Journal of Applied Pharmaceutical Science Vol. 11(07), pp 110-116, July, 2021
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2021.110712
ISSN 2231-3354

Lyophilized autologous serum stabilized with sucrose promotes the proliferation and migration of keratinocyte

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ARTICLE INFO

Received on: 11/01/2021
Accepted on: 22/04/2021
Available online: 05/07/2021

Key words:
Autologous serum, cell proliferation and migration, freeze-drying, hEGF, sucrose, vitamin E.

ABSTRACT

The autologous serum is widely used as therapy for epithelial cell treatment and dry eye syndrome due to its epitheliotropic properties, which are similar to tears. The serum contains human epidermal growth factor (hEGF) and retinol, which play important roles in cell proliferation and differentiation. Freeze-drying improves the stability of therapeutic proteins for long-term storage. Sucrose is known to stabilize protein during freeze-drying and storage. Also, antioxidants such as vitamin E can maintain unstable retinol levels. This study aims to investigate the effect of the addition of sucrose to preserve dried autologous serum during freeze-drying and storage, as well as the effects of the serum on keratinocyte proliferation and migration. The addition of sucrose to autologous serum could preserve the dried autologous serum components above the minimum requirement for substitute tears after 6 months of storage. The highest cell proliferation was achieved by the addition of 25 ng/ml hEGF and 1% vitamin E, while the highest migration activity was achieved by the addition of 25 ng/ml hEGF. Freeze-drying autologous serum with the addition of sucrose and storage at low temperatures can extend its shelf life. Also, the addition of exogenous hEGF and vitamin E to autologous serum can increase the proliferation and migration of keratinocyte cells.

INTRODUCTION

Autologous serum is recommended as eye drops for ocular surface disorder treatments, such as Sjörgen syndrome, a tear production disorder, and non-Sjörgen syndromes, such as graft-versus-host, neurotrophic keratitis, epithelial persistent defect, and superior limbic keratoconjunctivitis (Koffler, 2006). It is widely used in ophthalmology as a tears substitute, not only to lubricate the ocular surface but also to provide other components not present in patients with ocular surface disorders (López-García *et al*., 2007). Autologous serums are prepared from blood; so, they are natural, do not cause allergic reactions, and have biomechanical and biochemical properties similar to tears. Some components of autologous serum that are similar to tears are epidermal growth factor (EGF), transforming growth factor-β (TGF-β), immunoglobulin A, vitamin A, fibronectin, cytokine, and lysozyme (Geerling *et al*., 2004; Quinto *et al*., 2008), which are important to maintain the cornea and conjunctival epithelium health (Koffler, 2006). However, autologous serum in drop form has a shelf life of 1 month, 3 months if stored at −20°C (Herrero-Vanrell & Molina-Martínez, 2008). Therefore, another storage method is needed to enhance stability over time, while also maintaining its convenience and portability.

Freeze-drying, also known as lyophilization, can improve the long-term stability of therapeutic proteins (Tang & Pikal, 2004), as chemical and physical degradation is inhibited in freeze-dried solid form (Carpenter *et al*., 1997). Besides its advantage in terms of stability, lyophilized solid forms are also much easier to handle, package, and transport (Tang & Pikal, 2004). Protection of the conformation of the protein during freeze-drying through the

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appropriate control of the process and optimization of the material is important to preserve the pharmacological effects and reduce the immunogenicity of the product (Hermeling *et al*., 2004). Nonreducing sugar, like sucrose, acts as stabilizers, protecting the protein from chemical and physical degradation in aqueous solution during freeze-drying and subsequent storage (Carpenter & Crowe, 1989; Wang, 2000).

Vitamin A is essential for vision as well as cell growth and differentiation (Amann *et al*., 2011), with as much as 95% of vitamin A in the blood in the form of retinol (Catignani *et al*., 1983). Inside the cell, it is oxidized to retinoic acid which plays an important role in cell growth and differentiation (Amann *et al*., 2011; Zasada & Budzisz, 2019). Retinol in dried autologous serum has a relatively short shelf life due to oxidation (Maksum *et al*., 2016) but can be stabilized by the addition of vitamin E (Maksum *et al*., 2018). However, the effect of vitamin E added to the activity of autologous serum is still unknown.

Human epidermal growth factor (hEGF) also plays an important role in the proliferation and repair of the epithelial cells (Sriwidodo *et al*., 2019), with the addition of hEGF, increasing the stability of autologous serum activity, improving the process of epithelialization, and healing can occur faster (Agung *et al*., 2016). Previously, we successfully produced hEGF in *Escherichia coli* but did not test its effects on epithelialization (Indriyani *et al*., 2019). Therefore, the activity of autologous serum, as well as the effect of the addition of vitamin E and recombinant hEGF in the treatment of epithelial damage, should be assessed.

