ORIGINAL ARTICLE

Stabilization of eye drops containing autologous serum and recombinant human epidermal growth factor for dry eye syndrome

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ABSTRACT

Human epidermal growth factor (hEGF) and autologous serum are considered safer and more effective in treating dry eye syndrome. However, suitable formulas and preparation methods are needed to obtain eye drop containing autologous serum and hEGF, which are stable during storage and use. Therefore, this study aimed to develop a stable and effective eye drops containing autologous serum and hEGF. Stabilization of autologous serum and hEGF was done by adding lyoprotectant and antioxidant agents, and then prepared using the freeze-drying method. The clarity, pH, sterility, and endotoxin content of the preparation were evaluated. The effectiveness of the preparation was assessed by a cell viability test using a WST-8 reagent. Based on the results, all formulas produce preparations that are isotonic, clear, sterile, stable, and free from endotoxins. Cell viability test shows the addition of 25 μg/mL hEGF increased epithelial cell proliferation by up to 197%. It can be concluded that eye drops containing autologous serum and 25 μg/mL hEGF can be a promising therapy for dry eye syndrome.

Key words: Autologous serum, dry eye syndrome, lyophilization, recombinant human epidermal growth factor

INTRODUCTION

As many as, 5%–30% of people aged over 50 years suffer from dry eye syndrome, the number of sufferers will continue to increase every year. Dry eye syndrome occurs owing to decreased fluid output or increased tear evaporation due to meibomian gland dysfunction. Dry eye syndrome can cause vision problems, inflammation, and intensify osmolarity of the tear film.[1,2]

One of the conventional therapies for treating dry eye syndrome is artificial tears, which can increase the ocular surface moisture and lubricate the eyes. However, this therapy can destroy the natural biological components of the eye caused by chemical preservatives used in the preparations. Artificial tears can also cause allergic reactions, irritation, and inflammation.[3]

Another recommended therapy and currently being developed is autologous serum. The autologous serum is considered to be safer, more effective, and efficient in treating dry eye syndrome.[4,5] The serum can originate

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from the patient himself, thereby minimizing the risk of an immune reaction occurring in the patient's body and avoiding the risk of disease transmission.[6] Besides, autologous serum has many similarities in content with natural tears, including lysozyme, Vitamin A, fibronectin, transforming growth factor-β, epithelial growth factor (EGF), and immunoglobulin A.[7]

In dry eye syndrome patients, EGF levels in their eyes continue to decrease with decreasing the number of tears.[6] EGF has a function in maintaining ocular and corneal wound healing process.[8-10] In the healing process of corneal wounds, EGF will form a tissue granulation by stimulating cell migration and proliferation. The research conducted by Agung *et al*. showed that the addition of exogenous EGF by 25 μg/mL could increase cell proliferation activity to enhance and accelerate the healing process of corneal wounds.[6] Therefore, the presence of EGF needs to be maintained through the addition of exogenous EGF in autologous serum.

Autologous serum in eye drops have been used since the 1990s to treat dry eye syndrome in moderate-to-severe level.[11] The nature of autologous serum that is easily damaged requires the storage of preparations at low temperatures to maintain the stability of the active substance. The autologous serum has a short usage time limit. Autologous serum eye drops that are more practical in terms of storage and use are beneficial for patients with dry eye syndrome who need a long-term therapy. One way to extend the shelf life of autologous serum is through the addition of lyoprotectant, which is then lyophilized by the freeze-drying method to become a dry autologous serum. The drying method can increase the stability of autologous serum for up to 3 months of storage.[12,13] Therefore, this research aimed to find out the effectiveness of eye drops containing autologous serum and human epidermal growth factor (hEGF) prepared through the lyophilization process. The hEGF used is a recombinant product using *Escherichia coli* (BL21) obtained from the previous studies.[8,14-16]

METHODS

Materials

The material used in this study is two bags of blood samples (Palang Merah Indonesia [PMI]), sucrose (Sigma Aldrich, USA), physiological NaCl (Sigma Aldrich, USA), aquadest (IKA), recombinant hEGF from the previous study,[8,14-16] sodium pyrosulfite (Sigma Aldrich, USA), Vitamin E (Arrow Pharm), tween 80 (Sigma Aldrich, USA), fluid thioglycolate medium (FTM) (Sigma Aldrich, USA), tryptone soya broth (TSB) (Sigma Aldrich, USA), limulus amebocyte lysate (LAL) (Pyrotell, Cape Cod Inc., East Falmouth, USA), NIH3T3 fibroblast cell (ATCC® CRL-1658™, ATCC Inc., Manassas, Virginia), and WST-8 Reagent (Dojindo Molecular Technologies Inc., USA).

Preparation of autologous serum

Two blood samples from PMI were centrifuged for 10 min at 4000 g. Then, the serum (supernatant) was separated. After the serum was separated, it was diluted with physiological NaCl, and then 2% sucrose was added.

Preparation of eye drops

Autologous serum formulations were carried out aseptically in laminar airflow and sterilized through a filter membrane for bacteria. The formula compounds are shown in Table 1.

