ABSTRACT

Background: Radical surgery in cancer patients might result in complications like lymphedema. Tissue transfer procedure and lymphatic reconstruction could be done to manage lymphedema. Few biomarker expressions were identified to be associated with lymphangiogenesis and improved lymphatic system function after dissection. This study aims to evaluate the development of new lymphatic vessels by adding flap tissue after lymphatic dissection, assessed by VEGF-C expression, macrophage infiltration, and fibrotic tissue development.

Methods: This is a non-randomized clinical trial at Animal House Skill Laboratory, Faculty of Medicine, Universitas Indonesia, conducted from January to March 2018. Sprague clawley male mice were aged 8-12 weeks were used and divided into control and trial groups. Each mouse underwent inguinal dissection and was randomized to receive additional flap tissue procedures and then evaluated two months after. Methylene blue dye was used to assess the lymphatic flow. Histopathology examination was used to assess the development of fibrotic tissue. Data analysis was done by using SPSS version 20.0 for Windows.

Results: 18 mice were included in the analysis and divided into two groups. Eight out of 9 mice (88.9%) in the trial group, which received flap tissue, showed lymphatic flow from the visualization of methylene blue, compared to two mice (22.2%) from the control group (p=0.15) two months after surgery. Histopathology examination showed wider fibrotic tissue of the lymphatic system from the trial group (p=0.05). Immunohistochemistry analysis also showed higher VEGF-C (p=0.01) and CD68 expression in the trial group (p=0.15).

Conclusion: The addition of flap tissue might improve lymphatic flow, proven by increased lymphatic circulation and VEGF-C expression.

Keywords: CD68, Flap Transfer, Lymphedema, Lymphatic Vessels Reconstruction, VEGF-C.


INTRODUCTION

Breast cancer is the second most common cancer worldwide. It is the most common cancer in women, with an estimated 1.67 million new cases diagnosed in 2012 (accounting for 25% of the total number of cancers).1 In Indonesia, during 2010-2013, breast cancer was one of the three most common diseases, along with cervical cancer and lung cancer, at Dharmais National Cancer Hospital in Jakarta, with the number of new cases due to cancer continuing to increase.2 Up to now, surgical intervention has been the main therapy for breast cancer. Axillary dissection levels I and II are the standard therapy for all cancer stages, and complete axillary dissection might be considered in certain circumstances. Surgery involving dissection of the lymph nodes can lead to devastating complications, such as lymphedema.3,4

Lymphedema is a chronic condition caused by the abnormal accumulation of protein-rich fluids in the interstitial space-related drainage inadequate lymphatic.5,6 In the study by Hayes SC et al. in 2007, at first 18 months after the breast cancer diagnosis, at least one in 10 women experienced lymphedema. Furthermore, at 18 months after surgery, at least 30% of breast cancer survivors have or have had lymphedema. Among them, 40% experience lymphedema that lasts more than three months, with or without intermittent periods of improvement.7

Lymphedema is caused by the direct effects of breast tumors or by the indirect effects of anti-neoplastic drugs. Treating tumors by removing the lymphatic nodes can cause injury in the lymph vessels and nodes. Radiation therapy can also cause fibrosis around lymphatic structures and interfere with functioning.8,9 Lymphedema that occurs after mastectomy could be managed by a surgical procedure that has been around for more than a century.10 Surgery can be done by doing tissue transfer or by performing Anastomosis or lymphatic reconstruction.11 This means...
recreating the pathways of lymphatic vessels that have been damaged by mastectomy. Recent research has shown lymphatic regeneration after tissue transfer caused by spontaneous reconnection of the lymphatic system with the transferred tissue. This process can occur due to the expression of VEGF-C (vascular endothelial growth factor-C), which is the most important growth factor for lymphatic vascularization.

One of the important regulators of lymphangiogenesis is macrophages. VEGF-C excretion produced by macrophages is the main regulator in tumor lymphangiogenesis and during inflammation after renal transplantation or corneal injury. In addition, macrophages are thought to contribute directly to the formation of lymphatic vessels by trans-differentiation to lymphatic endothelial cells.