Herein, we prepared dry autologous serum by freeze-drying with the addition of sucrose. The stability of the serum components, EGF, TGF-β1, fibronectin, and lysozyme, was assessed before the effects of the autologous serum with the addition of hEGF and vitamin E on the proliferation and migration of keratinocyte cells were determined.

MATERIALS AND METHODS

Materials

The materials used in the present study were human blood obtained from Indonesian Red Cross (PMI), recombinant hEGF from our previous study (Indriyani *et al*., 2019), vitamin E (Sigma Aldrich), methanol (Genetika Science), ethanol (Genetika Science), *n*-hexane (Genetika Science), diethyl ether (Merck), aquabidest (Genetika Science), standard retinol (Retinol lot BCBL 139V *synthetic*, ≥ 95% HPLC *crystalline* (Sigma Aldrich), kanamycin (Sigma Aldrich), keratinocyte cell culture from human cell line HaCat, RPMI-1640 media, dimethyl sulfoxide (DMSO) (Merck), (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) reagent (Merck), phosphate-buffered saline (PBS) (Merck), sucrose (Sigma Aldrich), *Micrococcus lysodeikticus* (Sigma Aldrich), sodium chloride (Merck), and *Platinum* ELISA *Ready-to-use Sandwich* ELISA (eBioscience).

Preparation of autologous serum

The blood samples were allowed to clot at 4°C for 2 hours and then centrifuged at 5,000 g for 10 minutes to obtain the serum, which was diluted with sterile physiological sodium chloride solution to give a final concentration of 20% (*v/v*). The diluted serum was then divided into two groups; sucrose was added to one group to a final concentration of 60 mM. The serum samples were freeze-dried and stored at 4°C and room temperature. The content of EGF, TGF-β1, and fibronectin and also lysozyme activity were analyzed at 0, 1, 3, and 6 months after storage. For activity assays, the diluted serum was filtered using a 0.2 µm syringe, before the addition of vitamin E [0.5%; 1% (*v/v*)], hEGF (10, 25 ng/ml), and both vitamin E and hEGF (1%; 25 ng/ml). Untreated diluted serum was used as a control. The serum was added to RPMI-1640 media to a final concentration of 20% (*v/v*).

Determination of EGF, TGF-β1, and fibronectin content by ELISA

EGF, TGF-β1, and fibronectin content were determined using eBioscience™ Platinum ELISAs with a specific primary antibody for each protein according to the manufacturer's protocol.

Determination of lysozyme activity by the turbidity method

Each freeze-dried serum sample was diluted with aquabidest until a final volume of 1 ml. *M. lysodeikticus* cell suspension (0.01% *w/v*) in reaction buffer was used as a substrate. For the blank, 800 µl of *M. lysodeikticus* cell suspension was added to cuvette and mixed with 30 µl reaction buffer, and then the A_{450} was monitored. The A_{450} of the *M. lysodeikticus* cell suspension should be at 0.6–0.7. For the standards and samples, 30 µl lysozyme solution and 30 µl serum solution were added to 800 µl of *M. lysodeikticus* cell suspension and then mixed and the A_{450} was recorded every minute for 5 minutes.

Determination of vitamin A content by HPLC

The serum (100 µl) was mixed with 100 µl *n*-hexane and centrifuged at 800 g for 5 minutes to collect the supernatant, which was evaporated in a water bath at 60°C. The residue was suspended in 25 µl diethyl ether and 75 µl methanol. Standard retinol was dissolved with ethanol. Samples and standards were analyzed by analytical HPLC, equipped with a UV/Vis detector and 250 mm C_{18} column, using methanol and water as the mobile phase [methanol: water = 95:5 (*v/v*)]. The flow rate was set at 1 ml/min and retinol was detected at 320 nm.

MTT proliferation assay

Keratinocytes were cultured in RPMI-1640 media containing 5% calf serum and kanamycin (100 µg/ml). Cells were seeded in 96-well plates at a density of 2×10^4 cell cm^{-3} and incubated for 24 hours. The culture media were removed from the well, and then the well was washed with 100 µl of 1× PBS before the addition of 100 µl of the serum sample. The plates were incubated in a CO_2 incubator for 48 hours. The wells were washed with 1× PBS, and then MTT reagents were added into each well for 10µL per 100 µL medium and incubated for 4 hours. The reaction was stopped by the addition of DMSO and the absorbance was measured in a microplate reader at 450 nm. The percent proliferation was determined as follows:

$$\% \text{ Proliferation} = (A_{\text{Treatment}}/A_{\text{Without Treatment}}) \times 100\%$$

