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Research Article

THE IMPACT OF STORAGE DURATION OF FRESH HUMAN AMNIOTIC MEMBRANE WITH THE LEVEL OF EGF, TGF-β, AND BFGF GROWTH FACTORS

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Abstract

Background: Human amnion membrane has been used in many regenerative medicine. It has been reported that fibroblast and other epithelial cells lost its viability after going through cryopreservation. The objective of this study is to prove the effect of storage duration of the fresh amnion membrane to the levels of EGF, TGF-β and bFGF growth factors. **Methods**: Eighteen fresh human amnion samples stored at Dr. Soetomo General Academic Hospital's Centre of Biomaterial and Tissue Bank was involved in this study. Each group of 6 is selected from different storage times at -20° C (1 week, 3 months, and 6 months). The levels of EGF, TGF-β and bFGF growth factors at different storage times were analysed using ELISA. **Result**: From ELISA analysis, EGF mean value at 1 week storage duration was 8.43±5.13 pg/mL, 3 months storage was at 16.59±22.20 pg/mL and 9 month storage was 4.52±2.04 pg/mL. From TGF-β analysis it was 140.41±25.77 pg/mL at 1 week storage time, 3 months storage time was 140.58±50.62 pg/mL, and at 9 months storage it was 115.75±35.91 pg/mL. From the EGF and TGF-β analysis, it shows that the difference of the growth factor levels are negligible between the 3 storage duration groups, (p= 0.462) and (p=0.331) respectively. Whereas in bFGF , the mean value at 1 week storage duration was 57.60± 26.90 pg/mL, 3 months was 37.94±13.11 pg/mL, and at 9 months storage time it was 147.66±89.93 pg/mL. In comparison to 1 week group against 9 months, and 3 months against 9 months, results showed significant changes at p=0.028 and p=0.010 respectively (p<0,05). Whereas at 1 week against 3 months storage duration, the result was insignificant at p= 0.273 (p>0,05). **Conclusion:** There were no significant changes in the value of growth factor between different storage times at 1 week, 3 months, and 9 months.

Keywords: Fresh human amnion, EGF, bFGF, TGF-β, Storage time.

INTRODUCTION

Amnion membrane is the deepest layer in the placenta that protects the foetus. This membrane is thin at an average of 0.02 to 0.5 mm of thickness, transparent in colour and very durable to protect the foetus in the uterus[1,2,3]. It forms a sac and filled with amniotic fluid. Human amniotic membrane has been used in regenerative medicine and its application varies in many field of medicine. Its unlimited source and low cost makes it a very potential resource for regenerative medicine[4].

In vitro study has proven that amnion membrane secretes growth factors (GF) that contributes to angiogenesis, re-epithelization and immunomodulation. Using Enzyme-Linked Immunosorbent Assay (ELISA) method, growth factors were extracted from fresh amnion membrane and it contains many growth factors such as bFGF, TGF-α, TGF-β1, -β2, EGF, KGF, and HGF[5]. In healing phases, basic Fibroblast Growth Factor (bFGF) is responsible for granulation, re-epithelization and tissue remodelling, while Transforming Growth Factor (TGF-β) stricts the degradation of Extracellular Matrixs (ECM) and increases collagen production[3]. Epidermal Growth Factor (EGF) is well known to facilitate re-epithelization by stimulating proliferation and keratinocyte migration and increases tensile strength of newly formed tissue. Other advantageous characteristics of amnion membrane are its anti-adhesive, bacteriostatic, antiangiogenetic, antiinflammation, anti-cicatrix, high tensile strength, wound coverage and reduces pain on the wound[4]. Fresh amnion membrane has been constantly used in regenerative medicine. Its original structure, preserved growth factors and viable epithelial cells makes it a promising material in regenerative medicine. However after going through preservation, fibroblast and epithelial cells lost its viability. Ihsan P (2009) conducted an experimental study on 13 human amniotic membranes. Each human amnion membrane was divided into two parts, fresh amnion membrane stored at 4°C for 24 hours and freeze-dried amnion that have been cryopreserved at -80°C for 24-36 hours before being freeze dried and gamma radiated at 25 kGy. EGF was extracted from both groups and analysed using ELISA. Results showed the average value of fresh amnion membrane and freeze dried amnion membrane were 122.756 ± 11.591 pg/g and 100.436 ± 9.690 pg/g respectively. The average degradation concentration was 22.320 ± 15.353 pg/g with percentage between 11.04-23.96%. Fresh amnion membrane was shown to have more significant concentration of EGF compared to freeze dried amnion membrane[4]. Wagner et al (2018) examined bFGF levels in fresh amnion membranes preserved in glycerol media and stored in sodium chloride 0.9% for 0.5, 3, and 6 months. The results showed that bFGF levels decreased significantly (p=0.006) in the three storage groups[6]. Based on some of the literatures above, this study aims to measure the levels of EGF, TGF-β, and bFGF in fresh amnion membranes stored in the Centre of Biomaterial and Tissue Bank at Dr. Soetomo General Academic Hospital, Surabaya with storage duration of 1 week, 3 months, and 9 months. Supply of fresh amnion membrane tend to accumulate overtime and some may reach 1 year of storage in the storage

