08. Cytotoxicity test for the use of freeze-dried amniotic membranes against

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Research Article

Cytotoxicity test for the use of freeze-dried amniotic membranes against viability, proliferation, and apoptosis on brain cell culture: An in vitro study

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ABSTRACT

Objective: This study aimed to conduct a cytotoxicity test in determining biocompatibility of freeze-dried amniotic membranes on brain cell culture.
Methods: An in vitro study was carried out on rat's brain cell culture. Samples were divided into three groups: control, conditioned medium, and direct amniotic membrane exposure. Each group was stained with MTT, DAPI and Annexin-V for its viability, proliferation, and apoptosis, respectively. Statistical analysis was conducted using ANOVA, post-hoc, or the Kruskal-Wallis test with CI 95%.
Results: Viability test using MTT staining showed viable cell in conditioned medium group of 76.99 ± 10.19, and direct amniotic membrane exposure group of 90.36 ± 23.31 ($p = 0.001$). DAPI staining showed a median value of 5.32 (1.97-16.53) for control group, 6.00 (2.42-16.62) for conditioned medium group, and 3.53 (1.32-8.69) for direct amniotic membrane exposure. Annexin-V single staining showed mean value of 10.28 ± 2.43 (control group), 10.07 ± 0.97 (conditioned medium), and 10.42 ± 4.07 (direct amniotic membrane; $p = 0.969$). Double staining by Annexin V + DAPI showed mean value of 10.43 ± 1.82 (control), 10.01 ± 1.07 (conditioned medium), and 12.40 ± 3.67 (direct amniotic membrane exposure; $p = 0.148$).
Conclusion: Amniotic membrane exposure affected rat's brain cell culture viability in tolerable limit, while proliferation and apoptosis do not get affected.

1. Background

Dural defects are problems often faced by neurosurgeons. This defect can occur due to iatrogenic or pathological process. Dural closure is primarily an ideal method that needs to be conducted to prevent cerebrospinal fluid (CSF) leakage [1]. If primary closure is not possible, the surgeon will need to close the defect with a graft from another tissue. A good dura mater substitute must be biocompatible in terms of acceptability, inflammatory reaction, and tissue adherence [2]. Various biomaterial choices are available for grafting, which are grouped into: autologous materials [3], allograft [4], xenograft [5,6], and artificial or synthetic materials [7].

Despite graft usage, post-operative evaluation has reported CSF leakage, wound infection, asymptomatic pseudo meningocele, hematoma, adhesions, brain herniation, tension pneumocephalus, epilepsy, bacterial or chemical meningitis, all of which result in longer hospitalization after duraplasty. A study of dural closure using waterproof seams showed an average CSF leakage of 7.7%, an infection rate of 7.5%, asymptomatic pseudo meningocele diagnosed using MRI and or CT scan of 11.8% [8]. The use of synthetic absorbable biomaterials made from microporous polyester non-woven fine fiber material shows the potential for transmigration of fibrinopurulent and malignant cells into dura mater transplants at the closure of defect. Local inflammation can create local immunosuppression. In addition, graft implantation stimulates IL-10 and or TGF-β, increasing immunosuppression and malignant cell infiltration [9].

One of the materials which is currently being examined as dural patch is human amniotic membranes. Several studies have shown satisfying results in both experimental and clinical study settings. Amniotic membrane has been proposed as an ideal material due to its

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absorbance rate and its microscopic profile in facilitating adequate membrane formation. Amniotic membrane is considered as the right material as a graft duraplasty. There are several beneficial characteristics with its anti-inflammatory effect and low immunogenicity in the area of concentration. Amniotic membrane which has been preserved by freeze-dried will function as scaffold in the process of dural defect closure. The low level of two potential inflammatory mediators shows minimal inflammatory reaction in the cortex. Active fibroblast cells are vital in dural healing for scar tissue formation, including the process of gliosis in brain. Tomita et al. examined the potential of amniotic membrane as biomaterial option, showing satisfying result in both experimental and clinical study settings [10,11].