Scratch migration assay

Keratinocytes were cultured and seeded into 96-well plates as before, then a scratch was made in the cell monolayer

using a sterile tip and visualized. The culture media were removed from the well and the cells were washed with 500 µl of 1× PBS, before the addition of 500 µl of each serum sample. The plates were then incubated and observed after 18, 24, and 42 hours of incubation. The data were analyzed using TScratch software, with the percentage migration calculated as follows:

% cell migration = (area of the final stroke/scratch area at 0 hour) × 100%

RESULTS

Preparation of autologous serum

Autologous serum was separated from the blood cells (Fig. 1A), with the amount of serum collected from different donors ranging from 24 to 42 ml (Geerling *et al*., 2004). The autologous serum was then diluted with sterile physiological sodium chloride, before the addition of sucrose. The serum samples were freeze-dried to yield a white-yellowish solid (Fig. 1B), which was then stored until further analysis.

Preservation of autologous serum components during storage

The content of EGF, TGF-β1, and fibronectin of native autologous serum was 731.63 pg/ml, 62.33, and 235.13 µg/ml, respectively, while lysozyme activity was 1,133.33 Unit/ml. The freeze-dried autologous serum was stored in both the refrigerator (4°C) and room temperature. The EGF, TGF-β1, and fibronectin content and lysozyme activity were determined after 0, 1, 3, and 6 months (5 months for fibronectin) of storage (Fig. 2). Storage at a low temperature was more effective at preserving the autologous serum components compared to room temperature with or without the addition of sucrose. The addition of sucrose to autologous serum maintained a high EGF concentration throughout storage (Fig. 2A). Lysozyme activity and EGF concentration remained stable up to 6 months of storage in the refrigerator with or without the addition of sucrose (Fig. 2D), whereas TGF-β1 and fibronectin concentration decreased after several months of storage (Fig. 2B and C), and the presence of sucrose suppressed the decrease in the concentration of each component during storage. Hence, the addition of sucrose could preserve the freeze-dried autologous serum components for up to 6 months.

Figure 1. Preparation of autologous serum from donor blood: (A) centrifuged blood, the autologous serum is in the upper layer, and (B) freeze-dried autologous serum.

Vitamin A content in autologous serum

The vitamin A content in the form of retinol was determined in autologous serum by reverse phase HPLC analysis (Fig. 3). Standard retinol has a retention time of 8.980 minutes, with the chromatogram of autologous serum from individual I and individual II characterized by a single peak at 8.709 and 8.723 minutes, respectively. A summary of retinol content determination is presented in Table 1, showing that there are differences in retinol levels in autologous serum from each individual, which is consistent with previous studies (Fischer *et al*., 2012; Geerling *et al*., 2004; Kanďár *et al*., 2014; Quinto *et al*., 2008).

EGF content of autologous serum

The serum hEGF levels prior to the freeze-drying process are presented in Table 2, showing a difference between individuals. According to Geerling *et al*. (2004), serum hEGF levels are 0.5 ng/ml in serum and 0.2–0.3 ng/ml in tears. The serum hEGF of each individual has a concentration that meets the hEGF concentration range in the tears. Therefore, the autologous serum could provide sufficient hEGF as a substitute for tears.

Proliferation activity of autologous serum

The percentage keratinocyte proliferation treated with autologous serum from each individual is shown in Figure 4, revealing that adding autologous serum alone to cells achieved 76.53% and 78.16% proliferation for individual I and individual II, respectively. The addition of hEGF or variation in vitamin E concentration in the autologous serum samples increased cell proliferation compared to autologous serum alone. Autologous serum with the addition of 1% vitamin E and hEGF 25 ng/ml induced the most proliferation, 89.82% and 96.55% for individual I and individual II, respectively. The pattern of cell proliferation was similar for both donors, although an increase in the percent cell proliferation was dependent on the individual autologous serum components.

The effect of autologous serum on keratinocyte migration

The percentage migration of keratinocytes induced by autologous serum was determined using TScratch software. The progression of the scratch width after 0, 18, 24, and 42 hours of incubation is shown in Figure 5. The scratches were completely closed after 42 hours of incubation, indicating that the addition of autologous serum could induce the migration of keratinocytes. The percentage migration of keratinocytes treated with autologous serum from individual I and individual II with various treatments after 18, 24, and 42 hours of incubation is shown in Figure 6. The addition of hEGF 10 and 25 ng/ml to autologous serum increased cell migration compared to autologous serum alone. The addition of 0.5% vitamin E decreased cell migration, while 1% vitamin E increased cell migration comparable to the addition of 10 ng/ml hEGF. Keratinocytes treated with autologous serum containing 25 ng/ml hEGF and 1% vitamin E had 78.57% and 82.77% cell migration for individuals I and II, respectively, which was lower than autologous serum with the addition of 25 ng/ml hEGF. The pattern of cell migration was similar for both individuals I and II but the slight differences that arose between individuals I and II may be due to differences in their autologous serum content (Geerling *et al*., 2004).

