DIFFERENCES OF EPIDERMAL GROWTH FACTOR (EGF) CONCENTRATION BETWEEN UNPRESERVED AND PRESERVED AMNIOTIC MEMBRANE
R. Gunawan Effendi1, Gatut Suhendro1, Indro Handoko2
1Department of Ophthalmology, Faculty of Medicine, Airlangga University
2Department of Clinical Pathology, Faculty of Medicine, Airlangga University

ABSTRACT
Previous studies have provided good evidence in supporting the strong opinion that amniotic membrane is good for medical treatment. This is due to growth factors contained in the above mentioned amniotic membrane. Amniotic membrane can be used in the form of none preserved as well as preserved. Preservation processes including cryopreservation, however could reduce the viability of cells as well as the concentration of growth factors. This decrease will influence the function of amniotic membranes. The objective of this study is to measure the difference of EGF concentration in between fresh amniotic membranes and with preservation (cryopreservation) in 16 amniotic membranes. We divided each amniotic membrane into two parts. The first part was extracted in fresh forms and the second part underwent preservation (cryopreservation) with glycerol 50% and was stored at - 80° C during 1 month before extraction. Both parts of the membrane were extracted using ultrasonic disintegrator and concentration of EGF was measured from the obtained extract using ELISA method. Results showed that the average concentration of EGF in the fresh amniotic membrane was 122.76 ± 11.59 pg/g while the average concentration of EGF in the amniotic membrane underwent preservation (cryopreservation) was 99.34 ± 9.49 pg/g. Average degradation of EGF concentration due to preservation (cryopreservation) is 18.49% ± 10.20%. So, we conclude that EGF concentration in fresh amniotic membrane is significantly higher than the EGF concentration in amniotic membrane underwent preservation (cryopreservation) (p = 0.000). Degradation of EGF concentration due to preservation (cryopreservation) at 95% confidence interval is 12.33% to 24.66%.

Keywords: Epidermal growth factor, amniotic membrane, preservation, cryopreservation, ultrasonic disintegrator, ELISA.

Correspondence: R. Gunawan Effendi, Department of Ophthalmology, Faculty of Medicine, Airlangga University

INTRODUCTION
Amniotic membrane is the innermost layer of the placenta, consists of three layers, one layer of epithelial layer, a thick basal membrane containing collagen type IV, V, VII, fibronectin and laminin, and the stroma that contains no blood vessels. In addition amniotic membranes also contain a variety of growth factors, epidermal growth factor like (EGF), keratinocyte growth factor (kgf), hepatocyte growth factor (HGF), fibroblast growth factor (FGF), transforming growth factor-α (TGF-α), and transforming growth factor-β (TGF-β). (Dua & Azuara-Blanco 1999, Letko et al. 2001, Sippel et al. 2001, Gray & Tseng 2002, Two et al. 2004, Souza et al. 2004, Fernandes et al. 2005).

Human amniotic membrane has been widely used in medicine, among others: corneal ulcer, bullous keratopathy, conjunctival surface reconstruction, persistent epithelial defect, and pterygium surgery. This is because the amniotic membrane is able to facilitate the adhesion and migration of basal epithelial cells, preventing apoptosis, repair of epithelial phenotype, stimulating the epithelial process, reducing inflammation, reducing angiogenesis and scar process and have antimicrobial effects (Dua & Azuara-Blanco 1999, Sippel et al. 2001, Gray & Tseng 2002, John et al. 2003).

Amniotic membrane has also been used in the field of eye medicine, among others: corneal ulcer, bullous keratopathy, conjunctival surface reconstruction, persistent epithelial defect, and pterygium surgery. This is because the amniotic membrane is able to facilitate the adhesion and migration of basal epithelial cells, preventing apoptosis, repair of epithelial phenotype, stimulating the epithelial process, reducing inflammation, reducing angiogenesis and scar process and have antimicrobial effects (Dua & Azuara-Blanco 1999, Sippel et al. 2001, Gray & Tseng 2002, John et al. 2003).

