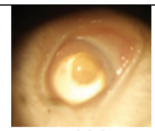
Group	Corneal haziness grading, number of samples			
	0	1	2	3
Negative control group (normal)	 10 rabbits	none	none	none
Positive control group (Stromal pocket)	 9 rabbits	none	none	 1 rabbit
Treatment group (Stromal pocket + intrastromal implantation)	 2 rabbits	none	 6 rabbits	 2 rabbits

Table 3. Differences of corneal haziness grading between groups

Group	N	Corneal haziness grading (mean ± Standard deviation)	p
Negative control group (normal)	10	0	0.000
Positive control group (Stromal pocket)	10	0.30 ± 0.949	
Treatment group (Stromal pocket + intrastromal implantation)	10	1.80 ± 1.033	

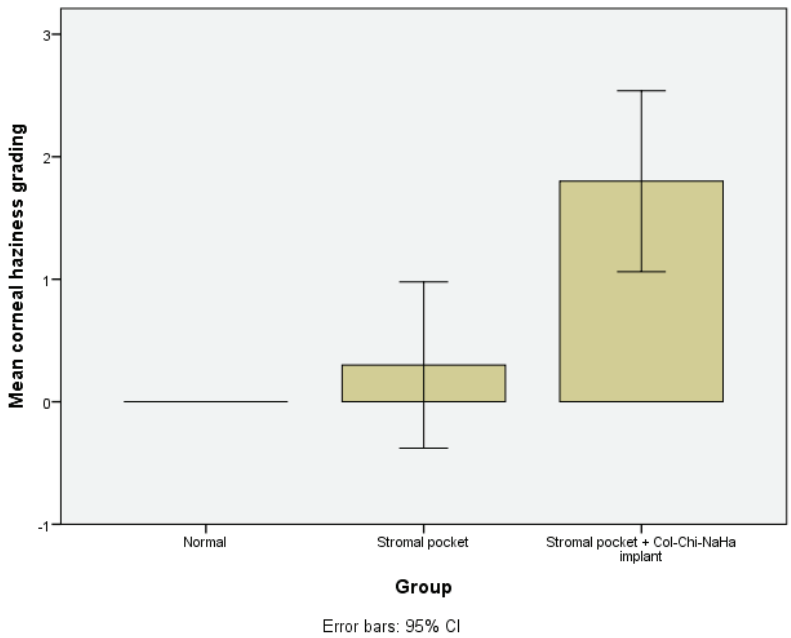


Fig 1. chart comparison of corneal haziness grading between group

The Effect Of Collagen-Chitosan-Sodium Hyaluronates Intrastromal Implantation On TGF-β expression

Distribution statistical test using saphiro-Wilk showed a normal distribution in the TGF-β expression data (p = 0.093, p> 0.05). Test with the one way ANOVA method showed a significant difference between the positive control group and the treatment group (6.00 ± 0.66 with 4.10 ± 0.73, P = 0.00; P <0.05) and the positive control group with the negative control group (6.00 ± 0.66 with 2.10 ± 0.73). , P = 0.00; P <0.05).

Table 4. Differences of TGF- β immunoreactivity score between groups

Group	N	Mean ± Standard deviation	Minimum	Maximum	p
Negative control group (normal)	10	2.10 ± 0.73	1	3	0.00
Positive control group (Stromal pocket)	10	6.00 ± 0.66	5	7	
Treatment group (Stromal pocket + intrastromal implantation)	10	4.10 ± 0.73	3	5	