However, there have been no studies examining the safety of amniotic membranes usage against surrounding brain tissue. This study aimed to conduct a cytotoxicity test for the use of freeze-dried amniotic membranes against viability, proliferation, and apoptosis of brain cell culture.

2. Methods and materials

2.1. Amniotic membrane preparation

Amniotic membrane material was obtained through consenting labor patient at Dr. Soetomo General Academic Hospital. The placenta was prepared by the tissue bank. Amniotic membrane was first washed using normal saline solution followed with 0.05% chlorine. The membrane was then irrigated to remove all chlorine solution. The tissue was stretched on glass board, separated from the chorionic layer, and catalogued. The membrane was further stored in a −80 °C freezer followed with sublimation process to evaporate all remaining water content using lyophillizer for 6–7 h in a −105 °C temperature.

2.2. Cell culture and characterization

Primary rat brain cell culture in 3rd-4th passage was obtained from Wistar rat (Animal Laboratory of Institute of Tropical Disease; Surabaya, Indonesia). The surgical process began with general anaesthesia using ketamine for animals, followed by a craniotomy process. After the rat brain was exposed, tissue was taken for further culture. Rat brain cell was cultured in a 35-mm dishes at 70% confluence in Dulbeco's Modified Eagle Media (DMEM) medium with 20 mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% fetal bovine serum, and 100 ml streptomycin with 100U/ml penicillin in 37 °C and 95% humidity and 5% CO_2. Procedures to the animals complied with the approval of the Ethical Committee of Faculty of Veterinary, Universitas Airlangga, Surabaya, Indonesia (Ethical number: 2.KE.034.02.2019).

Cell culture were divided into three groups: control, conditioned-medium, and direct amniotic exposure. Culture in control group received no amnion treatment. The second group consisted of cell culture in a conditioned-medium for 24 h. Medium for cell culture were then washed and replaced with conditioned medium. Conditioned medium was made by submersing amniotic membrane in culture medium for 1 h at 37 °C [12]. The third group consisted of cell culture with direct exposure to crude amniotic membrane. Pieces of amniotic membrane was submerged directly into the cell culture medium, incubated for 24 h and was taken out afterwards. Sample was taken from each group to be processed further for characterization followed by cytotoxicity test using immunohistochemistry staining.

2.3. Characterization

Characterization of cell culture was performed to confirm cell type. The process was carried out by immunocytochemistry technique using GFAP, NESTIN, and SOX-21 Polyclonal Antibody, purchased from Bioss Antibodies Inc., Woburn, MA, USA, to confirm neuronal cell characterization.

2.4. Cytotoxicity test

The cytotoxicity of dura mater material was assessed in this study by measuring cell viability, proliferation, and apoptosis. Study material was introduced using two types of intervention: direct exposure of amniotic membrane to cell culture by submersion and amniotic-membrane-treated conditioned medium. Cell culture viability was estimated with colorimetric assay using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) staining, purchased from (Sigma-Aldrich Corp., St. Louis, MO, USA). Proliferation and apoptosis were evaluate with immunocytochemistry staining technique analysis using DAPI (4′,6-diamidine-2′-phenylindole, dihydrochloride), purchased from Seracare Life Sciences Inc., Milford, MA, USA, and Annexin-V, purchased from eBioscience, San Diego, CA, USA, respectively.

2.5. MTT staining

Cells seeded in parallel 96-multiwell plates were used to calculate the number of cells/well at different post-plating. Cell was taken form CO_2 incubator in 80–90% confluence for harvesting. Cell was incubated in a 37 °C, 5% CO_2 environment for 24 h, distributed into each well and incubated further for 20 h. 25 μL/well of 5 mg/mL MTT was used prior to the third incubation for 4 h. Medium was discarded following the third incubation. DSMO of 200 μL/well were added. Cell was put under ELISA reader (Multi Reader Promega GM35000) on 595 nm wavelength for reading. Absorbance graph was made to be further compared to concentration. Percentage of viable cell and IC50 value were calculated using linear regression of log concentration. Three types of absorbance were obtained: cultured medium with cell and Dimethyl Sulfoxide (DMSO), controlled medium, and study cultured medium with cell and study material. Peak concentration of DMSO was observed from highest concentrated sample [13].