Ghanta S et al., in their study, found that depletion of macrophages was associated with an increase in collagen deposition, fibrosis, impaired lymphatic function, and decreased expression of VEGF-C. How macrophage mechanisms regulate the process of fibrosis in lymphedema until now is unknown and still needs further research. Our earlier study in 2017 found the formation of a new lymph flow which was visually assessed by looking at the blue dye flow flowing towards the inguinal in 13 of the 16 mice that were inguinal dissection with the addition of tissue flaps. These studies only assessed the formation of new lymph flows by looking at the flow of blue dye flowing towards the inguinal of mice. Based on the data above, researchers want to prove the formation of new lymph vessels by evaluating the increase in VEGF-C expression, macrophage infiltration, and fibrosis tissue formation after the dissection the lymph nodes with the addition of tissue flaps.

**METHODS**

This is a non-randomized controlled trial conducted in the Animal House Skill Laboratory at the Faculty of Medicine, Universitas Indonesia, from January to March 2018. The experimental trial was done by purposive sampling, which involved Sprague clawley mice with the following inclusion criteria: male, aged 8-12 weeks, weighing 250-300 grams, had normal behavior and activity, and no anatomical abnormalities. The exclusion criteria were mice with the following characteristics: dull hair, experiencing hair loss, or less active; abnormal exudate from the eyes, mouth, anus, and genital; more than 10% weight loss after the adaptation period in the laboratory; died during adaptation.

Mice that met the inclusion and exclusion criteria underwent inguinal dissection under general anesthesia with ketamine 50mg/kg and xylazine 5mg/kg intramuscular. Methylene blue (STEROP, Belgium) 0.2 ccs is injected subcutaneously 1 cm from the tail base. An incision was made above the inguinal 30-45 minutes after the anesthesia. Lymphatic vessels and lymph node identification were done by the methylene blue visualization. The identified lymph nodes were dissected, leaving a 1 x 1 cm tissue defect. Flap tissue was marked by polypropylene 3.0 sutures.

The second surgery was done two months following the first surgery. After the same anesthesia and methylene blue injection procedure were done, the mice were euthanized. Midline abdominal incision was done through cutaneous and subcutaneous layers. A flap was made in the direction of the dissection. The lymph flow was evaluated using the marked axillary lymph nodes and vessels. The second specimen was taken on the prior dissected tissue, including the suture markings and extending its size by 0.5 cm on each side.

**Histopathology examination**

Histopathology evaluation with hematoxylin-eosin (HE) staining was done to assess the development of fibrotic tissue. At the same time, IHC evaluated VEGF-C expression and macrophage infiltration (CD68 expression) on the dissected inguinal tissue of both groups from the first surgery and the specimen from the second surgery. Interpretation of the IHC is evaluated under the binocular light microscope (Leica DM500) with 40 times magnification and captured by a built-in camera (Leica ICC50HD) by a laboratory assistant specialized in animal tissue IHC evaluation. Each slide is randomly taken in five different fields of view in 2048 x 1536 pixels. Protein expression is analyzed with an IHC profiler on Image-J software, a freeware program developed by the National Institute of Health. Color deconvolution and computerized intensity measurement were used, resulting in an intensity score automatically from each picture. IHC Profiler would result in semi-quantitative data categorized as strongly positive, positive, weakly positive, or negative. Thus, IHC Optical Density Score was used to calculate the score. The difference of VEGF-C and CD68 expression between baseline and two months for surgery were also calculated, defined as delta.

**Data analysis**

Data were collected and documented in Microsoft Excel. The association between tissue flap and the presence of lymphatic flow is analyzed using chi-square. The mean difference between the width of fibrotic tissue, VEGF-C and CD68 expression between the control and trial groups is analyzed using the dependent T-test. All results were expected to be significant at a p-value <0.05. Statistical analysis was done using SPSS version 20.0 for Windows.