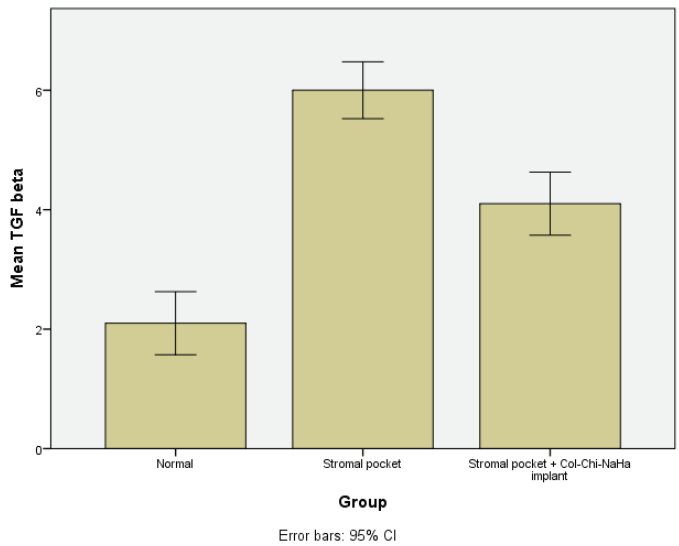


Fig 2. Mean TGF- β immunoreactivity score between groups

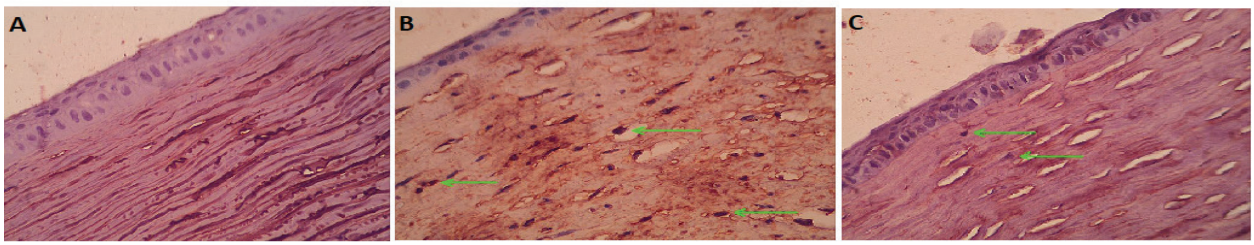


Fig 3. Immunohistochemistry staining under a light microscope at 400x magnification. (A) Negative control group (normal), (B) Positive control group (Stromal pocket), (C) Treatment group (Stromal pocket + intrastromal implantation). Green arrows show macrophage cells expressing TGF β, which is marked with a silvery brown color around the macrophages.

Discussion

The cornea is a transparent avascular tissue that functions as a protective and refractive structure for the eye and responsible for transmitting light. The corneal stroma is a structure that makes up almost 90% of the total thickness of the cornea, so it is imperative to maintain the transparency of the stroma in order to be maintained. Changes in the corneal stroma structure due to disease, trauma, or scarring can cause the loss of corneal transparency[12]. Corneal stromal abnormalities cause millions of cases of blindness worldwide and currently there is an imbalance between corneal donors and the needs of corneal donors that are needed. A synthetic stromal substitute that can replace the endogenous regeneration ability of the stroma, however, until now there has not been found a synthetic material that can replace the corneal stroma because of the complex structure of the stroma consisting of the extracellular matrix, stromal cells and proteoglycans which are difficult to replicate in vitro. Therefore, research on synthetic materials is currently directed at helping stromal regeneration so that scar tissue that causes corneal haziness does not occur. Synthesis materials currently being researched are corneal tissue donors or other tissues such as lip mucosa, acellular corneal scaffold with bioengineering, adhesive tissue, 3-dimensional bioprinting and stromal stem cell therapy[4,13]. Currently, many biomaterials are developed to help heal the cornea. Biomaterials have the advantage of inducing less immune reactions and can be fabricated in the laboratory, so the availability is relatively fast compared to waiting for donors' availability. Good biomaterial can also function as a scaffold for additional cells and medication which are expected to accelerate healing even further[14].

Corneal opacity is a relatively easy modality for assessing the corneal reaction to foreign bodies. In this study, the level of corneal haziness was examined using an ophthalmic surgery microscope and a handheld slit lamp at the second postoperative week using Sonoda and Streilein grading with a value scale of 0-4[15]. In this study, there was a significant difference in the mean level of corneal haziness between the positive control and treatment groups. In previous studies, intrastromal implanted biomaterial began to appear at the fifth month. Chen's study examined the collagen chitosan and sodium hyaluronic composite biomaterials implanted intrastroma with different percentages. Each group showed a postoperative reaction in conjunctival infection and corneal edema on two postoperative days, which disappeared on the seventh day. At 15-25 days postoperatively, a second inflammatory reaction occurred in the form of corneal edema and neovascularization, which subsided after 30 days. After five months, the biomaterial was degraded entirely, and the cornea is back to its transparent stage[5]. In vivo implantation in Widiyanti's study of Col-Chi-NaHa with composition of 20% collagen, 10% chitosan and 0.5% sodium hyaluronic in the cornea of New Zealand rabbits with full thickness implantation technique for eight weeks, the central pupil is clear and there is no exudate and inflammation. Histopathological examination shows corneal epithelial cells can grow on implanted biomaterial[31].

Implantation of intrastromal biomaterials in rabbit eyes with fish scales as the main ingredient showed no biomaterial degradation until 54 weeks after surgery, and there were minimal corneal opacities around the implant site. In vitro biomaterials with fish scales can support the growth of stromal and epithelial cells. So that biomaterials that are difficult to degrade are more suitable for use to help stromal healing rather than replace the corneal stroma as a whole[14]. Implantation of rabbit intrastromal biomaterials with collagen and Hydroxypropyl methylcellulose (HPMC) shows a mild inflammatory reaction two weeks post-op and neovascularization one-month post-op, which disappeared one week after. Furthermore, observations for seven months showed that the implanted biomaterial was well received without implant extrusion and the corneal condition was clear[6].

Thus, higher corneal haziness result in the treatment group is due to the short time of observation that causing implanted biomaterial to not fully absorbed and causes corneal haziness.

In this study, we also found 1/5 of the population in the treatment group had completely absorbed biomaterials resulting in transparent corneal condition after 14 days of implantation. The rate of degradation of the Col-Chi-NaHa biomaterial has been previously investigated in vitro by immersing the Col-Chi-NaHa biomaterial in 5 ml PBS and 0.05-gram collagenase for one week. With the results of biomaterial degradation of 0.68% per week[16]. There has been no research on the degradation rate of the Col-Chi-NaHa biomaterial implanted intrastromally, further research is still needed on the timeline of Col-Chi-NaHa biomaterial degradation in vivo.