2.6. 4′,6-Diamidino-2-phenylindole (DAPI) staining

Cell culture were fixated and washed three times with PBS + Tween 0.05%. Cell seeker were conducted using syringe procurement, followed by diluting DAPI solution 1:1000 in PBS for 5 min in dark room with room temperature of 27 °C. Remaining cell were washed three times for three minutes [14,15].

2.7. Annexin-V – Fluorescein isothiocyanate (FITC) conjugated staining

Cell culture were fixated and washed three times with PBS + Tween 0.05% and rinsed. Cell permeability were increased with three times application of PBS and Triton-X 0.1%. Blocking buffer was carried out with 30-minute incubation in PBS and BSA 1% to prevent non-specific protein. Specimen then washed three times in 4 °C. Primary antibody of 1:100 was put into blocking buffer for 1 h in dark room with room temperature, followed by rinsing for three times and counterstaining with DAPI [16–18].

2.8. Image analysis

The immune reaction product was observed with immunofluorescence microscope (Fluorescence Microscope Olympus IX71, 100x magnification). Images were captured then processed by ImageJ program to calculate intensity of colour spectrum, giving quantitative number of pixels as output. High resolution images taken by fluorescence microscope were processed by ImageJ program to obtain quantify percent coverage. The threshold colour was put on dark background and measured for percent area of threshold. Percent area of coverage for multiple region within each sample was calculated as average pixels [19].

2.9. Statistical analysis

The results of data collection were presented in the form of mean ± standard deviation (SD) or median (minimum–maximum) and percentage (%). The research results were also displayed in the form of pictures or tables. Statistical analysis used IBM SPSS Statistics software version 23.0 (IBM Corp., Armonk, NY, USA). ANOVA test was conducted should the data in normal distribution, while Kruskal-Wallis method was performed should the data in abnormal distribution. The statistical test was followed with a post-hoc test between groups to look for significant differences between groups. A significant correlation was determined should the $p < 0.05$ with 95% CI.

3. Results

3.1. Cell culture viability on amniotic membrane exposure

Cell viability testing was conducted by MTT staining at each well (n = 48), followed by colorimetric reading on each well. Each well received treatment in the form of amniotic membrane direct exposure (n = 16), conditioned medium (n = 16), and control (n = 16). The data were processed to describe the percentage of living cells. The mean value of MTT reading results in each treatment group was reduced by the absorbance value of control group. From these calculations, cell viability was obtained in the form of a living cell percentage of 76.99% in the conditioned-medium treatment group and 90.36% in the amniotic membrane direct exposure group (Fig. 1).

The results showed the mean value of 0.44 ± 0.09 for the amniotic membrane group, 0.39 ± 0.04 for the conditioned medium group, and 0.48 ± 0.03 for the control group ($p = 0.001$). The mean value of the conditioned medium (0.39 ± 0.44) was significantly lower than the mean value of control group (0.48 ± 0.02; $p < 0.001$). It was concluded that cell culture still has good cell viability in amniotic membrane exposure in the form of direct exposure or in the form of condition-medium (Table 1).

3.2. Cell culture proliferation on amniotic membrane exposure

The results of ImageJ processing showed mean for the control group was 5.49 ± 2.6, while subjects in conditioned-medium was 6.10 ± 2.56

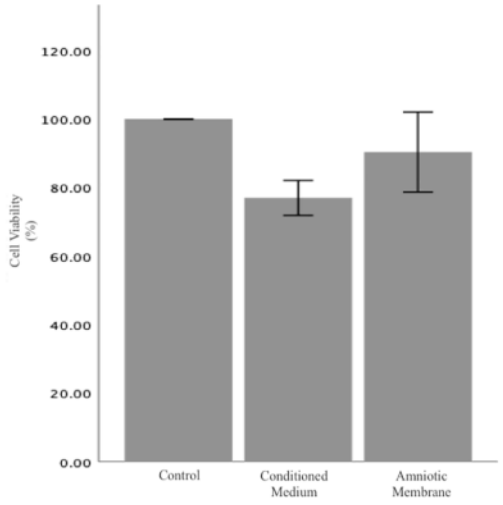

Fig. 1. Percentage of cell viability, control 100.00 ± 0.00, conditioned mediums76.99 ± 10.20, and amniotic membrane 90.36 ± 23.31 (p = 0.001; CI 99%).

