

Effects of different preparation protocols of blood serum eyedrops on corneal healing after alkali burn: Clinical evaluation and α -SMA expression

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ABSTRACT

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Background: In cases of alkali burn, blood serum eyedrops may supply protein and growth factors. A standard preparation protocol is necessary for consistency in epitheliotropic capacity.

Objective: We aim to evaluate how post-alkali-burn administration of blood serum eyedrops from different preparation protocols results in different outcomes on rabbits.

Methods: This randomized experimental study used three treatments: placebo, non-diluted serum, and 25% concentration serum. We analyzed the corneal haziness, neovascularization degree, histopathological examination of neovascularization scores, and alpha-smooth muscle actin (α -SMA) expression.

Results: Twelve rabbits (24 eyes) were split evenly into three treatment groups. On day 14, the group receiving the 25% concentration serum had the least corneal haziness ($p = 0.005$). No adverse effect was observed on the treated eyes. Histopathologically, no significant difference in neovascularization scores and α -SMA expression was observed.

Conclusions: Treatment using the 25% concentration serum resulted in less corneal haziness, smaller defect size, and greater healing rate. There was also no significant difference in histopathological outcomes among the three treatment groups.

INTRODUCTION

Ocular chemical trauma is an ophthalmology emergency with 11-13% prevalence out of all ocular trauma cases.¹ Chemical trauma caused by an alkaline solution, also known as alkali burn, is more prevalent than acidic trauma.² Alkali substance causes more extensive tissue damage by destroying the corneal stroma's proteoglycan and collagen bundles, with the proteases' continuous production from the damaged tissue.³ Severe chemical injuries may cause slowing epithelial regeneration, ulcers, neovascularization, and haziness on the cornea, which may lead to permanent visual loss or blindness. Ocular chemical trauma has consequential and extensive ramifications on the patient's visual functions and quality of life. Prompt management to promote

epithelialization and suppress inflammation is needed to prevent complications.⁴

Blood-derived products have been used in ocular surface diseases for decades.⁵ In cases of alkali burn, blood serum eyedrops are expected to supply blood derivatives to the avascular cornea, thereby accelerating epithelialization and preventing corneal neovascularization and haziness. Autologous serum (AS) from human peripheral blood serum contains various components (e.g., epidermal growth factor (EGF), transforming growth factor- β (TGF- β), matrix metalloproteinase-9 (MMP-9), vitamin A, albumin, fibronectin, and bactericidal components) that help biological cellular responses and prevent contamination and infection during epithelial healing process.⁶ Fibronectin plays an essential

role in guiding the morphogenesis of tissues for both the early provisional matrix protein fibrin and the permanent matrix protein collagen.⁷ Along with EGF, TGF- β stimulates myofibroblast differentiation and extracellular matrix production by keratocytes. However, TGF- β in high concentration may prevent the proliferation of corneal epithelial cells and may also stimulate excessive activity of myofibroblast. This phenomenon increases the activity of α -SMA, which is critical in signaling corneal fibrosis.

Following the Clinical Practice Guideline for handling chemical trauma in Cipto Mangunkusumo Hospital (Rumah Sakit Cipto Mangunkusumo = RSCM) Kirana, AS is given based on the grading of the trauma. Blood serum eyedrops were given from the onset until the early recovery phase (day 7 to 21) in grade III-IV chemical trauma based on the Roper-Hall classification.⁸ To date, there is excellent variability in regulatory oversight and preparation methods, with no accepted standard protocol worldwide for producing blood serum eyedrops.⁹ Many factors, such as freezing time, centrifugal speed, concentration, and diluent, may significantly affect the composition and epitheliotropic effects.¹⁰ In this study, we used a protocol that has not been followed before at RSCM Kirana to compare the clinical improvements that were proven with histopathological examination on the use of blood serum eyedrops. We aim to evaluate how post-alkali-burn administration of blood serum eyedrops from different preparation protocols results in different outcomes on rabbit test subjects.

METHODS

Study design

This study was a randomized experiment on rabbits to assess the effect of human blood serum eyedrops, prepared using a different protocol, on clinical and histopathological outcomes in cases of alkali burn. Our study was conducted from February to May 2023 at the Animal Research Facility, Indonesian Medical Education and Research Institute (IMERI). Histopathological examination was performed at the Department of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia, RSCM.