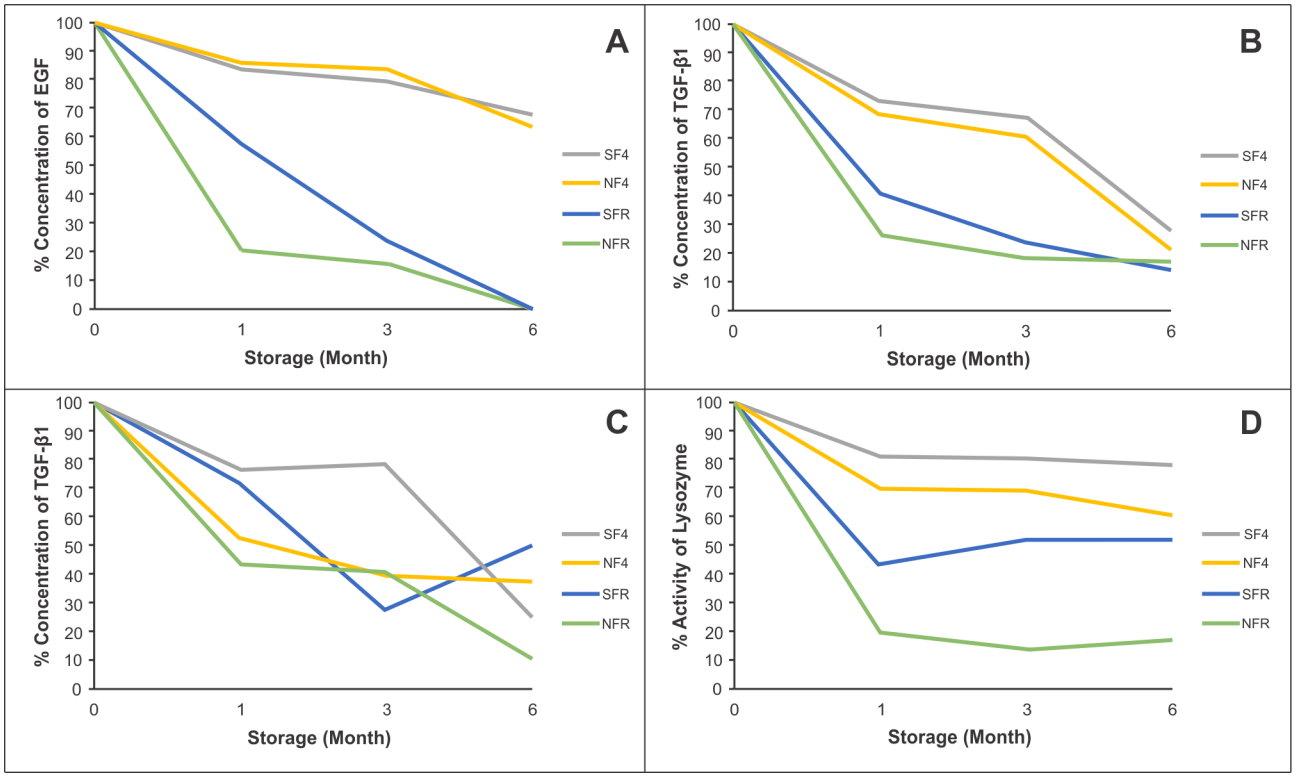

Figure 2. Stability of the autologous serum components during storage: (A–C) EGF, TGF-β1, and fibronectin and (D) lysozyme activity. SF4: dried serum with the addition of sucrose at 4°C, NF4: dried serum without the addition of sucrose at 4°C, SFR: dried serum with the addition of sucrose at room temperature, and NFR: dried serum without the addition of sucrose at room temperature.

