The Effect Of Fresh Human Amniotic Membrane Storage Time On Growth Factor Levels (EGF, TGF-β, bFGF) in Freeze-Dried Human Amniotic Membranes

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Abstract

Background: *Each preservation method can cause different levels of damage to tissue components and can affect the regenerative performance of the amniotic membrane. The concentrations of most of the cytokines were found to decrease during the preservation period. There are still no studies regarding the effect of fresh human amnion storage time on growth factor levels in freeze dried human amnion products.*
Method: *This study used an experimental post test group design.*
Result: *There was no significant difference in EGF levels of freeze-dried human amniotic membrane products resulting from 3 types of fresh human amniotic membrane storage (1 week storage with 8.49 ± 5.16 pg/mL, 3 months with 4.55 ± 1.98 pg/mL, and 9 months with levels of 4.04 ± 2.12 pg/mL). There was no significant difference in bFGF values between freeze-dried human amniotic membrane products resulting from 3 types of storage duration of fresh human amniotic membrane (1 week storage with levels of 97.86 ± 113.98 pg/mL, 3 months with levels of 109.69 ± 113.39 pg/mL, and 9 months with levels of 117.53 ± 73.46 pg/mL). There was a significant difference in the levels of TGF-β, namely in the product from storage for 1 week (97.93 ± 18.27 pg/mL) and 3 months (55.94 ± 28.67 pg/mL). Whereas TGF-β levels in freeze dried human amniotic membrane from the results of fresh human amniotic membrane storage at 1 week and 9 months, and 3 months and 9 months were found to have no significant difference.*
Conclusion: *There was no significant difference in EGF and bFGF levels in this study. There was a significant difference in the levels of TGF-β freeze-dried human amniotic membrane produced from fresh human amniotic membrane with higher growth factor levels at 1 week of storage compared to 3 months.*

Keywords : bFGF; EGF; TGF-β; freeze dried human amnion; storage time; preserve

1. Introduction

Human amniotic membranes have long been employed in plastic surgery for a range of therapeutic uses. Human amniotic membrane has been routinely used in numerous locations across the world to treat superficial and partial burns[1]. The amnion can also be utilized to treat chronic ulcers and pressure sores as a biologic dressing[2]. Human amniotic membranes can also be used to effectively treat donor skin graft regions. Fresh amniotic membranes have clinical, biological, and logistical constraints, including the fact that they are not durable, inefficient, and need time for serological testing since the evaluation must be repeated 6 months later for a potential window. The healing impact of fresh amniotic membranes is anticipated to be larger than that of preserved amniotic membranes because the preservation procedure might diminish active cells, particularly growth factors[3,4,5].

The Dr.Soetomo Hospital Tissue Banks preserve and handle all amnion biological materials collected from patients. Tissue Banks strictly monitor all storage and manufacturing procedures. There were multiple occasions when inventories of amnion biological material collected from patients gathered as a result of waiting for the order of the production process, necessitating long-term storage.

After dehydration, growth factor levels in the amniotic membrane were considerably lower (compared to fresh tissue). Previous research comparing dehydrated amnion to fresh or cryopreserved amnion discovered substantial differences in membrane structure[6,7]. Currently, no study has been conducted to determine the influence of fresh human amniotic membrane storage period on growth factor levels in freeze dried human amniotic membrane products. Fresh human amnion that has been preserved for several years was used to make this product. I hope this research can provide scientific evidence support to compile a standard operating procedure for the collection and storage of fresh amnion donors which will be processed into freeze-dried human amnions.

2. Method

This research is an in vitro experimental study with a post test only group design to determine the levels of growth factors (EGF, bFGF, TGF-β) in freeze dried human amniotic membranes originating from fresh human amniotic membranes that have been stored for several periods of time. The storage time range for fresh human amniotic membrane was selected in the range of 1 week, 3 months, and 9 months.

The sampling technique used in this study was consecutive sampling in one batch of amnion donors at a predetermined storage time, which consisted of three patients who met the selection criteria. The research subjects were 18 pieces of freeze-dried human amniotic membrane obtained from the Tissue Banks of Dr. Soetomo Hospital which comes from fresh human amniotic membrane which has been stored for 1 week, 3 months and 9 months. Each specimen with a size of 6x6 cm was collected and mashed in pulverized form then PRO-PREP enzyme (iNtRON Biotechnology, Burlington, MA) was added for growth factor extraction from the amniotic membrane. After extraction of the protein from the amnion membrane, EGF and bFGF levels were examined using the Human ELISA Kit specific for GF from Elabscience China and TGF-β examination using the ELISA Kit from BTLab. The obtained data were analyzed using ANOVA. Because the sample is <20, the normal distribution test uses the Shapiro-Wilks.