Population and samples

Male New Zealand White rabbits weighing

2,500-3,000 grams without any corneal abnormality were used as test subjects. The rabbits were provided by Royal Kelindo Rabbit Breeding Farm, Bogor. Before the study, the rabbits were acclimatized for seven days and treated following guidelines from The Association for Research in Vision and Ophthalmology (ARVO). The rabbits were kept in a separate room and were caged separately in 50 x 40 x 35 cm³ cages. Large-sized cages were used so the rabbits could freely move to minimize their stress level. Drinking water and rabbit pellets (2,600 kcal/kg) was given ad libitum. The sample size was calculated using the resource equation approach.¹¹ We categorized the subjects into three treatment groups, each with a minimum sample size of four, according to the ethical principle of 3R (replacement, reduction, refinement).

Interventions

After acclimatization, the rabbits were then randomized into one of the three treatment groups: Group A, in which both eyes were given sterile saline eyedrops 4x per day for 14 days as placebo; Group B, who received non-diluted blood serum eyedrops prepared using the protocol generally used in RSCM Kirana, or; Group C, who received 25% concentration blood serum eyedrops prepared using Liu et al.¹⁰ protocol. The rabbits were initially given anesthesia of a mixture of ketamine HCl (35 mg/kg) and xylazine (5 mg/kg) via intramuscular injection on the posterior thigh. Then, a piece of 6 mm Whatman filter paper, which had been soaked in NaOH 1M, was placed on the central area of the right cornea for 60 seconds. The paper was removed, and irrigation was done using 50 ml phosphate-buffered saline (PBS) for 2 minutes.¹²

The blood serum eyedrops were given to the rabbits four times per day, one drop (0.05ml) each time, for 14 days after the burn on both the left and right eyes. The non-traumatized left eye was used as a control to identify possible rejection or irritation in healthy eyes. The RSCM protocol serum was prepared by directly performing centrifugation (without blood clotting) at 1,780 g for 1 hour. Meanwhile, Liu et al. protocol serum included blood clotting for 120 minutes in preparation, followed by centrifugation at 3,000 g for 15 minutes. Then, it was diluted to reach 25% concentration using a balanced salt

solution (BSS). The human blood used to prepare the serum was obtained from whole blood of type O rhesus negative without anticoagulant. The blood was obtained from the Red Cross in Jakarta, screened beforehand, and prepared in the Laboratory of Pharmacology at the Faculty of Medicine, Universitas Indonesia.

Examinations

Clinical examinations were performed in all treatment groups on days 0, 1, 3, 7, and 14 post-chemical traumas. The eyeballs were examined under a slit lamp by the researcher, and images were taken using a camera integrated with the slit lamp with 10x magnification. Assessments on the degree of corneal haziness¹³ and neovascularization¹⁴ (Table 1) were done by cornea and refractive surgery consultants.

The rabbits were euthanized on day 14 after the burn to examine the formation of

neovascularization and inflammation at the peak of α -SMA expression. This procedure was done via subcutaneous injection of ketamine (300 mg/kg) and xylazine (30 mg/kg). Enucleation of both eyes was performed under the supervision of consultants, after which the samples were sent to the Department of Anatomical Pathology for excision of the cornea and limbus. After the preparation of the slides, staining was performed using two methods: hematoxylin-eosin (HE) and immunohistochemistry (IHC) with the specific antibody of α -SMA on the corneal tissue (MBS266274, My BioSource®, USA). Paraffin block was deparaffinized using xylol and then rehydrated using ethanol (100%, 95%, 70%, 50%). The antigen retrieval procedure was initiated with Tris EDTA (pH 9.0) in the decloaking chamber. The antibody was diluted in the ratio of 1:200 for 60 minutes.

Table 1. Grading of corneal haziness and neovascularization, neovascularization with HE staining, and intensity of α -SMA expression¹²⁻¹⁵

Parameter	Grade
Corneal haziness	0: No corneal haze 1: Mild stromal haziness, iris, and pupil detail visible 2: Moderate stromal haziness, pupillary margin visible, iris details partially visible 3: Severe stromal haziness, pupillary margin not visible, iris details sparsely visible 4: Completely opaque cornea
Corneal neovascularization	0: Without vessels in the cornea 1: Vessels <1 mm in the peripheral cornea 2: Vessels of 1-1.9 mm in the peripheral cornea 3: Vessels of 2-2.9 mm in the peripheral cornea 4: Vessels \geq 3 mm in the peripheral cornea
Number of blood vessels	0: None 1: Few 2: Moderate 3: Severe
Mean grey value	Negative: > 180 Weak intensity: 121-180 Moderate intensity: 61-120 Strong intensity: 0-60