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chamber waiting to be processed into freeze dried amniotic membrane sheets.

METHODS

Eighteen fresh human amnion membranes samples stored at Centre of Biomaterial and Tissue Bank at Dr. Soetomo General Academic Hospital, Surabaya were involved in this study. Each group of 6 was selected from different storage times (1 week, 3 months, and 6 months) at temperature -20° C. The levels of EGF, TGF-β and bFGF growth factors were analysed using ELISA. The growth factor extraction and ELISA analysis was done at Institute of Tropical Disease, Surabaya.
Fresh amion membranes were thawed for 24 hours after storage. The membranes were cleaned and immersed in sodium hypochlorite 0,05% (NaOCl 0,05%) for 10 minutes. The washing process using sterile water and repeated for 3 times to remove NaOCl 0,05% residual particles. The membrane was placed on a flat surface and cleaned from remaining blood and particles. After that, the membrane was rinsed again three times with sterile water. Samples of fresh amnion membrane were pulverised using mortars and the growth factors were extracted using PRO-PREP™ (iNtRON Biotechnology, Burlington, MA). The extract was analysed using Human ELISA Kit specific for each growth factors from Elabscience, China and BT-Lab, China. The ELISA used Sandwich ELISA method with monoclonal antibody specific for human with pg/mL as the unit. Data were analysed using ANOVA.

RESULTS

EGF Concentration

The EGF result after extraction in each storage group is shown in Table 1 below.

Table 1. EGF value in fresh amnion membrane

Sample	EGF Level (pg/mL)		
	1 Week	3 Months	9 Months
1	17.90	3.55	6.85
2	5.84	3.87	7.24
3	5.48	2.84	3.45
4	5.72	6.72	2.46
5	10.90	58.99	2.94
6	4.77	23.56	4.20
Mean	8.43	16.59	4.52
SD	5.14	22.20	2.04

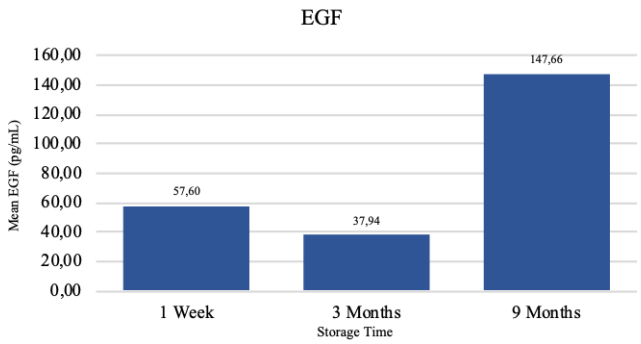

Figure 1. Mean EGF Value in fresh amnion membrane at 1 week, 3 months and 9 months storage time

EGF mean value at 1 week storage duration was 8.43±5.13 pg/mL, 3 months storage was at 16,59±22,20 pg/mL and 9 month storage was 4.52±2.04 pg/mL. Using Shapiro Wilk normality test, the EGF value at 1 week and 3 months were not normally distributed because it was less than 0.05 (significance value of 0.022 and 0.009 respectively). Hence, Kruskal Wallis test was used and found the value at 0.331 (significant value > 0.05). There were no significant difference between the 3 storage groups.