Use of amniotic membrane was first introduced by Davis in 1910 and Sabella in 1913 in the case of skin transplantation. In the area of the eye, was first introduced in 1940 by de Rötth, and then the fetal membranes (chorion and amnion) are used to repair conjunctiva defects in patients simblefaron. In 1946,

Amniotic membranes obtained from the placenta from the birth process with patients serology negative for hepatitis B and C, syphilis and HIV. The amniotic membrane can be used with or without preservation. One method of preservation membranes amnion is cryopreservation, in which the lining of the amnion was washed with antibiotic and stored in a solution of glycerol or dimethyl sulfoxide (DMSO), at a temperature of - 80 ºC (Prabhasawat et al. 2000, Tseng 2001, Gray & Tseng 2002, Souza et al. 2004).

Clinically, amniotic membrane without preservation has been widely used in the field of eye medicine. The presence of active cells in sufficient numbers, including growth factors and cytokines, has an important role. Amniotic membrane without preservation is used for the reconstruction of the surface of the eye. No inflammatory reactions, immunology and infection are found. In general, it is easy to use and safe technique (Gündüz et al. 2006, Mejia et al. 2000).

The emphasis of using amniotic membrane with cryopreservation is the function of the matrix. This is a result of the decrease of active cells in the amniotic membrane (Kruse et al. 2000, Prabhasawat et al. 2000). Department of Ophthalmology at dr. Soetomo Hospital been using amniotic membrane without preservation in some cases associated with abnormalities on the surface of the eye.

In the year 2007 there are 13 cases. Cryopreservation is defined as the maintenance of biological material at a temperature of -80 °C or below. Stage one starts from the stage cryopreservation hipotermia, glassy phase, and vice versa to melt back through the thawing process (Strong 2000, Baut 2007, Pegg 2007). The biological effects of this cooling caused by the freezing of the liquid cell.

The theory of freezing injury illustrates that cell damage can be caused by the formation of ice crystals in cells that will cause direct mechanical action, or the result of changes in concentration and composition of the fluids so that cells can cause interference with the process of cell diffusion and osmosis -cells (Pegg 1997, Meryman 1998, Strong 2000, Rama et al. 2001, Baut 2007, Pegg 2007, Taylor 2007).

Cryopreservation process can reduce the active cells, including several growth factors around 50%. This would cause a reduction in the effect of amniotic membrane on wound healing process (Dua & Azuara-Blanco 1999, Two et al. 2004, Gündüz et al. 2006, Hennerbichler et al. 2007). In the amniotic membrane without preservation, cells and growth factors are still more effective compared with amniotic membrane with preservation (cryopreservation) (Gündüz et al. 2006).

Based on the description above, then the identification problem is how far the effect of changes in cells that are activated as a result of the process of preservation with cryopreservation methods in conjunction with interests in medicine, particularly in the field of eye health on several factors that affect wound healing. Based on that basis, it needs an ongoing series of studies to investigate factors that affect wound healing growth. This study is one of the circuit, which would investigate the epidermal growth factor (EGF) associated with wound healing process which can be more beneficial in its use.

**MATERIALS AND METHODS**

This research is experimental research design with one-group pretest - posttest design. Experimental unit of this study is that amniotic membrane in the Biomaterials Center / Tissue Bank at dr. Soetomo Hospital, with the acceptance criteria, which are processed without amniotic membrane preservation and the preservation (cryopreservation) according to standard procedures at the Center for Biomaterials / Tissue Bank dr. Soetomo Hospital and criteria for rejection that is, without a preserved amniotic membranes for more than 24 hours.

This research conducted in two places, first in Biomaterials Center/ Tissue Bank at dr. Soetomo Hospital for taking samples, second place is in clinical Pathology Department, Faculty of Medicine, Airlangga University/dr. Soetomo Hospital for EGF examination. The study was conducted from December 2007 until August 2008.