HE: hematoxylin-eosin, α -SMA: alpha-smooth muscle actin

Data analysis

Blinding was applied whereby the researcher, assistant researcher, and pathologist were uninformed about the treatment groups. The blinding was subsequently removed post-data analysis. Both semiquantitative and quantitative measurements were done by looking at the sum average of seven microscopic fields in 400x magnification. Neovascularization was measured

by the number of blood vessels that were quantified and graded, as shown in Table 1. A quantified assessment of α -SMA expression was done using ImageJ software with color deconvolution. Images of five different representative vision fields with 400x magnification were transformed, and the staining intensity was measured using the mean grey value (MGV). The MGVs were then categorized according to a previous study, as shown in Table

1.¹⁵ The intensity of α -SMA expression was also measured via a pathologist's manual reading using a microscope. The staining intensity was divided into negative (unstained), weak intensity (pale brown), moderate intensity (light brown), and strong intensity (dark brown).

Ethics

Ethical approval was obtained from the Health Research Ethical Committee of the Faculty of Medicine Universitas Indonesia and RSCM, with the ethical clearance number KET-1275/UN2.F1/ETIK/PPM.00.02/2022.

RESULTS

We found no significant difference in the rabbits'

weight across all three treatment groups. Until day 14, no rabbit experienced death or significant loss in weight. Clinically, no infection or perforation was observed on the left or right eyes, and no side effect of irritation was found on the left eye. On days 1, 3, 7, and 14, we observed significant differences in corneal haziness across the three treatment groups, with the lowest median being found in the group that received 25% concentration serum (Table 2). Post hoc analysis showed a significant difference in haziness between the placebo and 25% concentration serum group since day 3 ($p = 0.036$). In addition, there was also a significant difference in haziness between the non-diluted and 25% concentration serum group from day 1 ($p = 0.013$) until day 14 ($p = 0.011$). Clinical

Table 2. Degree of corneal haziness and neovascularization in each treatment group

Groups	Degree of corneal haziness (median [min-max])				
	Day 0	Day 1	Day 3	Day 7	Day 14
Placebo	4 (4 - 4)	4 (2 - 4)	3.5 (3 - 4)	2 (2 - 3)	3 (3 - 3)
Non-diluted serum	4 (4 - 4)	4 (4 - 4)	3 (3 - 4)	2 (2 - 2)	3 (3 - 3)
25% concentration serum	4 (3 - 4)	3 (2 - 3)	2 (1 - 3)	1 (1 - 1)	2 (0 - 2)
p	0.368	0.033*	0.042*	0.007*	0.005*

Groups	Degree of corneal neovascularization (median [min-max])				
	Day 0	Day 1	Day 3	Day 7	Day 14
Placebo	0 (0-0)	0 (0-0)	1 (0-1)	1 (1-2)	2 (1-3)
Non-diluted serum	0 (0-0)	0 (0-0)	1 (0-1)	1 (1-2)	2 (1-3)
25% concentration serum	0 (0-0)	0 (0-0)	1 (0-1)	1 (0-2)	1 (0-2)
p	1.00	1.00	0.730	0.829	0.279