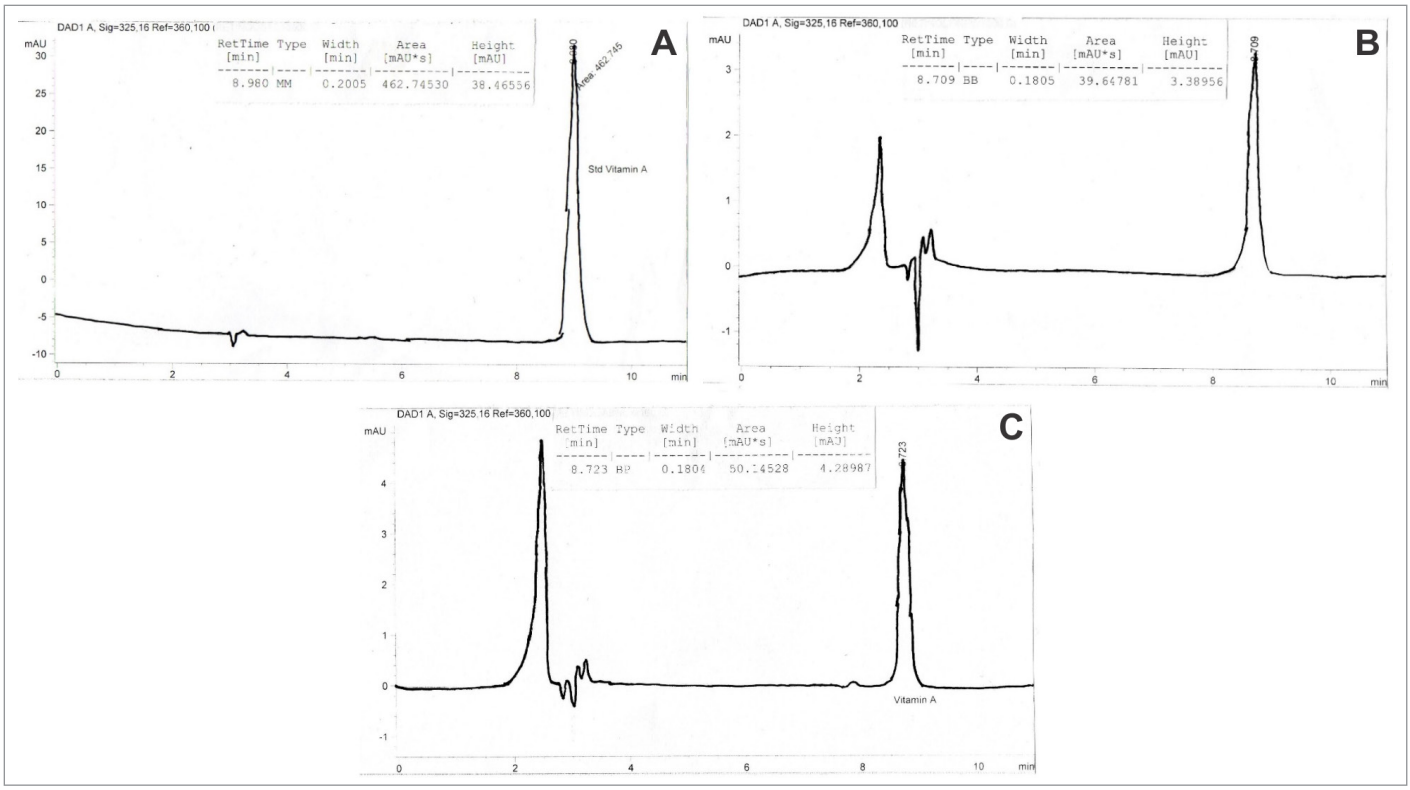

Figure 3. The chromatograms of retinol in autologous serum samples: (A) standard retinol 8 ppm, (B) autologous serum from individual I, and (C) autologous serum from individual II.

DISCUSSION

The protocol for autologous serum collection used in the present study was based on protocol suggested by Liu *et al*. (2005), who reported that the serum obtained contained more EGF and consequently was a better treatment for dry eyes. The variation between the serum obtained from different donors was expected (Geerling *et al*., 2004). The freeze-drying process was approximately 24 hours in total, yielding a white-yellowish solid, which was resolubilized with sterile double distilled water to its original volume so that all components were in their original concentration before analysis.

Storage temperature influences the protein stability of solids, with the physical and chemical stability lower at a higher temperature (Wang, 2000). The storage of dried autologous serum at 4°C preserves the content of EGF, TGF-β1, fibronectin, and lysozyme above the minimum concentration to replace the component in tears for up to 6 months. Sucrose acts as a stabilizer that can protect proteins in aqueous solutions, during both freeze-drying and subsequent storage. It can protect the conformation of proteins in solids by forming hydrogen bonds that replace water molecules around protein molecules (Cleland *et al*., 2001; Wang, 2000). The addition of sucrose to autologous serum was protective, combined with freeze-drying process and storage at 4°C, which could preserve the concentration of autologous serum component as a substitute for tears in long-term storage.

Serum extraction using *n*-hexane is the best for extracting retinol (Kitagawa & Hosotani, 2000), as evidenced by the peak in Figure 3. In line with previous studies, there was variation in blood serum retinol levels between individuals (Maksum *et al*., 2016, 2018).