TGF-β concentration

The results of TGF-β in fresh amnion membrane in different storage times are shown below (Table 2). From the analysis, the value of TGF-β was 140.41±25.77 pg/mL at 1 week storage time, 3 months storage time at 140.58±50.62 pg/mL, and at 9 months storage it was 115.5±35.91 pg/mL. Using ANOVA test, the significant value is 0.462 (significance value > 0.05) and therefore no significant difference found between the 3 storage time groups.

Tabel 2. TGF-β value in fresh amnion membrane

Sample	TGF-β Level (pg/mL)		
	1 Week	3 Months	9 Months
1	109.33	135.71	141.611
2	119.06	142.60	153.463
3	154.45	89.99	107.384
4	135.71	82.32	133.742
5	181.34	213.56	104.475
6	142.60	179.34	53.826
Mean	140.41	140.58	115.75
SD	25.77	50.62	35.91

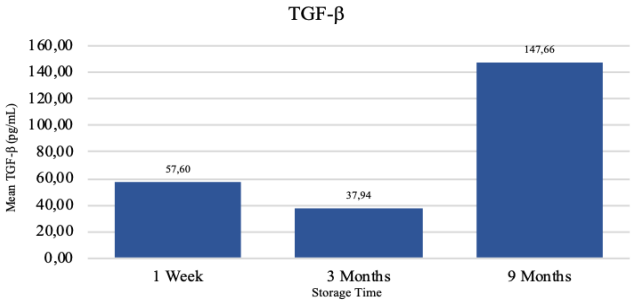

Figure 2. Mean TGF-β value in fresh amnion membrane at 1 week, 3 months and 9 months storage time.

bFGF Concentration

The results of bFGF in fresh amnion membrane in different storage times are shown below in Table 3. Post hoct test (Mann Whitney) were used to see the comparison between each group. When comparing the value of bFGF in 1 week storage time and the 9 months group (0,028) and between 3 months and 9 months (0,10), the difference is significant since the value is < 0.05. However, it was different between 1 week and 3 months group, the difference was not significant (0.273).

Table 3. bFGF value in fresh amnion membrane

Sample	bFGF Level (pg/mL)		
	1 Week	3 Months	9 Months
1	54.52	33.47	174.93
2	45.51	30.40	301.97
3	27.25	32.11	150.06
4	60.60	63.30	141.48
5	(-)	27.95	62.70
6	100.12	40.40	54.83
Mean	57.60	37.94	147.66
SD	26.90	13.11	89.93

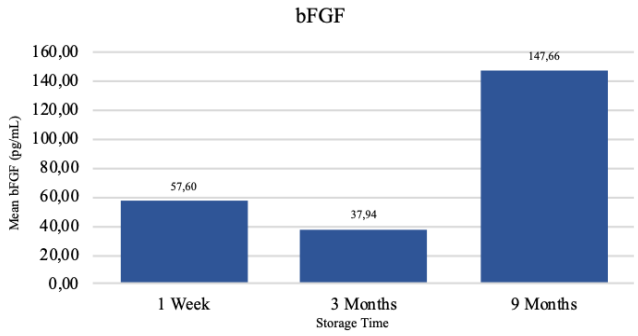

Figure 3. Mean TGF-β value in fresh amnion membrane at 1 week, 3 months and 9 months storage time