* $p < 0.05$ from Kruskal-Wallis analysis

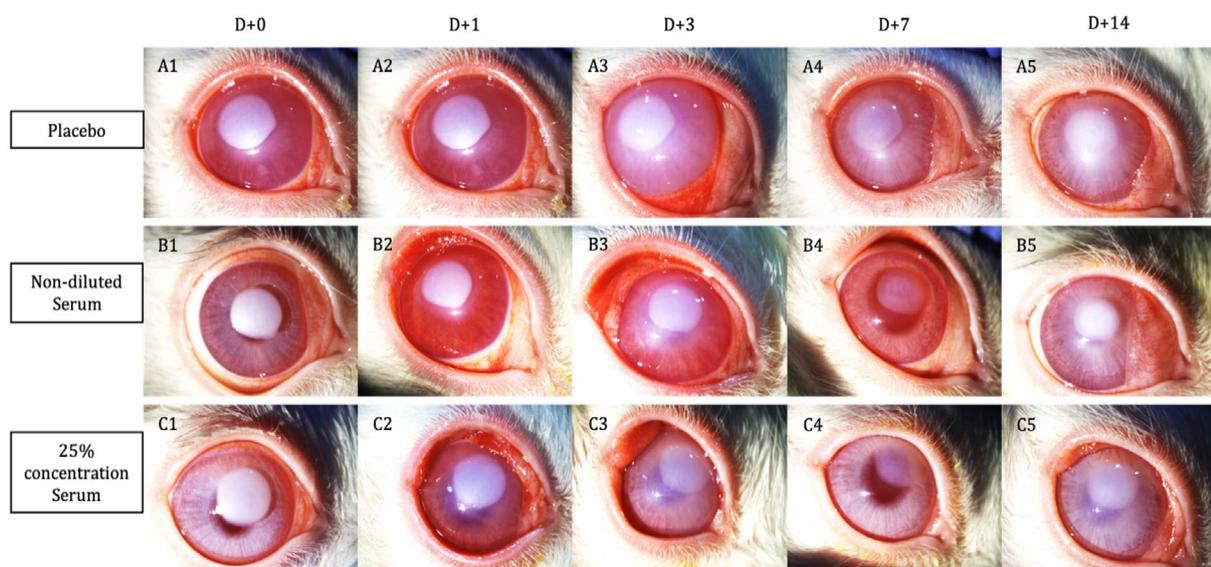


Figure 1. Clinical representation of corneal haziness in each treatment group. In all treatment groups, fourth-degree haziness was observed on day 0 (A1, B1, C1). On day 14, the placebo group (A5) and non-diluted serum group (B5) had third-degree haziness, whereas the 25% concentration serum group had second-degree haziness (C5).

representations of corneal haziness for each treatment group are presented in Figure 1.

Corneal neovascularization started to appear on day 3 in some rabbits in each treatment group. Rabbits that received 25% concentration serum had the lowest median degree of neovascularization on day 14, but it was not significantly different from the other groups (Table 2). Figure 2 presents a clinical representation of corneal neovascularization for

each group. The highest macroscopic degree of neovascularization was observed in the placebo group. In addition, histopathologically, the degree of neovascularization score in the 25% concentration serum group was low but not significant (Table 3).

The intensity of IHC staining was quantified from the binary threshold diaminobenzidine (DAB) images using the MGV scores. A lighter color