3. Result

This research was conducted experimentally using a posttest group design. We compared growth factor from each storage time of fresh human amniotic membrane. We expected a decrease in the growth factor levels gradualy from the shortest to the longest storage time. The results of measuring EGF levels in freeze-dried human amniotic membrane can be seen in Table 1.

Table 1. Freeze-dried human amniotic membrane EGF levels

Sample EGF	Kadar EGF (pg/mL)		
	1 Minggu	**3 Bulan**	**9 Bulan**
1	4,88	1,66	2,36
2	10,90	4,09	7,24
3	3,98	3,35	1,50
4	2,94	4,77	4,65
5	13,86	6,85	3,14
6	14,35	6,59	5,37
Rerata	8,49	4,55	4,04
SD	5,16	1,98	2,12
Nilai Max	14,35	6,85	7,24
Nilai Min	2,94	1,66	1,50

Based on the normality test using Shapiro Wilk, the significance value of EGF storage for 1 week, 3 months, and 9 months was all > 0.05, so it can be concluded that the EGF levels for each storage were normally distributed. For the next test of comparison of 3 storages using the Anova test. Based on the ANOVA test, a significance value of 0.078 was obtained where the value was > 0.05, so it can be concluded that there was no significant difference in EGF levels between 3 storages (1 week, 3 months and 9 months) which can be seen in figure 1.

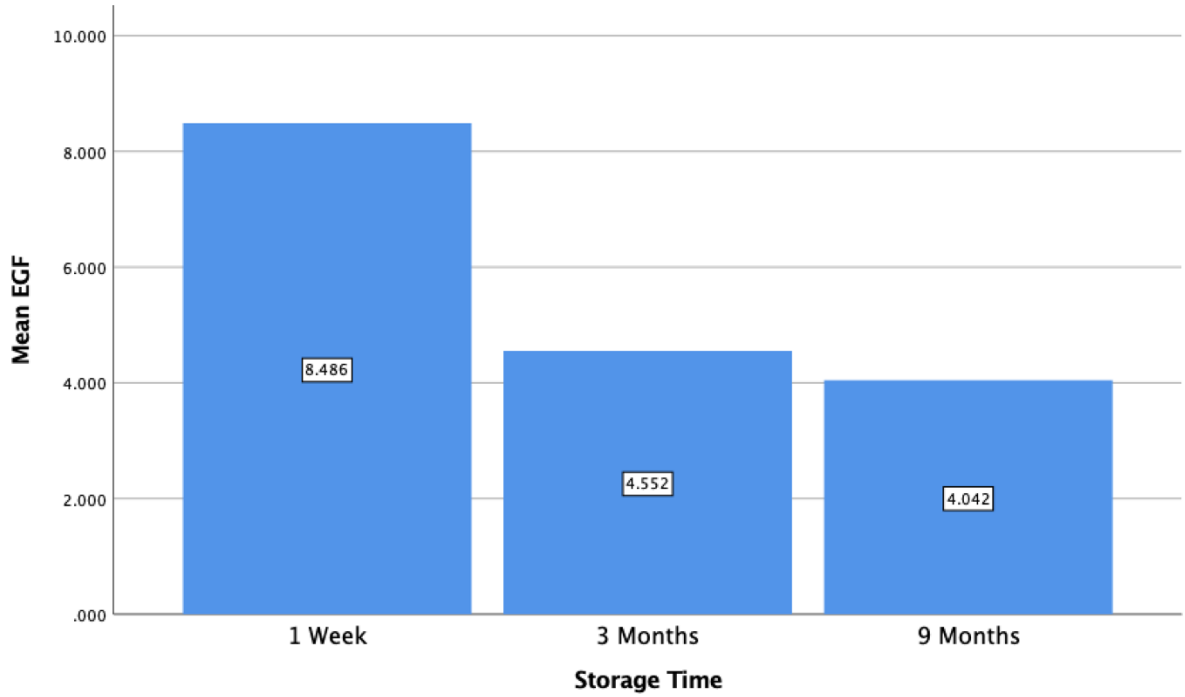

Fig 1. Graph of average EGF on freeze-dried human amniotic membrane processed from fresh amniotic membrane at 1 week, 3 months, and 9 months of storage.