Lyophilization of eye drops using the freeze dry method

The preparation was frozen in refrigerator for 24 h. After freezing, the serum was put into a tube and then it was freeze-dried. The drying process was carried out for 24 h to obtain serum in the form of dry powder. Furthermore, the serum powder was stored at 4°C.

Evaluation of eye drops

Clarity test

This evaluation was done visually under good lighting and had a black and white background. The preparations were observed and confirmed to be free of small floating particles, and then compared with distilled water. Observations were made on days 0, 1, 4, 7, 14, and 28th.

pH measurement

The pH measurement was carried out for 28 days. This observation was carried out to see the effect of the formula's components on the stability of the autologous serum during storage, marked by changes in pH.

Sterility test

The FTM and TSB media, which had been inoculated with *Bacillus subtilis* and *Candida albicans*, were defined as positive control groups. The negative control only contained FTM media and TSB media. Then, each formula (±1 mL) was put into FTM media and incubated at 30°C–35°C. Each

Table 1: Formula of autologous serum eye drops

Formula	A1	A2	A3	B1	B2	B3
Serum A (20%)	✓	✓	✓			
Serum B (20%)				✓	✓	✓
Physiological NaCl	✓	✓	✓	✓	✓	✓
Sucrose (2%)	✓	✓	✓	✓	✓	✓
hEGF (25 μg/mL)		✓	✓		✓	✓
Sodium pyrosulfite (0.1%)		✓			✓	
Vitamin E (0.1%)			✓			✓
Tween 80 (0.05%)			✓			✓
Tonicity (%)	1.06	1.12	1.06	1.06	1.12	1.06

Serum A : Serum originated from 1st individual, Serum B : Serum originated from 2nd individual

formula (±1 mL) was also inoculated in TSB media and incubated at 20°C–25°C. The observation of turbidity was done during the 14-day incubation period.

Endotoxin test

The endotoxin test was done by entering the test formula into a single test vial. Then, the LAL reagent was added and incubated at 37°C ± 1°C for 60 ± 2 min. Then, the tube was carefully turned 180°. A positive endotoxin reaction is indicated by the formation of a thick gel that will stick to the bottom of the tube when turned 180°.

Cell proliferation activity test using WST-8

Cell culture was taken from a liquid nitrogen tank and then resuspended in 3 mL Dulbecco's Modified Eagle's Medium (DMEM) +20% fetal bovine serum. The cell was then centrifuged for 5 min, and the supernatant was removed. Cell samples were resuspended in 10 mL DMEM and then incubated at 37°C for 24 h. The cell sample was transferred to a petri dish, and then 750 µL trypsin was added and incubated for 5 min at 37°C. Cell samples were resuspended again in 8 mL of DMEM media, transferred to a falcon tube, then centrifuged for 5 min, and the supernatant was removed. The cell sample was then added to 20 µL trypan blue, resuspended, and then, 20 µL of cell suspension was taken as a cell culture. The suspension was inserted on the slide, and the number of cells was counted. Cell samples were mixed in 970 µL DMEM media and added to 24 well then incubated at 25°C for 24 h.

After the cells were transferred into 24 wells, the autologous serum formula was inoculated into the well and incubated for 4 h. The medium was removed, and the well plate was washed with phosphate-buffered saline (PBS) 250 µL 3 times. Thirty microliter of WST-8 reagent and 270 µL of PBS were added to the well plate and then incubated for 30 min. Cell proliferation activity was figured by measuring absorbance at 450 nm and 655 nm wavelengths using a microtiter plate reader. The following formula used to calculate the percentage of cell viability:

$$\% \text{ Cell viability} = \frac{\text{Optical density of treated cell}}{\text{Optical density of control}} \times 100\%$$

RESULTS AND DISCUSSION

Preparation of autologous serum

The centrifugation of the serum at 4000 g for 10 min appeared in obvious separation between serum and blood plasma, without inducing hemolysis. The speed and duration of centrifugation will affect serum quality. At low centrifugation speeds, the platelet membrane will mix in the supernatant, and at high speeds, it will induce apoptosis.[17-19]

Preparation of eye drops

Eye drops are considered isotonic if their tonicity is equal to serum/blood plasma. The tonicity of the preparations is shown in Table 1. Based on these results, all preparations were still within the allowed tonicity range (1%–2%). In this range, eye drops can be used without causing significant interference to the eyes.[20]

The preparations that contain Vitamin E have slight turbidity; this is because oil-based Vitamin E is difficult to blend with water-based serum. Therefore, tween 80 was added to this formulation because it is considered safe and acceptable for eye preparations with limited concentration.[21,22]

Lyophilization of eye drops using the freeze dry method

In the lyophilization process, the removal of water molecules can occur, thereby reducing the occurrence of protein degradation in autologous serum. However, freeze-drying can cause damage to the structure of the protein when it is redissolved. Therefore, sucrose was added as a stabilizer. Sucrose is a nonreducing saccharide. Sucrose protects protein conformation by replacing water molecules around protein molecules and reducing chemical degradation by wrapping proteins in an environment with low molecular mobility.[23] The freeze-drying process takes approximately 48–56 h until the preparation turns into a dry serum.