**RESULTS**

At first, 20 mice were included in the study, 10 in each control and trial group. Control group mice were named K1B-K10B, and trial group mice were named P1B-P10B. However, one mouse in each group died before the study was finished. Thus, 18 mice were included in the analysis, consisting of nine mice in each group. In the trial group, eight (88.9%) mice had inguinal lymphatic flow, and lymph nodes were visualized and marked by methylene blue. However, in the control group, only two (22.2%) mice had positive inguinal lymphatic flow. Figure 1 depicts the trial and control mice two months after the first surgery. Chi-square analysis between flap surgery and the presence of lymphatic flow post-surgery was found to be statistically significant (p = 0.015), as seen in Table 1.
The fibrotic tissue was evaluated by HE staining; width measurement was done and analyzed statistically. Figure 2 shows the fibrotic tissue by HE staining on the control and trial mice two months after surgery. However, no significant difference in fibrotic tissue width between the two groups was found using dependent T-test analysis (p = 0.05), as seen in Table 2.

An immunohistochemistry examination was done to evaluate VEGF-C expression. Figure 3 shows the IHC results in control and trial mice specimens. Specimen from the trial mice showed a more dominant dark brown color than the control, indicating an increased expression of VEGF-C. The expression of VEGF-C two months post-surgery in the control and trial groups was 1.13±0.04 and 1.92±0.37, respectively. The independent T-test analysis showed a statistically significant mean difference in VEGF-C expression and VEGF-C delta (p=0.016), as seen in Table 3.

On the other side, CD68 expression two months post-surgery in the control and trial groups was 1.36±0.18 and 1.76±0.49, respectively. However, the mean difference is not statistically significant (p=0.151), as indicated in Table 4.

**DISCUSSION**

Currently, no known supportive or surgical treatment is available to prevent the progression or cure lymphedema. An advanced novel surgical technique called lymphatic reconstruction might decrease lymphedema volume by up to 67%. In both human and animal studies, prior studies which implemented flap surgery for lymphedema cases reported a better outcome and decreased risk of lymphedema by 36%.11,16 This result indicates lymphatic flow improvement in the breast cancer surgery area with flap surgery. Other surgeons also began to do lymphatic bypasses for the injured vessels. Improvement in lymphedema was seen and found that lymphatic rerouting occurred.13,17

Anthony JP et al. used skin and fatty tissue originating from excess abdominal tissue, which was placed on the injured tissue by reconnecting arteries and veins without lymphatic vessel anastomosis. The study reported that lymphedema improvement was seen six to eight weeks after microsurgical tissue transfers, which indicates a spontaneous regeneration of the lymphatic system.11 Yan A et al. conducted a trial on mice which reported the expression of VEGF-C was seen in the peripheral region of the skin graft two weeks after surgery, especially on the distal margin. After six weeks, VEGF-C expression was seen in the central part of the skin graft, and lymphoscintigraphy evaluation showed improvement in lymphatic flow.11 These findings conclude that lymphatic regeneration from skin transplantation occurred through spontaneous reconnection and ingrowth of new capillary lymphatic channels and support the hypothesis that spontaneous regeneration of lymphatic vessels from autogenous tissue may cause bypass of the injured lymphatic vessels and results in lymphatic flow and function.11

Another study also reported the success of vascularized lymph node transfer with or without skin paddle. The dermis is rich in capillary lymphatic vessels; thus, the skin paddle may support the recanalization of the lymphatic system between the recipient and the transferred flap. The lymphoscintigraphy evaluation confirmed the result by visualizing the lymphatic vessels’ connection between the recipient and the skin paddle.18 This study reports that most of the mice who underwent flap transfer had lymphatic flow compared to those who did not (88.9% vs. 11.1%, respectively). The association between flap transfer and the presence of lymphatic flow in the mice who underwent inguinal dissection was found (p=0.015). The findings support the prior studies regarding the advantage of flap transfer in improving lymphedema.11,13 Similar results