The results of measuring bFGF levels in freeze-dried human amniotic membrane can be seen in Table 2.

Table 2 Kadar bFGF *freeze-dried human amniotic membrane*

Sample bFGF	**Kadar bFGF membran amnion (pg/mL)**		
	1 Minggu	**3 Bulan**	**9 Bulan**
1	232,23	52,68	211,99
2	256,89	48,96	212,34
3	29,70	21,39	71,64
4	26,89	24,73	77,25
5	24,37	257,28	62,10
6	17,10	253,06	69,87
Rerata	97,86	109,69	117,53
SD	113,98	113,39	73,46
Nilai Max	256,89	257,28	212,34
Nilai Min	17,10	21,39	62,10

Based on the normality test using Shapiro Wilk, the significance value of bFGF for storage for 1 week, 3 months, and 9 months was all <0.05, so it can be concluded that the bFGF value for each storage was not normally distributed. For the next test of comparison of 3 storages using the Kruskal Wallis test. Based on the Kruskal Wallis test, a significance value of 0.484 was obtained where the value was > 0.05. It can be concluded that there was no significant difference between bFGF levels in freeze-dried human amniotic membrane products produced from 3 types of length of storage of fresh human amniotic membranes which can be seen in Figure 2.

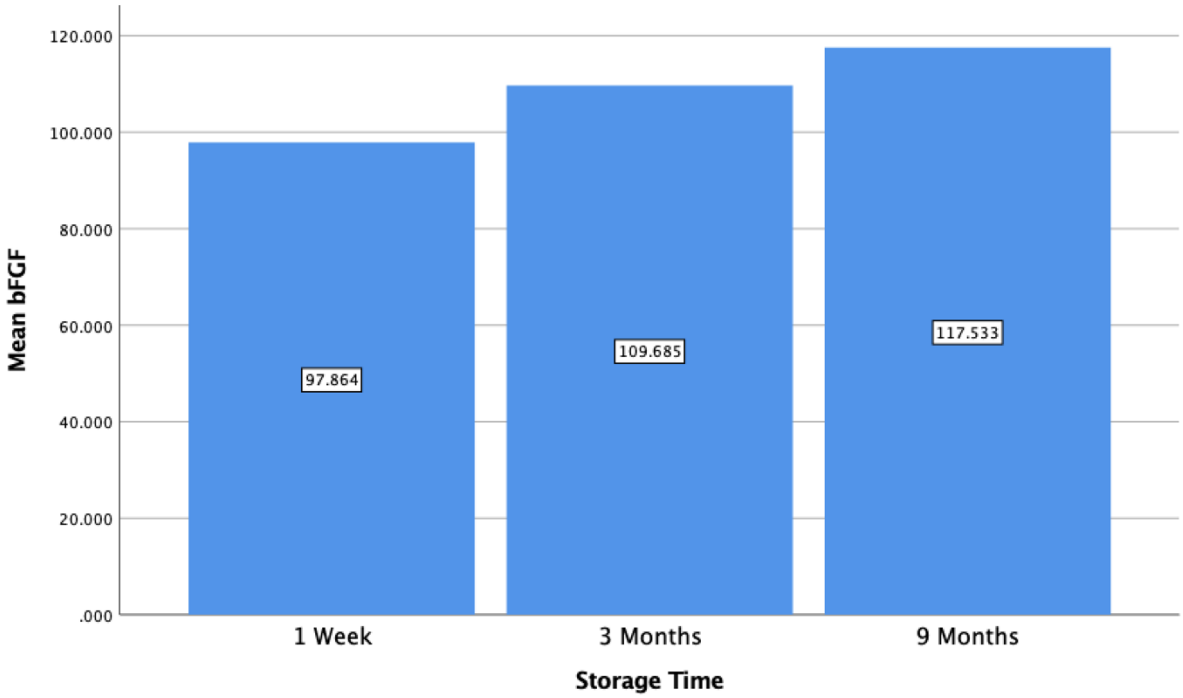

Fig 2. Graph of average bFGF in freeze-dried amnion membranes processed from fresh amniotic membranes at 1 week, 3 months, and 9 months of storage.

The results of measuring TGF-β levels in freeze-dried human amniotic membrane can be seen in Table 3.