**RESULTS**

This research is laboratory research to know the difference between the EGF levels of amniotic membrane without amniotic membrane with the preservation and preservation (cryopreservation). This research was performed on amniotic membrane 16 that have met acceptance criteria. Research conducted at the
Center for Biomaterials/ Bank Network dr. Soetomo and Section of Clinical Pathology, Airlangga University School of Medicine/ dr. Soetomo. Each of the subjects were divided into two parts, namely: amniotic membrane without preservation and other parts of amniotic membrane preservation process is carried out with a cryopreservation method in 50% glycerol solution at a temperature of -80 ° during the first month.

Individual wet weight of study subjects were weighed and measured its volume, and then the extraction process is carried out by amniotic membrane is cut into pieces as small as possible and crushed with a mortar until crushed, then destroyed again by an ultrasonic disintegrator to obtain extracts of amniotic membranes. Extracts obtained, is then performed with ELISA kits EGF EGF (RayBiotech). Three subjects were not included in the study because during the extraction process without the preservation of amniotic membrane to decrease the volume of spill resulting from the extract solution.

**EGF Concentration**

It can be seen in Table 1 EGF levels of amniotic membrane without amniotic membrane with the preservation and preservation (cryopreservation). In the amniotic membrane without a preservation group found that mean = 122.76 pg / g with SD = 11.59 pg / g. The highest levels were 143.80 pg / g and the lowest levels were 108.95 pg / g. In the group with amniotic membrane preservation (cryopreservation) found that mean = 99.34 pg / g with SD = 9.49 pg / g. The highest levels were 115.26 pg / g and the lowest levels were 81.42 pg / g.

**Normality Test**

EGF concentration data from both treatment groups: group without preservation of amniotic membrane and amniotic membrane groups with preservation (cryopreservation) test for normality with Kolmogorov-Smirnov test. Results of normality test on amniotic membrane group without preservation is p = .879. In the group with amniotic membrane preservation (cryopreservation) got p = .470. Based on the results of normality test, the two treatment groups had a normal distribution (p> 0.05).

Table 1 Concentration amniotic membrane EGF

<table>
<thead>
<tr>
<th>Replication</th>
<th>EGF Concentration (pg/g)</th>
<th>EGF Concentration decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Without preservation</td>
<td>Preservation (Cryopreservation)</td>
</tr>
<tr>
<td>1</td>
<td>111.41</td>
<td>102.71</td>
</tr>
<tr>
<td>2</td>
<td>111.61</td>
<td>100.21</td>
</tr>
<tr>
<td>3</td>
<td>113.72</td>
<td>101.23</td>
</tr>
<tr>
<td>4</td>
<td>112.84</td>
<td>108.23</td>
</tr>
<tr>
<td>5</td>
<td>112.42</td>
<td>96.28</td>
</tr>
<tr>
<td>6</td>
<td>124.24</td>
<td>115.26</td>
</tr>
<tr>
<td>7</td>
<td>129.64</td>
<td>92.68</td>
</tr>
<tr>
<td>8</td>
<td>137.69</td>
<td>113.29</td>
</tr>
<tr>
<td>9</td>
<td>128.32</td>
<td>102.29</td>
</tr>
<tr>
<td>10</td>
<td>133.13</td>
<td>89.88</td>
</tr>
<tr>
<td>11</td>
<td>108.95</td>
<td>81.42</td>
</tr>
<tr>
<td>12</td>
<td>128.07</td>
<td>92.10</td>
</tr>
<tr>
<td>13</td>
<td>143.80</td>
<td>95.85</td>
</tr>
</tbody>
</table>

Mean: 122.76, SD: 11.59, CI (95%): 11.59 - 23.42, %: 10.20

Max: 143.80, Min: 108.95, Lower limit: 12.33, Upper limit: 24.66
Paired two sample t test

Both treatment groups had a normal distribution, so the test will be the difference with two sample paired t test. The result of two sample paired t test showed that the levels of amniotic membrane without EGF and with preservation preservation (cryopreservation) has a significant difference ($p = 0.000$). EGF levels of amniotic membrane without preservation is significantly higher ($122.76 \pm 11.59$ pg/g) compared with amniotic membrane preservation (cryopreservation) ($99.34 \pm 9.49$ pg / g).