The addition of autologous serum alone promoted keratinocyte proliferation, probably due to the endogenous hEGF content (Maksum *et al*., 2017; Sriwidodo *et al*., 2017). However, this was less than the percentage of cell proliferation induced by hEGF alone. The autologous serum contains other proteins in addition to hEGF which regulates gene expression, such as TGF-*β*. Cell proliferation is carefully controlled with EGF regulating *cyclin D* gene expression involved in proliferation (Pecorino, 2012), while TGF-β functions as a growth inhibitor (Zhang *et al*., 2017). As expected, the addition of hEGF (10 and 25 ng/ml) increased keratinocyte proliferation more than autologous serum alone (Agung *et al*., 2016).

Autologous serum with the addition of vitamin E also has increased cell proliferation compared to autologous serum or hEGF alone. Vitamin E is an excellent antioxidant, preserving the retinol content in serum to achieve maximum stimulation of cell proliferation (Maksum *et al*., 2018; Niki, 2015). Inside the cell, retinol undergoes oxidation to retinoic acid, which is important for gene transcription related to cell growth and differentiation (Amann *et al*., 2011; Zasada & Budzisz, 2019). The addition of 1% vitamin E to autologous serum promoted more cell proliferation than 0.5% vitamin E, consistent with previous work that showed that 1% vitamin E is better at stabilizing the retinol content during storage of dried autologous serum (Maksum *et al*., 2018). The addition of 25 ng/ml hEGF and 1% vitamin E to autologous serum promoted the most keratinocyte proliferation, providing optimal conditions for cell proliferation.

The scratch assay was a well-known technique for cell migration assessment. The application of a scratch to

Table 1. Retinol concentration of autologous serum samples**.**

Sample	Retention time (minutes)	Area (mAU.s)	Retinol concentration (ppm)
Standard	8.980	462,745	8
Individual I	8.709	39.6478	0.6854
Individual II	8.723	50.1453	0.8669

The retinol concentration was determined using HPLC (with Waters 2998 photodiode array detector; Waters 1525 binary HPLC pump; column RP-18 endcapped 5 µm Purospher STAR LiChroCART 250-4.6; flow rate 1 ml/minutes; mobile phase being methanol : water = 95:5).

Table 2. The hEGF levels of autologous serum samples. The hEGF levels were determined using ELISA.

Sample	Absorbance	hEGF (ng/ml)
Individual I	0.1142	0.2964
Individual II	0.1367	0.5731

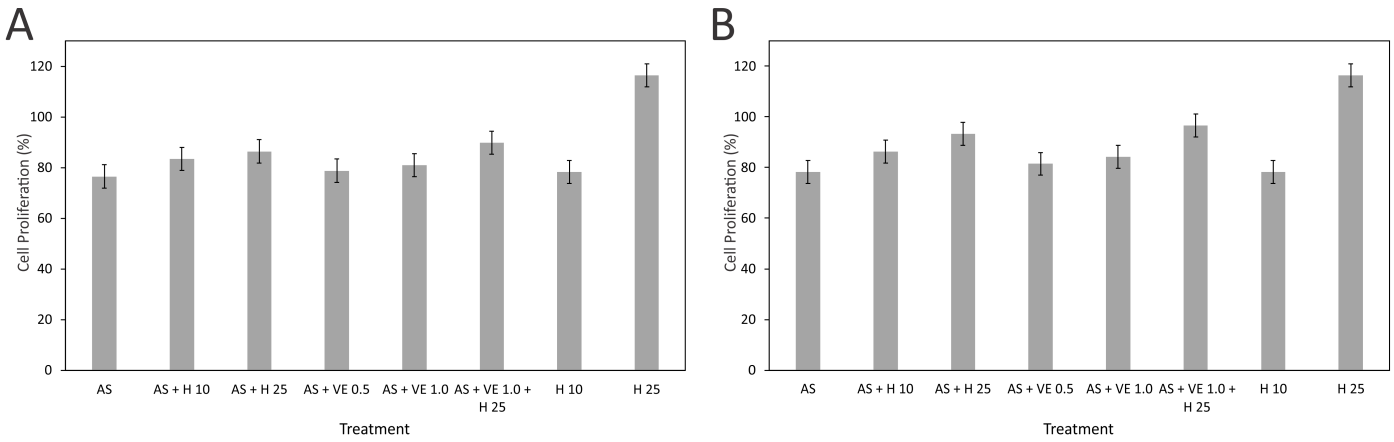

Figure 4. The effects on individual autologous serum on the percentage keratinocyte proliferation: individual I (A) and individual II (B). SO = autologous serum; SO + H 10 = autologous serum added with 10 ng/ml hEGF; SO + H 25 = autologous serum added with 25 ng/ml hEGF; SO + VE 0.5 = autologous serum added with 0.5% vitamin E; SO + VE 1 = autologous serum added with 1% vitamin E; SO + VE 1 + H 25 = autologous serum added with 1% vitamin E and 25 ng/ml hEGF; H 10 = 10 ng/ml hEGF; H 25 = 25 ng/ml hEGF. Data are means of triplicate readings; error bar was represented.