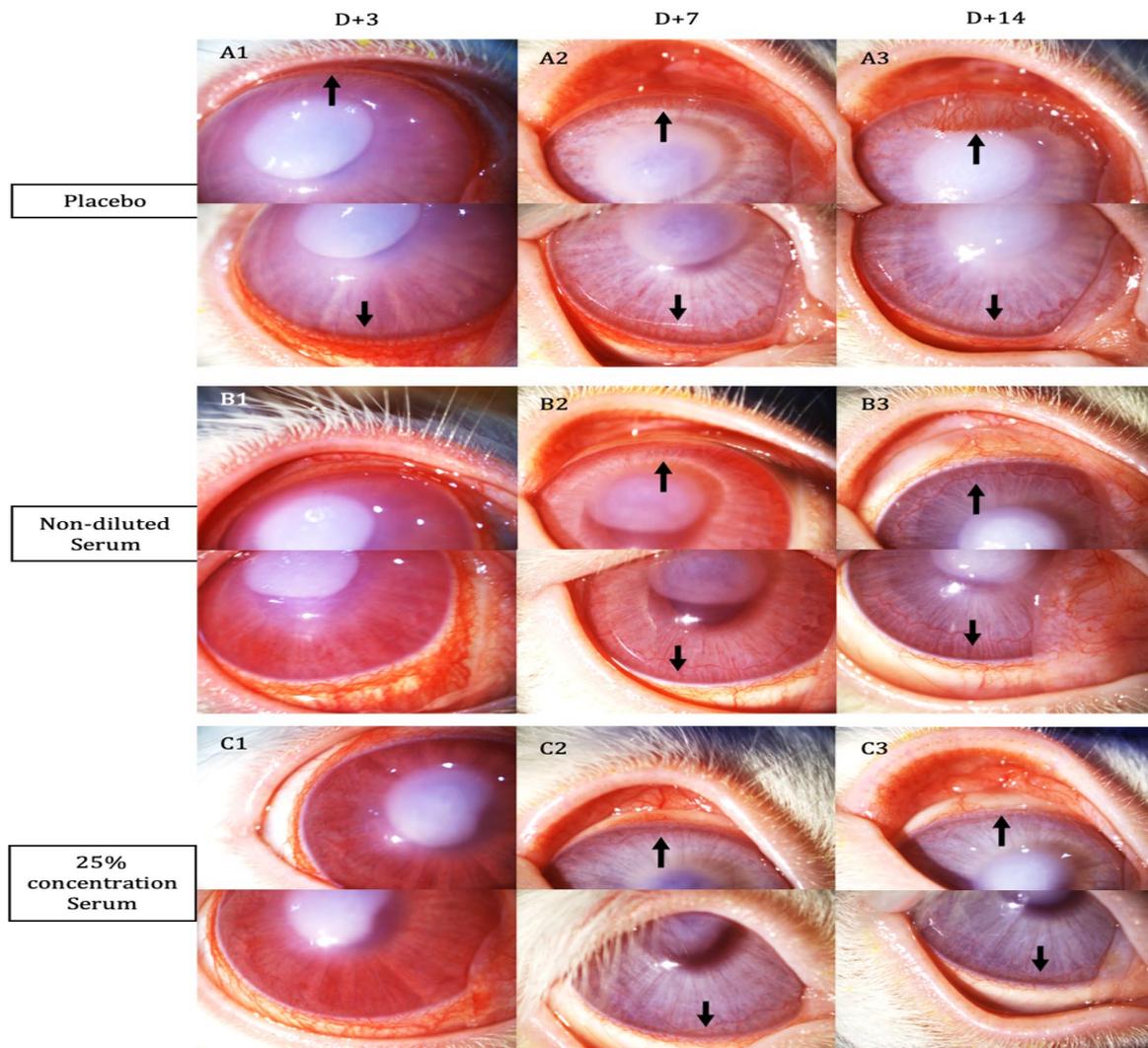


Figure 2. The clinical representation of corneal neovascularization in each treatment group. The black arrows indicate neovascularization. In the placebo group, first-degree neovascularization started to appear on day 3 (A1), which became third-degree on day 7 (A3). In both the non-diluted and 25% concentration serum group, first-degree neovascularization appeared on day 7 (B2, C2) and remained until day 14 (B3, C3).

Table 3. HE staining scores in each treatment group

Groups	Neovascularization scores on HE staining, median (min-max)
Placebo	2 (1-2)
Non-diluted serum	2 (1-2)
25% concentration serum	1 (1-2)
<i>p</i>	0.4

p value from Kruskal Wallis analysis, HE: Hematoxylin-eosin

intensity shows a higher MGV score, indicating lower α -SMA expression. In the 25% concentration serum group, the mean MGV score was higher than the other groups (25% concentration serum group = 145 ± 11.46 ; non-diluted serum group = 134.59 ± 23.67 ; placebo = 119.97 ± 14.04).

Those results meant that α -SMA expression in 25% concentration serum was lower than the other treatment without significant differences (Figure 3B). In the semiquantitative grading of IHC staining intensity, all slides in the 25% concentration serum group were graded as weak intensity (Table 4).