The lower corneal clarity in the treatment group could also occur because, in the second week postoperatively, the corneal wound healing phase was still in the remodelling phase. In general, the timeline for wound healing in the corneal stroma is that apoptosis of cells around the wound and neutrophil infiltration begins at six hours post-injury and reaches a peak at 24 hours after injury. Activation of keratocytes started at two days after injury and fibroblast and myofibroblast differentiation occurred 3-5 days after the injury occurred. Myofibroblasts also produce an extracellular matrix whose fibre direction is irregular, causing cloudiness in the cornea. Myofibroblasts have the contractile ability to go to the corneal wound and close the wound. The fibrosis expression gene will be detected within two weeks after injury, and remodelling and scar tissue deposition will occur 14-28 days after the first wound occurs[17,18]. If the healing goes well and the wound is completely closed, the number of myofibroblasts in the stroma will decrease. In clinical conditions, this is characterized by the disappearance of corneal opacities and this occurs an average of 30-40 days after the injury occurs[11]. We suggest more extended observation to assess the effect of Col-Chi-NaHa implantation on corneal healing.

Transforming growth factor (TGF-ß) is a cytokine that has a significant role in healing corneal stromal wounds. TGF-ß will activate the cornea's wound healing pathway, including differentiate keratocytes into fibroblasts and myofibroblasts, induce changes in the extracellular matrix, and support the proliferation and migration of corneal epithelial cells. However, overexpression of TGF-ß also negatively affects the cornea by causing the accumulation of myofibroblasts in the stroma and producing an irregular extracellular matrix leading to post-injury corneal opacification. Inhibition of TGF-ß results in more transparent cornea after corneal alkali burn[19]. Mice wounded by corneal alkali burn and given TGF-ß inhibitor also had less inflammatory cell infiltration and myofibroblasts than the control group and had more transparent corneas and less neovascular at day 20 observation[20].

The primary ingredient of this biomaterial is collagen, a natural protein found throughout the body. Collagen is brittle and needs to be cross-linked with various ingredients. Mixing collagen with other ingredients can increase mechanical strength and defend collagen from enzyme degradation[21]. Collagen-chitosan composites are reported to have good mechanical strength and clarity and can regenerate epithelial cells, stroma, and eye nerves after being implanted for 12 months in pig eyes[21,22]. Chitosan is mixed with collagen to improve mechanical strength so that the biomaterial does not degrade quickly before the wound closes. Clinically, chitosan and its derivatives have been shown to aid in eye healing and are cytologically compatible with various cells in the eye. Administration of chitosan to rabbits with keratoconjunctivitis due to alkaline trauma can decrease symblepharon formation and increase collagen formation in the conjunctiva[23]. Chitosan also accelerates the proliferation of epithelial cells and inhibits the proliferation of stromal cells in rabbits whose corneal epithelium is damaged due to chemical trauma. The cornea that is given chitosan treatment is more transparent due to rapid wound closure, and the stroma is still regular in structure[24]. Chitosan mixed with n-acetyl cysteine in the form of eye drops has also been shown to accelerate the closure of corneal defects that occur due to manual scratching in experimental rabbits[25]. Chitosan also aids in corneal healing by inducing extracellular signal-regulated kinases (ERK) pathway[27]. Another ingredient in this biomaterial is sodium hyaluronic that

is present naturally in the extracellular matrix in various organs so that it has non-toxic and non-immunogenic properties. Hyaluronic acid can accelerate corneal healing by reducing pro-inflammatory cytokines, including CD44, IL-1β and MMP9[26]. Combining these three materials in the form of a biomaterial composite in this study was shown to reduce TGF-beta levels on day 14 compared to the control group, which is thought to occur because it accelerates healing and reduces pro-inflammatory cytokines.

Limitation

This study limitation is the short observation period and was not conducted serially to compare the level of corneal opacities at each phase of wound healing. This study also requires many other variables to be investigated to evaluate the chol-Chi-NaHa biomaterial composite's effectiveness and investigate the inflammatory pathways this composite can inhibit.

Conclusion

In this article we reported on our study of Col–Chi–NaHA composite and its effect on corneal clarity and TGF-β level. After 14 days after stromal wounding, the results of the study showed that corneal clarity in the implanted group was lower than that of the control group, but this was due to the short observation period which causing biomaterial not completely degraded. The long-time effects of this biomaterials in cornea still need to be studied. TGF-β level in the implanted group was lower than control. Examination of other parameters, especially other pro-inflammatory cytokines and pro-inflammatory cells count can evaluate the effectiveness and further understand how the Col-Chi-NaHa composites work.

Acknowledgement: The author would like to thank Imam Susilo for the assistance in immunohistochemistry reading of TGF-β and Budi Utomo for the assistance of statistical analysis in this study.

Ethical Clearance: Given by the animal care and Use Committee of the Faculty of Veterinary Airlangga University (Surabaya, Indonesia).

Source of Funding: Self-funding.

Conflict of Interest: Nil.

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