Estimated percentage decrease in levels of EGF Can be seen in table 1 the percentage decrease in levels of EGF result from the process of preservation (cryopreservation). The mean decline was 18.49% with SD = 10.20%. The highest level decrease was 33.35% and the lowest level decrease is 4.09%. Estimated percentage decrease in levels of EGF result from the process of preservation (cryopreservation) with a confidence interval (CI) 95% is between 12.33% to 24.66%.

<table>
<thead>
<tr>
<th>Intervention</th>
<th>Mean (pg/g)</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
<th>Pair t Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without preservation</td>
<td>122.76</td>
<td>11.59</td>
<td>108.95</td>
<td>143.80</td>
<td>t = 5.901</td>
</tr>
<tr>
<td>Preservation (Cryopreservation)</td>
<td>99.34</td>
<td>9.49</td>
<td>81.42</td>
<td>115.26</td>
<td>p = 0.000*</td>
</tr>
</tbody>
</table>

Note: * Significance at $\alpha = 0.05$

DISCUSSION

Reliability amniotic membrane peptide extraction method

Epidermal Growth Factor (EGF), like other growth factor is a polypeptide molecule were also found in amniotic membranes. Amniotic membrane is a solid material, so that for the ELISA test can be performed, the amniotic membrane requires the extraction process first. Amniotic membranes were used as samples dried on paper dryer, then weighed and measured its volume weight.

Peptide extraction performed by first destroy and squash the amniotic membrane with penghacur tool. This method is a modification of the method by Zhang et al. (2001), et al Baharvand. (2007) and Munadziroh (2007) for protein in amniotic membranes. Modifications made by chopping amniotic membrane as small as possible and after crushed, added to PBS solution containing EDTA, then crushed with an ultrasonic disintegrator.

To obtain the desired peptide samples that had been destroyed then performed ultracentrifugation and the supernatants were taken. Peptides contained in the supernatant were then precipitated by the addition of PEG would result in peptide molecules tend to interact with each other, until the peptide precipitated. Precipitate that happens again dissolved in PBS to the

volume is equal to the volume of tissue prior to this process. Based on the above, then the reliability of peptide extraction as performed in this study need not doubt.

The relevance of growth factor tested in this study

Some research states that EGF has a strong activity in supporting the process mitogenik in various forms of cells in vivo and in vitro. EGF will bind EGF-R, and that bond will activate the enzyme tyrosine kinase, which is a protein phosphorylase, will then induce the phosphorylation cascade reaction, cascade reaction, and eventually brought the final signal to the nucleus to stimulate DNA synthesis and to activate cell proliferation, differentiation and migration. In the field of eye health, EGF plays a role in the reconstruction process of the conjunctiva and cornea. Addition of EGF in the management of the conjunctiva and cornea defect will support the process of healing the defect. Election growth factor tested is based on the strong role of EGF on epithelial proliferation process, as explained above.

Reliability of ELISA used in this study

To determine a peptide, the tests were performed using the ELISA test. The results of amniotic membrane extraction has been done is composed of various peptides including EGF. To determine one of the peptides contained in extracts of amniotic membranes,
we need a test that is sensitive and specific ELISA to the peptide to be tested, in this case is EGF. Based on these reasons, this study used the EGF ELISA with double antibody sandwich method streptavidine biotin test. Use of human EGF ELISA with double antibody sandwich method streptavidine biotin test, with reason: an ELISA with two antibodies and the use streptavidine-biotin in enzyme labeling, so that sensitivity can be relied upon, and an ELISA test with monoclonal antibodies against human EGF, so that specificity can be relied upon.