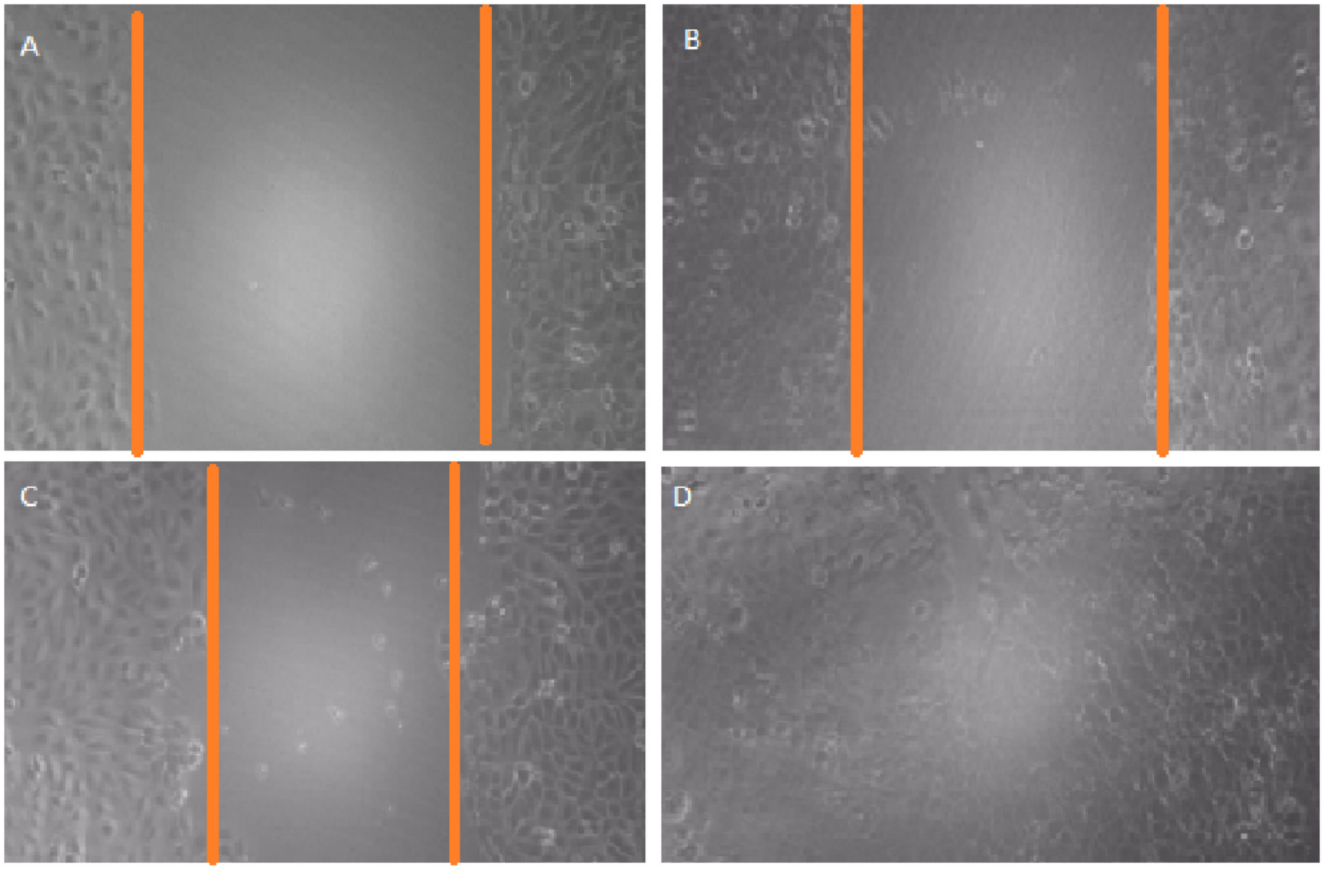

Figure 5. The effects of autologous serum from individual II containing 25 ng/ml hEGF on keratinocyte migration assessed by the scratch assay: A = scratch at 0 hour, B = scratch at 18 hours, C = scratch at 24, and D = scratch at 42 hours.