DISCUSSION

There was no significant difference in EGF value between the 3 groups. This study however contradicts the study did by Wagner et al 2018 where bFGF, as the chosen representative growth factor among the rest due to its high content in a fresh amnion membrane, was found to be lower significantly. In this study, Wagner compared bFGF in fresh amnion membrane preserved in glycerol medium and straight storage without any medium for the duration of 1 week, 3 months and 6 months. Results show that bFGF was significantly lower (p= 0.006) in all three storage duration groups[6]. Despite the difference in the result, it proves that EGF is still detected in the 9 months storage duration group and hence it can still be used. In TGF-β analysis, it was shown that there was no significant difference between all the storage groups (p=0.462). This is also contradictory with the study conducted by Wagner previously mentioned where bFGF was decreased along with longer storage duration time[6]. Whereas in bFGF, from post hoc test it was shown that there was significant increase between 1 week and 6 months storage against 9 months storage at p=0.028 and 0.010 respectively (p<0.05). There was no significant difference between 1 week and 3 months (p=0.273).

The result is different from what is stated by Wagner where bFGF level decreases along with the longer storage duration in -80 °C. Wagner used glycerol mixed with Dulbecco's Modified Eagle Medium (DMEM) and also straight storage at fresh amnion membrane stored for 1 month, 3 months, and 6 months. This study shows that bFGF is more preserved in glycerol mixed with DMEM medium rather than using straight storage preservation in the 3 months group although there was no significant difference between 1 month and 6 months storage time group. Cell viability at 3 months group were also higher than 1 month and 6 months group, this corresponds to the bFGF level in the same 3 months group where it was higher than the rest of the group[6]. In this study, the temperature used for storage was -20°C whereas in the study conducted by Wagner et al was stored at -80 °C. According to the study by Witt et al (2022), the value of bFGF in human amniotic membrane stored in -28 °C (1063.2 ± 680.3 pg/g; range 369.2–2534.2) was not significantly lower than the -80 °C group[7]. Therefore, it emphasizes that the temperature used for amniotic membrane's storage at -20 °C is as good as storage in -80 °C. There are plenty of factors that can affect the value of growth factors contained in fresh amnion membrane. Inter-donor variability is very high for human amnion membrane as donor's age, gestation period, and anatomy of amnion membrane itself affects the value of growth factors[8]. Other than that, different preparation techniques and preservation methods of the fresh amnion membrane also affects the value of the growth factors[9,10,11]. In this study, growth factor proteins were extracted using PRO-PREP, a detergent agent for extracting proteins. It was claimed by Gicquel *et al.*, (2009) that using detergent agent is better than using Phospathe Buffered Saline (PBS) like the study done by Koizumi et al[11,12]. Pulverizing the amnion membrane without using liquid nitrogen were found to be more difficult as the samples were not able to be broken down into smaller pieces hence lesser growth factors to be extracted. The difference of growth factors between each storage duration group in fresh amnion membrane in this study is negligible. This contradicts with the hypothesis where growth factors were predicted to decrease along with the longer storage duration time. The hypothesis correlates with the study by Kubo *et al.* (2001) where it states that there were a decrease in the value of viable epithelial cells by 50% after cryopreservation in -80°C storage for 2 months in a DMSO medium[13]. The number of epithelial cells correlates with the amount of growth factors it contains. Small sample size and limited availability of fresh human amnion samples are the limitations of this study. Furthermore, more trials with bigger sample size with longer storage duration and using pre and posttest study design are required to minimize the bias from high inter-donor variability the human amniotic membrane.

Conclusion

there was no significant changes in egf, tgf-β, and bfgf levels between fresh human amniotic membrane after 1 week, 3 months, and 9 months of storage. these findings showed the efficacy of biologic characteristic of fresh amnion membrane and support that fresh amnion membrane can be used even after 9 months of storage time.