Table 3. Freeze-dried human amniotic membrane TGF-β levels

Sample TGF-β	Kadar TGF-β membran amnion (pg/mL)		
	1 Minggu	3 Bulan	9 Bulan
1	128,84	54,77	80,40
2	100,60	37,00	71,81
3	80,40	26,85	66,11
4	91,92	49,13	71,81
5	105,44	58,54	76,58
6	80,40	109,33	89,99
Rerata	97,93	55,94	76,12
SD	18,27	28,67	8,35
Nilai Max	128,84	109,32	89,99
Nilai Min	80,40	26,85	66,11

Based on the normality test using Shapiro Wilk, it was found that the significance value of TGF-β at 1 week, 3 months, and 9 months of storage were all > 0.05, so it can be concluded that the TGF-β levels for each storage were normally distributed. For the next test of comparison of 3 storages using the Anova test. In the ANOVA test, a significance value of 0.009 was obtained where the value was <0.05, so it can be concluded that there was a significant difference in TGF-β levels between the 3 storages.

To see the comparison test for each storage, a post hoct test (LSD) was carried out. Based on the results of the post hoct test, it was found that there was a significant difference in TGF-β levels, namely in products from 1 week storage (97.93 ± 18.27 pg/mL) and 3 months (55.94 ± 28.67 pg/mL) with a significance value of 0.003 (significance value <0.05), while the product from fresh human amniotic membrane storage at 1 week to 9 months, and 3 months to 9 months did not have a significant difference which can be seen in Figure 3.

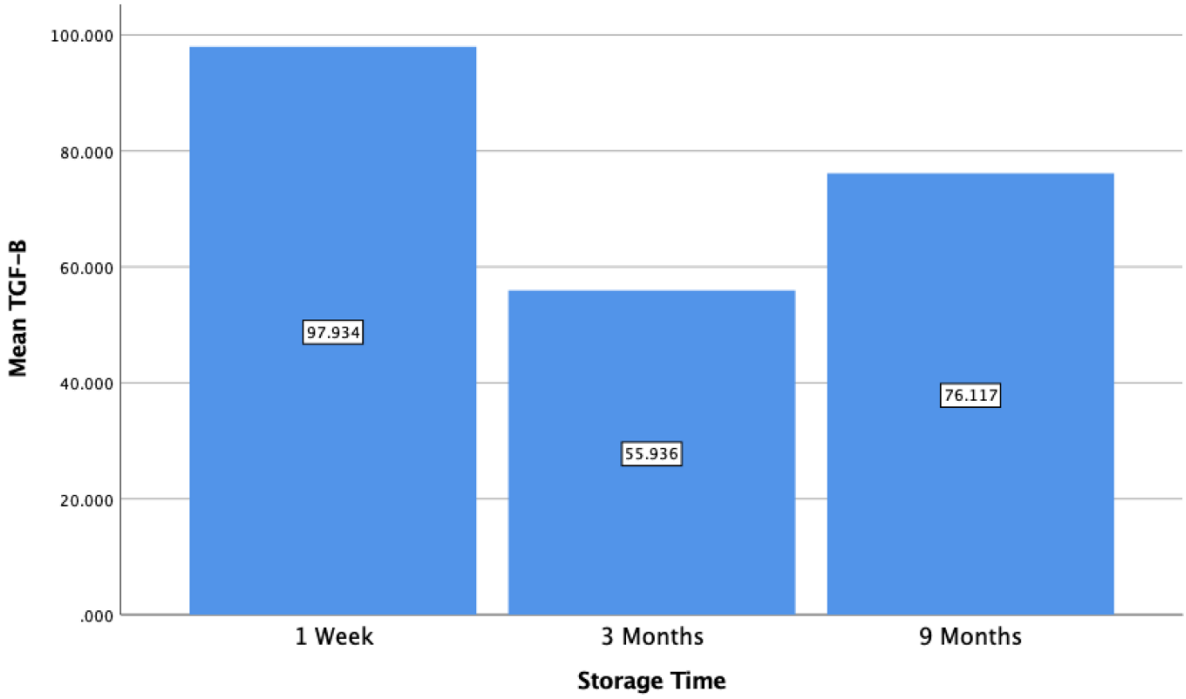

Fig 3. Graph of average TGF-β on freeze-dried amniotic membranes processed from fresh amniotic membranes at 1 week, 3 months, and 9 months of storage.