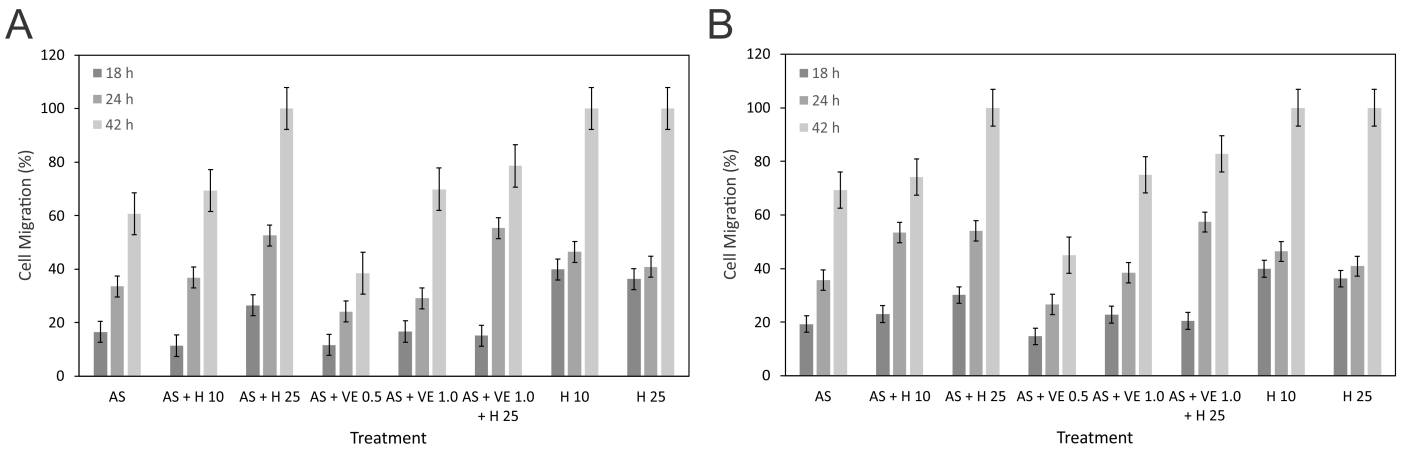

Figure 6. Percent cell migration with the addition of serum from individual I (A) and individual II (B) after incubation for 42 hours. SO = autologous serum; SO + H 10 = autologous serum plus 10 ng/ml hEGF; SO + H 25 = autologous serum added with 25 ng/ml hEGF; SO + VE 0.5 = autologous serum added with 0.5% vitamin E ; SO + VE 1 = autologous serum added with 1% vitamin E; SO + VE 1 + H 25 = autologous serum added with 1% vitamin E and 25 ng/ml hEGF; H 10 = 10 ng/ml hEGF; H 25 = 25 ng/ml hEGF. Data are means of duplicate readings; error bar was represented.

keratinocyte cells created a scratch area, inducing cell migration as well as cell proliferation to close the area (Agung *et al*., 2016; Asmus *et al*., 2019; Javer *et al*., 2020). The autologous serum from both donors induced cell migration as evidenced by the closure of the scratch, which also occurred with autologous serum containing 25 ng/ml hEGF, as well as autologous serum containing 25 ng/ml hEGF and 1% vitamin E. The addition of 10 and 25 ng/ml hEGF as control induced scratch closure after 42 hours. According to Agung *et al*. (2016), the optimal concentration for the addition of hEGF alone for wound closure is 10 ng/ml, while in this study 25 ng/ml hEGF also provided optimal migration activity.

The differences in cell proliferation and migration induced by the serum from individuals I and II are related to

differences in the autologous serum content, with individual II having higher levels of hEGF and retinol than individual I (see Tables 1 and 2), so that with the same treatment, there is a difference in the percentage proliferation.

CONCLUSION

In summary, in the present study, we found that the freeze-drying process can be useful to prolong the shelf life of autologous serum. Based on the content of EGF, TGF-β1, and fibronectin as well as lysozyme activity analysis, the addition of sucrose can be useful to preserve the autologous component. The freeze-drying process and addition of sucrose are preserving serum components above the minimum requirement to substitute tears after 6 months of storage. The autologous serum itself can induce proliferation and migration of keratinocyte cells. The addition of 25 ng/ml hEGF and 1% vitamin E to autologous serum can help increase its activity, indicated by increasing proliferation and migration of keratinocyte cells.

ACKNOWLEDGMENT

This work is supported by Academic Leadership Grant (ALG) Universitas Padjadjaran (no. 1427/UN6.3.1/LT/2020).

CONFLICT OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

PUBLISHER'S NOTE

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How to cite this article:
Maksum IP, Jubaedah S, Yosua Y, Melpin R, Rondonowu M, Enus S, Rachman SD, Sriwidodo S, Subroto T. Lyophilized autologous serum stabilized with sucrose promotes the proliferation and migration of keratinocyte. J Appl Pharm Sci, 2021; 11(07):110–116.