The relevance of the samples tested in this study
Amniotic membrane has been used in the field of Health Sciences Eye, and hopefully with the active ingredients and the growth factor in it will help the healing process. In the activity above is utilized and the active ingredients of growth factor contained in amniotic membrane. Based on the above reasons, hence in this study used as the sample is amniotic membrane, so that the results obtained from the growth factor (EGF in this case) is right there on the amniotic membrane and not affected by EGF contained in any other organ.

Research Results

This research using amniotic membrane 13 EGFnya measured levels of amniotic membrane group without preservation and then carried out cryopreservation. EGF content of amniotic membrane without preservation is 122.76 ± 11.59 pg / g. The highest levels of EGF were 143.80 pg / g and the lowest EGF concentration was 108.95 pg / g. This result is different from the results of a study by Koizumi et al. (2000) which was obtained by 1.0 TB EGFnya levels 0.6 pg / mg and the study conducted by Gomes (2005), was obtained for 12 EGF concentration 3 pg / ml. These different results may be due to different treatment of samples during the study. Nevertheless there are similarities to the trend of decreasing levels of EGF in amniotic membranes with preservation (cryopreservation) of this.

Concentration without preserved amniotic membrane EGF

EGF levels without preserved amniotic membranes in this study was obtained from amniotic membranes before 24 hours after childbirth, after cleaning with subsequent antibiotic solution was extracted and measured by ELISA. Average concentrations of EGF on amnion membrane without preservation is TB 122.76 ± 11.59 pg / g. The highest levels of EGF were 143.80 pg / g and the lowest EGF concentration was 108.95 pg / g. This result is different from the results of a study by Koizumi et al. (2000) which was obtained by 1.0 TB EGFnya levels 0.6 pg / ng and the study conducted by Gomes (2005), was obtained for 12 TB EGF concentration 3 pg / ml.

Differences in EGF levels of amniotic membrane without the preservation of other research that can be caused by possible differences during the process of extraction and ELISA are used. EGF levels in this study employs a unit of pg / g tissue. This is due to the material used is the amniotic membrane which is a solid object, so it is more appropriate to use units of pg / g tissue.

EGF Concentration amniotic membrane with preservation (cryopreservation)

EGF levels in amniotic membrane preservation (cryopreservation) in this study was obtained from the amniotic membrane is washed with antibiotic solution and then soaked in 50% glycerol solution and stored in a temperature-80TA C for one month, then extracted and measured by ELISA. The mean levels of EGF in amniotic membrane preservation (cryopreservation) is TB 99.34 ± 9.49 pg / g.

The highest levels of EGF were 115.26 pg / g and the lowest EGF concentration was 81.42 pg / g. This result is different from the results of research conducted by Gomes which obtained for seven levels EGFnya TB 8 pg / ml. These different results may be due to different treatment of samples during the study. Nevertheless there are similarities to the trend of decreasing levels of EGF in amniotic membranes with preservation (cryopreservation) of this.

Will generally decrease cell viability and growth factor levels as a result of cooling. Freeze injury that occurred due to cooling will damage cells and growth factors including EGF so will cause a decrease in the levels of EGF. This phenomenon is also found in studies conducted by previous researchers, such as Burgos and Faulk (1981), Kruse et al. (2000) and Gomes (2005).

Differences EGF levels of amniotic membrane without preservation and the preservation (cryopreservation)

All data obtained from measurements in both groups were tested for normality prior data with Kolmogorov-Smirnov test. Results of normality test showed that both treatment groups had a normal distribution (p> 0.05). This study aims to determine differences in levels of EGF ELISA between amniotic membrane without amniotic membrane with the preservation and preservation (cryopreservation).

Table 2. shows the results of two sample paired t test which showed a significant, meaning there is a difference between the levels of EGF without preservation of amniotic membrane with amniotic membrane with preservation (cryopreservation) (p = 0.000). Test results also indicate that the amniotic membrane without EGF levels significantly higher preservation (TB 122.76 ± 11.59 pg / g) compared with
EGF levels in amniotic membrane preservation (cryopreservation) (99.34 ± 9.49 pg/g).