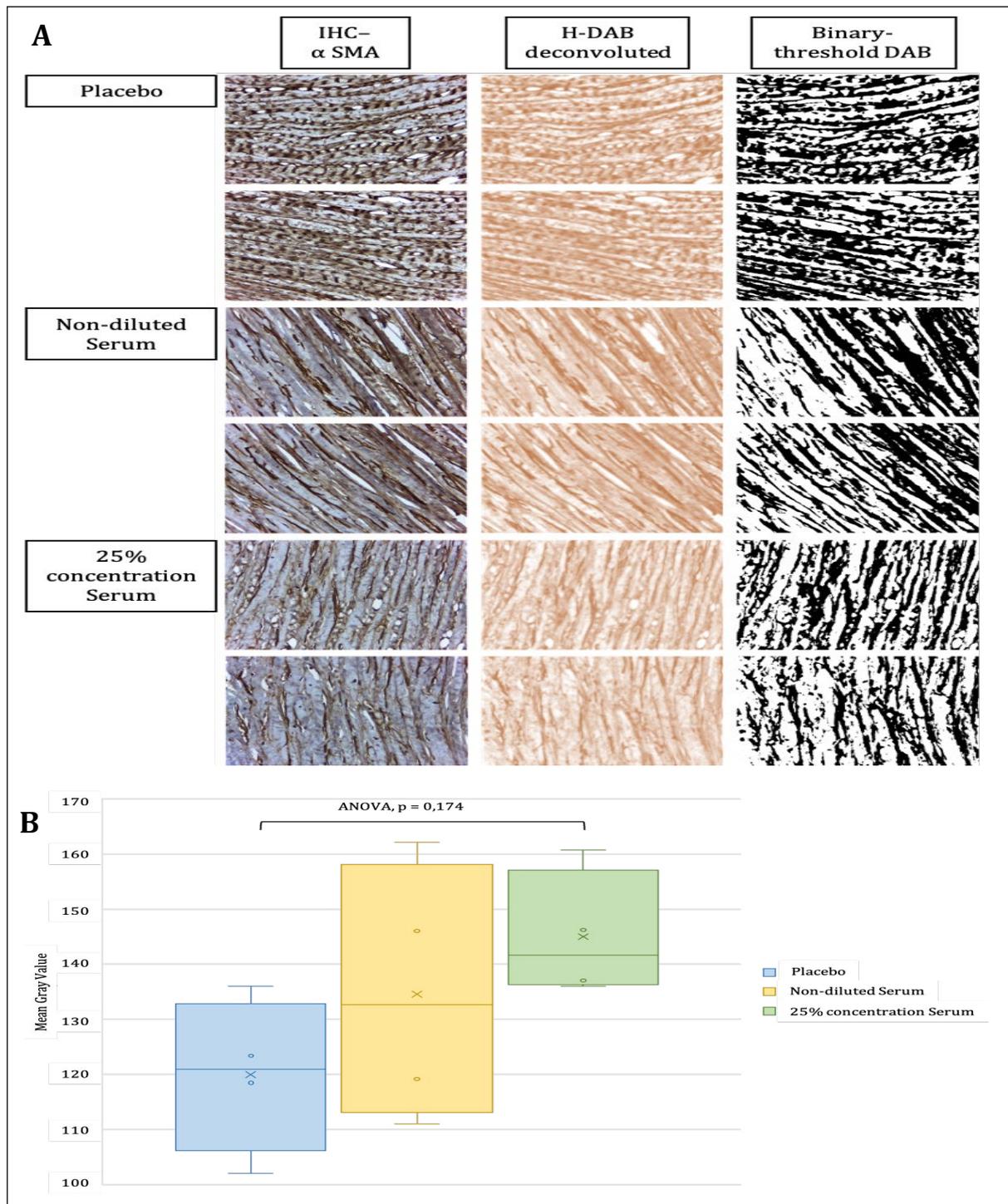


Figure 3. A. IHC staining with anti- α -SMA antibody on sample tissues from each treatment group. The assessment was done using ImageJ, in which the images were processed through deconvolution and binary threshold for the Quantification of α -SMA expression. B. Quantification of α -SMA expression in Mean Gray Value scores. IHC: Immunohistochemistry; α -SMA: alpha-smooth muscle actin; H-DAB: Hematoxylin and Diaminobenzidine; DAB: Diaminobenzidine, ANOVA: analysis of variance

We also measured TGF- β 1 levels in each serum via enzyme-linked immunosorbent assay (ELISA), which showed 534.04 ng/ml in non-diluted serum and 117.40 ng/ml in 25% concentration serum.

DISCUSSION

We observed significant differences in corneal haziness across all three treatment groups, with the lowest degree in the group that received 25% concentration serum. In previous studies

Table 4. Intensity of α -SMA expression using ImageJ

Groups	Intensity category			
	Negative n (%)	Weak intensity n (%)	Moderate intensity n (%)	Strong intensity n (%)
Placebo	0	2 (50%)	2 (50%)	0
Non-diluted serum	0	2 (50%)	2 (50%)	0
25% concentration serum	0	4 (100%)	0	0

α -SMA: alpha-smooth muscle actin

on using blood serum eyedrops with rabbit test subjects, none analyzed the difference in corneal haziness.^{12,16} In this study, the lower degree of corneal haziness observed in the group receiving 25% concentration serum was believed to be closely related to the lower concentration of TGF- β . Corneal haziness is highly affected by healing processes in the cornea, especially the stroma.¹⁷ Corneal stroma predominantly comprises regularly arranged collagen fibrils (I, III, V), keratocytes, and glycosaminoglycan ground substance. Following chemical injury, the keratocytes are mobilized to repopulate the cornea, starting from the most posterior part of the stroma.¹⁸ Fibroblast activity and transformation of keratocytes into myofibroblast are influenced by various cytokines, in which TGF- β is one of the main pro-inflammatory cytokines in corneal fibrosis.¹⁹