4. Discussion

The EGF examination with the ANOVA test obtained a significance value of 0.078 where the value was > 0.05, so it can be concluded that there was no significant difference in the EGF levels of freeze-dried human amniotic membrane products resulting from 3 types of length of storage of fresh human amniotic membrane (1 week storage with levels of 8.49 ± 5.16 pg/mL, 3 months with levels of 4.55 ± 1.98 pg/mL, and 9 months with levels of 4.04 ± 2.12 pg/mL). This study is in accordance with research conducted by Pereira et al[8] where there was no significant difference in EGF levels on days 1, 7, 60, and 180. This study also addressed the low levels of EGF and the lack of major alterations that may occur as a result of the mechanical manipulation performed to prepare the amniotic membrane, which removed most of the EGF and reduced its concentration[9].

In the bFGF examination it was concluded that there was no significant difference in bFGF values between freeze-dried human amniotic membrane products produced from 3 types of long storage fresh human amniotic membrane (1 week storage with levels of 97.86 ± 113.98 pg/mL, 3 months with levels of 109.69 ± 113.39 pg/mL, and 9 months with levels of 117.53 ± 73.46 pg/mL). The results of EGF and bFGF levels in the studies that have been conducted are the same as those conducted by Pereira et al[8] where researchers evaluated cytokine concentrations (eg, EGF, bFGF, HGF, KGF, TGF-β, IL-4, and IL- 10) by ELISA assay on fresh and cryopreserved amniotic membranes and reported a temperature of −80°C as optimal for amniotic membrane storage to maintain the concentrations of the cytokines tested. Another study found that the average concentration of bFGF contained in hAM that had been stored for 8.2 ± 2 months (range 7–12 months) at −28 ◦C (1063.2 ± 680.3 pg/g; range 369.2–2534.2) did not significantly lower than at −80 °C (1312.1 ± 778.2 pg/g; range 496.2–2442.7; p = 0.11)[9]. Different results were shown by Wagner (2018) who in his research results showed a significant decrease in bFGF levels in the process of storing fresh amnion at −80 °C for 1 month to 3 months, and 1 month to 6 months[10].

On examination of TGF- β levels it was concluded that there was a significant difference between the 3 storages (1 week, 3 months and 9 months). Next, a post hoct test (LSD) test was carried out which found that there was a significant difference in TGF- β levels, namely in products from 1 week of storage (97.93 ± 18.27 pg/mL) and 3 months (55.94 ± 28 .67 pg/mL). The decrease in growth factor levels was also mentioned in Pereira's study[8] that the concentrations of most of the cytokines were found to decrease during the preservation period, although there were some samples that had a slight increase in concentrations on the first day; for example, TGF-β is preserved in media to which TC199 is added at 0°C. This might be due to TGF-β release from the cell membrane where the cytokine is bound, or it could be owing to cell death during the preservation process[8]. The high standard deviation values obtained in this research may be due to the use of amnion from several different

donors at each storage period, resulting in considerable interdonor variability in cytokine concentrations. This is consistent with studies conducted by Gicquel et al[11] and Hopkinson et al[12] who found that the concentrations of EGF and TGF- β1 varied substantially between different placental segments[11,12]. Whereas TGF- β levels in freeze dried human amniotic membrane from the results of fresh human amniotic membrane storage at 1 week and 9 months, and 3 months and 9 months were found to have no significant difference.

One of the study's weaknesses is the minimal number of donors that can be analyzed. Nonetheless, according to multiple research, the sample size is adequate to indicate an association between growth factor levels in freeze dried human amniotic membranes and the length of time fresh human amniotic membranes have been stored/preserved[8,11].

All of the growth factors analyzed in this study have a relationship to the clinical usage of the amniotic membrane, especially accelerating epithelialization, enhancing angiogenesis, and lowering the risk of hypertrophic scars or keloids[4,13,14,15]. As a result, variability in growth factor levels in the amniotic membrane caused by donor variances may result in varying clinical efficacy.

5. Conclusion

There was no significant change in EGF and bFGF levels between freeze-dried human amniotic membrane and fresh human amniotic membrane after 1 week, 3 months, and 9 months of storage. There is a substantial difference in TGF- freeze-dried human amniotic membrane levels between fresh human amniotic membrane and freeze-dried human amniotic membrane, with greater growth factor levels after 1 week of preservation compared to 3 months. TGF- levels in goods stored for 1 week to 9 months and 3 months to 9 months did not change significantly.

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