Table 1
ANOVA analysis of cell culture on MTT staining.

Group	Mean ± SD	p
Amniotic membrane	0.44 ± 0.09	0.001
Conditioned medium	0.39 ± 0.04	
Control	0.48 ± 0.03	

and amniotic membrane was 3.75 ± 1.53 (Fig. 2).

The results showed that the median for the control group was 5.32 (1.97–16.53), while median for conditioned medium was 6.00 (2.42–16.62) and amniotic membrane direct exposure was 3.53 (1.32–8.69). The examination was continued by conducting a different test on 3 groups. The comparison results showed significant differences between the three groups, with $p = 0.001$ (Table 2).

Significantly different test results were achieved by comparing amniotic membrane treatment group to control ($p = 0.005$) and between amniotic membrane treatment group against the medium condition ($p = 0.001$; Table 3).

3.3. Cell culture apoptosis on amnion membrane exposure

The results of cell culture staining (Fig. 3) with Annexin V showed that the mean value for the control group was 10.28 ± 2.43, while conditioned medium was 10.428 ± 4.073 and amniotic membrane group was 10.06 ± 0.97. In the group with Annexin V + DAPI staining showed a median value for the control group of 10.19 (8.69–12.68), while conditioned medium was 12.78 (7.21–21.22) and amniotic membrane treatment group was 9.68 (8.58–11.67; Fig. 4).

The calculation of normal data distribution on Annexin V staining was processed for data analysis using ANOVA to look for significant differences between the 3 groups. The results showed no significant difference ($p = 0.97$) in the comparison of each treatment group. The results of analysis showed no significant difference ($p = 0.148$) in the comparison between treatment groups (Table 4).

4. Discussions

Amniotic membrane potential as biomaterial had been proven many times in some previous studies. Currently, amniotic membrane is also used as a biomaterial to cover dural defects. These applications have been investigated in several in vivo and clinical studies [10,11]. Ideally, as a basis for usage, biomaterial is necessary to go through stages that prove safety, starting from the in vitro research stage and continuing in vivo. Only if proven safe, it will be continued in human research [20]. There are no in vitro studies examining the toxicity of amniotic membranes to brain tissue [21–23].

Neurotoxicity study is recommended in vitro to find out the underlying processes of normal functioning of the nerve and its dysfunction. The neurotoxicity seen at the cellular level results from bioactivation of the response to the test metabolite, whereas in the in vivo level test, visible toxicity can also occur due to the response from non-neuronal or systemic cells [24]. Neurotoxicity assessment using cell culture media [24,25] aims to evaluate the potential for biochemical neurotoxicity in the form of experimental tests. The results obtained are measured to determine changes in receptors, growth, physiological and nerve cell basal function. The parameter evaluation includes evaluating macromolecular synthesis, oxidation, mitochondrial activity, apoptosis, DNA integrity, regulation and cell function, enzyme changes, to cell integrity [24].

Amniotic membrane as intrauterine embryonal protector provides mechanical barrier in suspensive stress from good tensile resistance from condensed interstitial collagen I, II and elastin, and adequate elasticity from collagen III. Its healing profile had been proven by Haugh et al. [26], and widely used in reconstructive surgery with satisfying result [10]. A study conducted by Turchan et al., also showed amniotic