Although required to initiate corneal healing, the high concentration of TGF- β may possess an antiproliferative effect that suppresses the healing process on the eye's surface. There are three isoforms of TGF- β , namely TGF- β 1, TGF- β 2, and TGF- β 3, in which TGF- β 1 is the most dominant mediator for fibrosis response in the cornea through TGF- β 1/Smad2/3 pathway.²⁰ Higher speed and shorter duration of centrifugation in the 25% concentration serum preparation led to a lower concentration of TGF- β 1. At higher speeds, the serum contains fewer leukocytes, affecting the concentration of TGF- β 1, as the residual leukocytes in the serum release TGF- β 1 into the supernatant.¹⁰ In the group receiving 25% concentration serum, wound healing still occurred, and corneal fibrosis, indicated by corneal haziness,

was reduced. Overconcentration of TGF- β may induce the excessive activity of myofibroblasts, leading to excessive α -SMA expression and, thus, fibrosis.²¹

Additionally, the TGF- β 1 concentration in the 25% concentration serum was almost five times lower than that in the non-diluted serum. This study also reflected the lower concentration in the better clinical and histopathological outcomes. However, it was still higher than the concentration in the original experiment conducted by Liu et al.¹⁰ The concentration of TGF- β in human peripheral blood serum is associated with multiple activities in the human body, such as early embryonic development and organogenesis, immune supervision, tissue repair, and adult homeostasis.²² Hence, TGF- β 1 concentration may differ from one individual to another depending on the disease or condition. In previous studies, TGF- β 1 concentration was higher in patients with cancer or autoimmune diseases.^{23,24} Meanwhile, the blood donors in this study were only screened for standard infectious diseases (human immunodeficiency virus (HIV), Hepatitis C, Hepatitis B, and syphilis). Therefore, they might be suffering from previously unknown diseases affecting the TGF- β 1 concentration in their blood samples.

There were significant differences in the degree of corneal haziness among the three treatment groups from day 1, day 3, day 7 until day 14. In all treatment groups, the degree of haziness improved on day 7 compared to day 3 but worsened on day 14. In a previous study on alkali burn on rabbits, corneal haziness increased between day 7 and 14 due to more significant thickening of the stroma. In that study, a gradual increase in the corneal

opacity was achieved using Pentacam[®], in which the cornea became more opaque and fibrous along with neovascularization.²⁵ This phenomenon explains why the degree of corneal haziness was lower on day 7 compared to day 14 in all treatment groups.

In this study, no significant difference in the degree of corneal neovascularization was observed among the three treatment groups, both clinically and histopathologically. In a previous study, neovascularization was only observed histopathologically, and the neovascularization score was compared across three treatment groups: control (healthy eyes), placebo, and autologous serum. The neovascularization scores differed significantly across all groups; however, the result of that study is incomparable with this study.¹² This is because observations on the healthy eyes were also analyzed in the previous study, and post hoc analyses were not subsequently performed to identify the group pairings with significant differences. Corneal neovascularization is divided into three phases: sprout phase, which starts on day 3; vigorous phase, which reaches the peak on day 7; and regression phase, which begins after day 14.¹⁷ This theory is in line with the result of this study, in which neovascularization started to appear on day 3 after the alkali burn was applied on all rabbits, and then gradually worsened until day 14. In some cases, if the neovascularization process does not enter the regression phase after day 14, fat deposits accumulate in the stroma, which causes irregularity on the corneal surface, known as lipid keratopathy, and ultimately impairs visual acuity.²⁶

The use of blood serum aims to suppress neovascularization by accelerating epithelial healing and reducing myofibroblast activity through TGF- β concentration. Myofibroblasts hinder the post-injury regeneration of nerves by secreting TGF- β that boosts the phosphorylation of the collapsin response mediating protein 2 (CRMP2) in the regrowing neurons.²⁷ Other than TGF- β 1, neovascularization is also triggered by the imbalance between pro-angiogenic (e.g. vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-1 (IL-1), platelet-derived growth factor (PDGF)) and anti-angiogenic (e.g. inhibitory Per/ARNT/Sim, endostatin, angiostatin, arrestin, restin, tissue inhibitor metalloproteinase 3) factors. Molecules

of pro-angiogenic factors are released by corneal epithelium but undergo sequestration on the basal membrane, which also contains anti-angiogenic factors. Upon damage to the basal membrane, anti-angiogenic factors will disappear, and pro-angiogenic factors are released to the stroma, causing neovascularization.²⁸ Blood serum eyedrops may assist in the proliferation, migration, and differentiation with trophic effects on corneal epithelium and limbus, hence accelerating the regeneration of basal membrane and suppressing further neovascularization.²⁹ This theory concurs with the findings in this study, whereby the placebo group had a higher neovascularization score than the other groups who received blood serum treatments.