Evaluation of eye drops

Clarity test

Based on the results of the clarity test can be seen that there was no change in all formulas during the 28 days of observation. The characteristics of the autologous serum eye drops are clear pale yellow, characteristic odor, and no visible foreign particles.

pH measurement

During the 28 days of observation, the pH of formula A underwent a significant change. Compared to formula A, formula B maintains the pH of the preparation better. This shows that different serum sources affect the stability of autologous serum preparations. Based on the test results, the formula for autologous serum eye drops obtained from the 2nd individual (serum B) was declared stable because the pH value was in the pH range of hEGF stability (pH 6–8).[24] The pH of each formula during storage is shown in Figure 1.

Sterility test

Based on observations, it was found that serum A and serum B did not experience turbidity either on FTM media or TSB media during 14 days of incubation. This indicates that the sterility of the serums can be maintained during the formulation process.

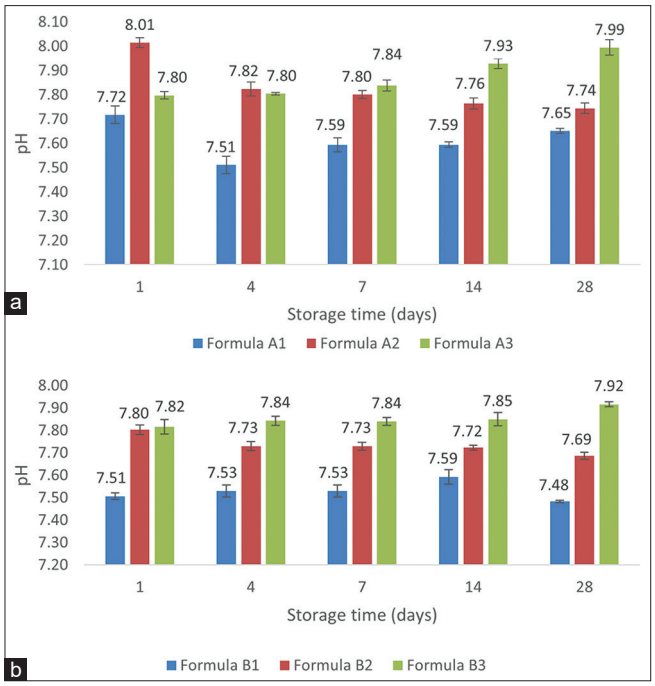

Figure 1: pH measurement of serum A (a) and serum B (b) during storage

Endotoxin test

The endotoxin test on the six formulas showed no gel formation at the bottom of the tube when rotated 180°. These results indicate that the concentration of endotoxin in all eye drops is smaller than the sensitivity of LAL reagent, which is 0.125 EU/mL. The results of the endotoxin test are shown in Figure 2.

Cell proliferation activity test using WST-8

Based on the calculation results, the highest cell viability was shown in autologous serum preparations containing sodium pyrosulfite (antioxidant) and 25 µg/mL hEGF. In the control group (formulas A1 and B1), the percentage of viable cell was 147%. This shows that blood serum also contains endogenous growth factors, so the percentage of cell viability obtained was more magnificent than the control group. The cell viability is show in Table 2.

In formulas containing Vitamin E, the percentage of cell viability was smaller than that in the control group. This may be due to Vitamin E being an antioxidant that contains a free thiol group and is a robust reducing compound. Therefore, Vitamin E can reduce the ability of reagents to form formazan which is the marker of living cell.

CONCLUSION

Based on the evaluation results, all autologous serum eye drops that were formulated met the requirement of eye drops preparations stated in the Indonesia Pharmacopoeia.

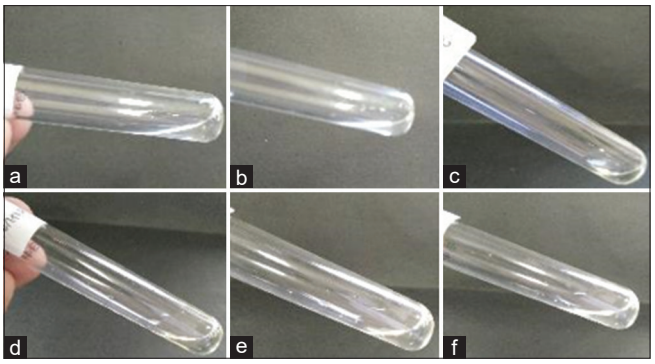

Figure 2: The results of the endotoxin test of formula A1 (a), A2 (b), A3 (c), B1 (d), B2 (e), and B3 (f)

Table 2: Viability of NIH3T3 cells

Konsentrasi hEGF (µg/mL)	Percentage cell viability	
	Formula B2	Formula B3
0	147	147
10	173	115
25	197	143

hEGF: Human epidermal growth factor

The preparations are isotonic, clear, sterile, and free from endotoxins. The addition of 25 µg/mL hEGF to the eye drops increased cell viability by up to 197%.

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Conflicts of interest

There are no conflicts of interest.

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