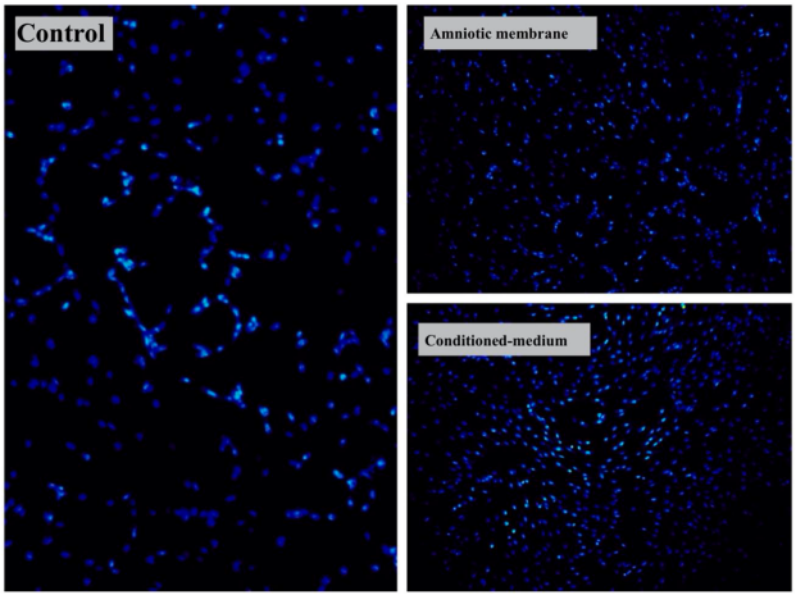

Fig. 2. DAPI staining in the control group, conditioned media and amniotic membrane. Visible fluorescent images with bluish DAPI in the cell nucleus. The reading was conducted in a dark room to measure the intensity of immunoreactive cells. (Olympus IX71 Fluorosity Microscope, 100x magnification).

Table 2
Median results for DAPI staining.

Group	Median (min – max)	p
Amniotic membrane	5.32 (1.97 – 16.53)	0.001
Conditioned medium	6.00 (2.42 – 16.62)	
Control	3.53 (1.32 – 8.69)	

Table 3
Comparative analysis for cell culture proliferation between DAPI stained groups using Mann-Whitney method.

Group	N	Mean Rank	p
Amniotic membrane	20	12,13	0.001
Conditioned medium	20	28,88	
Conditioned medium	20	16,68	0.307
Control	10	13,15	
Amniotic membrane	20	12,4	0.005
Control	10	21,7	

membrane effectiveness as scaffold in dural defect closure with no LCS leakage [11]. Adequate safety profile of amniotic membrane in using dehydrated human amnion for dural grafting was also reported [27]. These reports are in concordance with the results of this study, strengthening the basis of safety and cytotoxicity for clinical use. Extended study on amniotic membrane toxicology profile should be conducted to set basic cellular profile during amniotic membrane exposure on neuron cells.

Cell culture proliferation evaluation using DAPI staining showed blue colour, representing intact live cells followed by microscopic analysis to observe chromatin fragmentation in the nucleus and was read by ImageJ software for further processing [28,29]. DAPI staining in the amniotic membrane treatment group showed a low expression which was significantly different when compared to the control group and the conditioned medium. This could indicate that differences in cell proliferation of amnion membrane exposure group were not pended by apoptosis process. The result of low expression of DAPI in amniotic membrane exposure group could be due to the technical exposure of amniotic membrane to cell culture. A study by Leaw et al. reported a significant inhibitory effect of human amniotic membrane on apoptosis following brain injury in rat model [30]. Our study noted that amniotic membrane must be taken back when staining. The retrieval process can result in cell release which explained our result.

Annexin V has been widely used for apoptosis analysis based on membrane changes of asymmetrical loss. In viable cells, the phospholipid phosphatidylserine (PS) membrane is located in the inner part of plasma membrane, externalized and exposed on the cell surface [31]. The disadvantage of Annexin-V is the possibility of cell binding in undergoing necrosis [32]. The results of cell apoptosis assessment with Annexin-V staining showed a non-significant difference of apoptotic numbers between control and treatment groups. Rat's brain cell culture did not undergo extensive apoptosis during exposure. This non-significant difference strengthens amniotic membrane safety profile and can be used as basis for conducting further studies using brain tissue.