All IHC staining in this study was carefully performed by an anatomical pathology technician at the Laboratory of Anatomical Pathology, Faculty of Medicine, Universitas Indonesia. The pathologist has calculated the appropriate dilution concentration following staining optimization. However, excessive stromal staining was observed in most slides, making it difficult to perform manual assessment. It could happen due to several factors, such as excessive incubation, thick tissue section, antigen diffusion before fixation, undissolved deposit counterstain, inadequate irrigation, inadequate blocking, too much chromogen, as well as the existence of protein and endogenous pigment.³⁰

Expression of α -SMA is directly proportional to the contractile strength of myofibroblast. In the healing process of corneal stroma, myofibroblast assists in the regeneration and secretion of the extracellular matrix. Due to its contractile strength, however, myofibroblast may alter the cornea's shape, resulting in higher-order aberration. The concentration of crystalline protein in myofibroblast is lower, causing corneal haziness.¹⁹ Those theories align with our findings, in which all rabbits in the 25% concentration serum group had the lowest degree of corneal haziness and weak intensity of α -SMA expression. Meanwhile, greater corneal haziness and moderate intensity of α -SMA expression were found in 50% of the rabbits in the other two treatment groups. Previously, no study has ever analyzed the difference in intensity of α -SMA expression on rabbits with alkali burn, which were treated with autologous serums with different preparation protocols.

There are several limitations in this study. The scoring for histopathological assessment was adopted from another study, which might result in non-significant results. Furthermore, quantitative methods such as manual cell counting were not performed due to limitations. Also, during the examination of IHC staining, some overstaining was observed, which made it challenging to perform a visual assessment, and the alternative assessment using ImageJ might not produce the optimal result.

This study examines clinical and histopathological outcomes in post-treatment alkali burns. To our knowledge, no previous study has compared the outcomes of different human blood serum eyedrops prepared using different protocols. The Liu et al. protocol theoretically contains the optimal epitheliotropic concentration¹⁰, which was applied in vivo in this study. The use of animal subjects in chemical trauma models results in a gradation of trauma and similar epithelial size, making it easy to assess clinical outcomes and improvement more objectively. This study is expected to provide a more vivid illustration of the effectiveness of blood serum eyedrops with different preparation protocols so that it can become a baseline for further clinical trials.

CONCLUSION

In conclusion, the preparation protocol used in the 25% concentration serum produced more favorable results of less corneal haziness, smaller defect size, and a greater healing rate. There was no significant difference in the neovascularization degree. Histopathologically, no significant differences were observed in the epithelial, neovascularization, and PMN cell scores among the three treatment groups. Although not significant, the α -SMA expression was lower in the 25% concentration serum group. Further studies on the concentration of cytokines and other epitheliotropic factors will be required to understand the relationship between the serum contents and its outcome. The optimization protocol of IHC antibody staining needs to be adjusted to avoid excessive staining. Moreover, future studies on human subjects are needed to obtain a solid basis for producing blood serum eyedrops.

CONFLICT OF INTEREST

There is nothing to declare.

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AUTHOR CONTRIBUTION

Concept and design: TK, SW, MS, ML; data acquisition or sampling: TK, ES; analysis and data interpretation: TK; drafting the article: TK; critically revising and approving the final version to be published: SW, MS.

LIST OF ABBREVIATIONS

AS: Autologous serum; EGF: Epidermal growth factor; TGF- β : Transforming growth factor- β ; MMP-9: Matrix metalloproteinase-9; α -SMA: Alpha smooth muscle actin; RSCM: Rumah Sakit Cipto Mangunkusumo; BSS: Balanced salt solution; IMERI: Indonesian Medical Education and Research Institute; ARVO: The Association for Research in Vision and Ophthalmology; PBS: Phosphate-buffered saline; HE: Hematoxylin-eosin; IHC: Immunohistochemistry; DAB: Diaminobenzidine; MGV: Mean Gray Value; ELISA: Enzyme-linked immunosorbent assay, CRMP: collapsin response mediating protein, FGF: fibroblast growth factor; VEGF: vascular endothelial growth factor PDGF: platelet-derived growth factor; IL: interleukin

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