Lower levels of EGF in amniotic membranes with preservation (cryopreservation) is caused by the occurrence of freezing injury on the amniotic membrane. Freezing injury will provide the biological effects on cells due to freezing of the liquid cell. Damage to cells lining the amnion with preservation (cryopreservation) is caused by freezing injury can occur through direct mechanical action resulting from ice crystal formation in these cells, or the result of changes in concentration and composition of the liquid cell, that could cause disturbance in the diffusion process and the osmosis of those cells.

The changes resulting from cooling can cause the damage of these cells which in turn can lead to death of these cells. Another consequence will occur stress proteins to changes in temperature, this will cause denaturation of proteins caused by cold temperatures, including EGF. Freezing injury can be reduced by the addition of cryoprotectant materials such as glycerol, which reduces the formation of ice crystals and make the balance of osmotic fluid inside and outside the cell due to cooling (Pegg 1997, Meryman 1998, Strong 2000, Rama et al. 2001, Baust 2007, Pegg 2007, Taylor 2007).

The results are consistent with the theory of cryopreservation on top of and in accordance also with the results of some earlier research, as practiced by Burgos and Faulk (1981), Kruse et al. (2000) that suggest there is a decrease of cell viability around 50%. EGF levels of amniotic membrane without preservation is higher than the levels of EGF in amniotic membrane preservation (cryopreservation), as shown in this study can be taken up by the use of amniotic membrane for treatment of several diseases in the field of Eye Health Sciences, will be more perfect even if it is done with clinical research.

Decrease in levels of EGF

The preceding discussion has reviewed about the differences in levels of EGF amniotic membrane without preservation and preservation (cryopreservation). Statistical analysis showed a significant difference between EGF levels of amniotic membrane without preservation and preservation (cryopreservation), this means that levels of EGF without preservation of amniotic membrane is higher than the levels of EGF in amniotic membrane preservation (cryopreservation), or it can be said has been a reduction EGF levels in amniotic membranes with preservation (cryopreservation).

Decrease in EGF levels in amniotic membranes with preservation (cryopreservation) can be seen in table 1. Decrease in EGF levels in this study represents the difference between the levels of amniotic membrane without preservation EGF and EGF levels in amniotic membrane preservation (cryopreservation). The mean decline was 10.20% ± 18.49% TB. The highest level decrease was 33.35% and the lowest level decrease is 4.09%.

Estimated percentage decrease in levels of EGF result from the process of preservation (cryopreservation) with a confidence interval (CI) 95% is between 12.33% to 24.66%. Based on the calculation above, at 95% confidence interval of decreasing levels of EGF result from the process of preservation (cryopreservation) of 12.33% to 24.66%. The results showed a decrease in EGF levels due process of preservation (cryopreservation) as in some previous studies, although in this study the settlement was smaller. The results of this study can be taken up by the use of amniotic membrane for treatment of several diseases in the field of Health Sciences at the Eye and the medical field in general.

CONCLUSION

Based on the results of research on the differences between the EGF levels of amniotic membrane without amniotic membrane with the preservation and preservation (cryopreservation), the conclusions can be drawn are the mean levels of EGF in amniotic membrane without preservation was 122.76 ± 11.59 pg / g. The mean levels of EGF in amniotic membrane preservation (cryopreservation) was 99.34 ± 9.49 pg / g. There are differences between the levels of EGF without preserved amniotic membranes with amniotic membrane with preservation (cryopreservation), and showed that EGF levels without preservation of amniotic membrane is higher than the levels of EGF in amniotic membrane preservation (cryopreservation). The mean decrease in EGF concentration after the process of preservation (cryopreservation) was 18.49% ± 10.20%.

Based on the results of this study is recommended to conduct further research with the purpose of ascertaining levels of EGF in the process of preservation (cryopreservation) began to decline significantly. Conduct further research to determine clinical effectiveness of the use of amniotic membrane without amniotic membrane with the preservation and preservation (cryopreservation).
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