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REFERENCES

1. Dadkhah Tehrani, F., Firouzeh, A., Shabani, I., & Shabani, A., 2021. A Review on Modifications of Amniotic Membrane for Biomedical Applications. *Frontiers in bioengineering and biotechnology*, *8*, 606982. https://doi.org/10.3389/fbioe.2020.606982
2. Hilmy, N., Yusof, N., & Nather, A., 2018. Anatomy and Histology of Amnion. Human Amniotic Membrane: Basic Science and Clinical Application. Singapore: *World Scientific Publishing Co. Pte. Ltd*; 2018. P.87-99.
3. Suroto, H., Aryawan, D.M., Prakoeswa, C.A., 2021. The influence of the preservation method and gamma irradiation sterilization on TGF- β and bFGF levels in freeze-dried amnion membrane (FD-AM) and amnion sponge. *International Journal of Biomaterials*. vol. 2021, Article ID 6685225, 9 pages, 2021.
4. Ihsan, P., 2009. The difference of epidermal growth factor concentration between fresh and freeze-dried amniotic membranes. *Jurnal Oftalmologi Indonesia*. 5(3): pp. 62–66.

5. Gholipourmalekabadi, M., Farhadihosseinabadi, B., Faraji, M., & Nourani, M. R., 2020. How preparation and preservation procedures affect the properties of amniotic membrane? How safe are the procedures?. *Burns : journal of the International Society for Burn Injuries*, *46*(6), 1254–1271. https://doi.org/10.1016/j.burns.2019.07.005
6. Wagner, M., Walter, P., Salla, S., Johnen, S., Plange, N., Rütten, S., Goecke, T. W., & Fuest, M., 2018. Cryopreservation of amniotic membrane with and without glycerol additive. *Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie*, *256*(6), 1117–1126. https://doi.org/10.1007/s00417-018-3973-1.
7. Witt, J., Grumm, L., Salla, S., Geerling, G., & Menzel-Severing, J., 2022. Cryopreservation in a Standard Freezer: -28 °C as Alternative Storage Temperature for Amniotic Membrane Transplantation. *Journal of clinical medicine*, *11*(4), 1109. https://doi.org/10.3390/jcm110 41109.
8. López-Valladares, M. J., Teresa Rodríguez-Ares, M., Touriño, R., Gude, F., Teresa Silva, M., & Couceiro, J., 2010. Donor age and gestational age influence on growth factor levels in human amniotic membrane. *Acta ophthalmologica*, *88*(6), e211–e216. https://doi.org/10.1111/j.1755-3768.2010.01908.x
9. Thomasen, H., Pauklin, M., Noelle, B., Geerling, G., Vetter, J., Steven, P., Steuhl, K. P., & Meller, D. (2011). The effect of long-term storage on the biological and histological properties of cryopreserved amniotic membrane. *Current eye research*, *36*(3), 247–255. https://doi.org/10.3109/02713683.2010.542267.
10. Litwiniuk, M., Radowicka, M., Krejner, A., & Grzela, T., 2017. The influence of amniotic membrane extracts on cell growth depends on the part of membrane and childbirth mode selected: a proof-of-concept study. *Journal of wound care*, *26*(8), 498–503. https://doi.org/10.12968/jowc.2017.26.8.498.
11. Gicquel, J. J., Dua, H. S., Brodie, A., Mohammed, I., Suleman, H., Lazutina, E., James, D. K., & Hopkinson, A., 2009. Epidermal growth factor variations in amniotic membrane used for ex vivo tissue constructs. *Tissue engineering. Part A*, *15*(8), 1919–1927.. https://doi.org/10.1089/ten.tea.2008.0432.
12. Koizumi, N. J., Inatomi, T. J., Sotozono, C. J., Fullwood, N. J., Quantock, A. J., & Kinoshita, S. (2000). Growth factor mRNA and protein in preserved human amniotic membrane. *Current eye research*, *20*(3), 173–177.
13. Kubo, M., Sonoda, Y., Muramatsu, R., & Usui, M. (2001). Immunogenicity of human amniotic membrane in experimental xenotransplantation. *Investigative ophthalmology & visual science*, *42*(7), 1539–1546.

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