Amniotic membrane can also reduce inflammation from its immunosuppressive property. Ueta et al. showed its capabilities in suppressing T cell response in an in vitro cell culture study [33]. In addition, Walker et al. showed amniotic membrane capabilities to reduce adhesion and fibrosis for dural repair [25]. Reports show its axonal regeneration profile along with increased mRNA expression of neurotropic factor in animal model [34] and nerve scaring prevention [35].

Even though amniotic membrane has undergone a process of preservation, it still contains various growth factors that affect the condition of microenvironment. Therefore, it will create a more conducive condition to the wound healing process. The amniotic membrane contains growth factors such as EGF, FGF, GM-CSF, VEGF, and PDGF, which have an important role in wound healing. The complex role of this growth factor has been studied and reported to have an important role in adhesion, migration, proliferation, and cell differentiation [36,37]. This might explain the differences seen in the proliferation observed in this study, where cells using conditioned medium from amnion have a higher proliferation value compared to other groups. Results of this study showed that direct exposure to amniotic membrane is safe for amniotic membrane cell culture viability. The mean value of cell culture

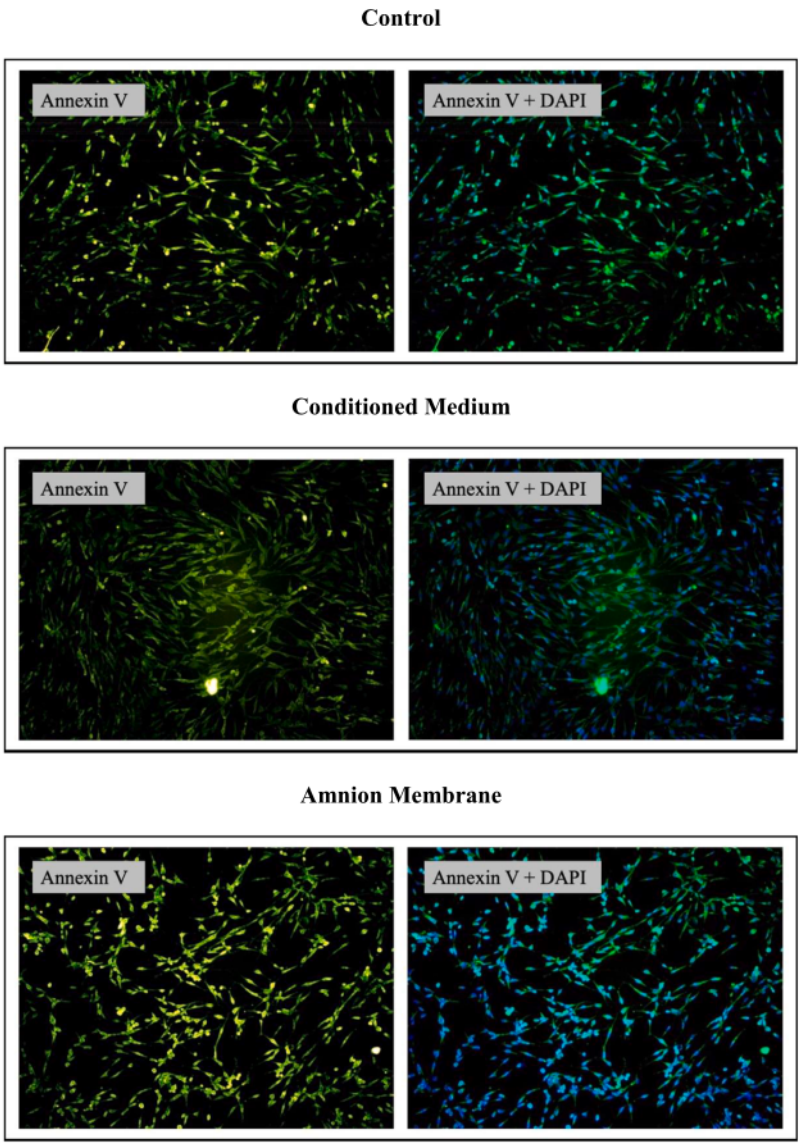

Fig. 3. The result of cell staining with Annexin-V (left lane) and addition of counter-stained with DAPI (right lane) in the control group, conditioned medium and amniotic membrane. (Olympus IX71 Fluorosity Microscope, 100x magnification).

in conditioned medium group was significantly lower than the mean value of control group. Conditioned medium group may have mediators and cytokines released and may have an effect on cell viability compared to direct exposure. A study of human amniotic epithelial cell (HAEC) conditioned medium of cultured neuronal cell of rats showed amniotic membrane regeneration profile postulated from the medium containing various expression of neurotrophic factor without neurotrophic effects on rat neurons [12]. Later, further evaluation of HAEC's neuroprotective effects by its conditioned medium suggested neuroprotective its potential [38]. Further studies are required to investigate the optimum usage of conditioned medium of amniotic membrane.

Amniotic membrane meets the ideal criteria for biomaterial and has valuable potential as dural graft material, but only a few studies exploring its safety and toxicity profile towards brain cell or tissue. Unlike other studies, this research examined the safety of amnion membranes in vitro against brain culture cells. This study should be followed by further in-depth researches related to cytocompatibility of freeze-dried amniotic membranes usage in duraplasty against brain tissue in animal model.

5. Conclusions

This study finds a difference in cell viability in amniotic membrane exposure, but still within tolerable limits. Amniotic membrane exposure does not affect apoptosis of rat's brain cell culture. Amniotic membrane is not toxic and is safe against rat's brain cell culture. The potential of amniotic membranes can be considered as an alternative material for the development of dural graft biomaterials in the duraplasty procedure. Further research is needed at the in vivo stage in animal models to assess inflammatory reactions in tissue reactions to foreign bodies.

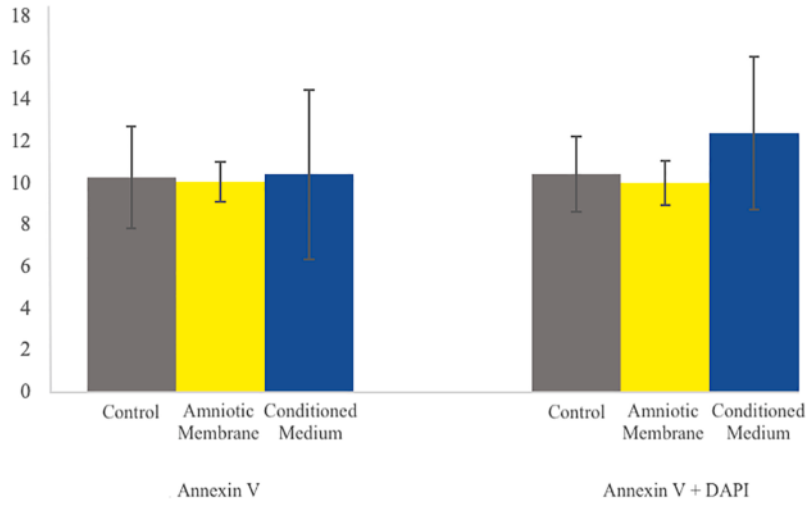

Fig. 4. Mean average of Annexin-V staining. Result was obtained from image reading on single staining and double staining with DAPI.

Table 4
Cell apoptosis data by Annexin V and annexin + DAPI staining.

	group I	II	III	p
Annexin V	10.28 ± 2.43	10.07 ± 0.97	10.42 ± 4.07	0.969
Annexin V + DAPI	10.43 ± 1.82	10.01 ± 1.07	12.40 ± 3.67	0.148

Ethical approval

All procedures performed in studies involving experimental animals were in accordance with the ethical standards of the faculty of veterinary medicine, Universitas Airlangga, Surabaya, Indonesia (Ethical number 2.KE.034.02.2019).

Author contribution

Rahadian Indarto Susilo was in charge on concept creation, research implementation, data collection and processing, writing manuscript. Joni Wahyuhadi conducted concept making, data processing, interpretation of results, writing manuscript. I Ketut Sudiana was in charge on concept making, interpretation of results, writing manuscripts. Fedik Abdul Rantam made concepts, interpreted results, and wrote manuscripts.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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PAGE 2

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PAGE 4

PAGE 5

PAGE 6

PAGE 7