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**Table 1. The presence of lymphatic flow in trial and control groups.**

<table>
<thead>
<tr>
<th>Variables</th>
<th>Presence of lymphatic flow</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yes (n=10)</td>
<td>No (n=10)</td>
</tr>
<tr>
<td>Trial group, n (%)</td>
<td>8 (88.89)</td>
<td>1 (11.11)</td>
</tr>
<tr>
<td>Control group, n (%)</td>
<td>2 (22.22)</td>
<td>7 (77.78)</td>
</tr>
</tbody>
</table>

*Statistically significant if p-value less than 0.05

**Figure 1.** The presentation of control and trial mice one day (A, B) and two months (C, D) after surgery. (A) Control mice K9A; (B) Trial mice P9A; (C) Control mice K9B; (D) Trial mice P9B.

**Figure 2.** Hematoxylin eosin staining of fibrotic tissue from control mice K3B (A) and trial mice P5B (B) two months after surgery.
were reported in the earlier study, in which flap transfer may contribute to developing a new lymphatic collateral pathway.\textsuperscript{15}

Only one mouse (11.1\%) which received flap transfer did not show lymphatic flow. Delay lymphatic regeneration process might explain this finding.\textsuperscript{13} However, no development of the new lymphatic pathway might occur, and the predisposing factors were still unknown. Yan A et al. reported that no lymphatic system improvement was found in the trial mice that underwent tail excision only compared to those who received additional skin grafts.\textsuperscript{11} Eventually, increased expression of VEGF-C, macrophage infiltration, and decreased thickness in the dermis layer and fibrotic tissue was found.\textsuperscript{11} Two mice (22.2\%) in the control group showed lymphatic flow, indicating the developed collateral pathway. Anatomy and physiologic variation or inadequate dissection technique leaving the remaining lymphatic vessels might be the reasons behind the findings. However, further research should be done to confirm this hypothesis.

From the histopathology examination using HE, fibrotic tissue width measurement was applicable to assess pathologic and physiologic changes in the mice tissue semi-quantitatively, as was done by Klopfleisch's study.\textsuperscript{19} On average, the trial mice were found to have wider fibrotic tissue than the control mice. However, no association was found to be statistically significant. This finding was supported by earlier research by Kinashi H et al. in which the inflammation process will induce cytokine that plays a major role in fibrosis named transforming growth factor beta 1 (TGF-β1).\textsuperscript{20} This profibrotic cytokine will stimulate mesothelial cells and macrophage, which would induce VEGF-C release, an essential cytokine for lymphangiogenesis. In summary, TGF-β1 will induce fibrosis and lymphangiogenesis at the same time. The role of TGF-β1 in adding tissue flap in the mice was still not yet known and needed further research. Our finding contrasts with another study that found an increased fibrotic tissue in the control mice.\textsuperscript{20,21}

Higher expressions of VEGF-C and CD68 were found from IHC evaluation, but the difference is not statistically significant. This indicates lymphatic regeneration and spontaneous lymphatic reconnection occurred after tissue transfer, in which VEGF-C was essential.\textsuperscript{11,12} On the other side, macrophages also function in the development of lymphatic vessels through lymphatic endothelial cell transdifferentiation.\textsuperscript{11}

We considered several limitations of the current study, such as no blinding and short-term follow-up. Notwithstanding, the findings in this study were expected to be initial research for further studies in patients who underwent dissection and the advantages of flap tissue to improve lymphatic flow in the dissected area, especially in cancer patients. Currently, the biomolecular aspects and gene mutation regarding secondary lymphedema remain unsolved, and the discovery of the related biomarker will further improve the prevention and management of secondary lymphedema. A better prospective design study is needed to confirm this result and further assess biomolecular aspects of lymphatic system regeneration, such as gene mutation discovery in secondary lymphedema or evaluation of

Table 2. The mean difference in fibrotic tissue width between the control and trial group two months after surgery.

<table>
<thead>
<tr>
<th>Group (N=18)</th>
<th>Fibrotic tissue width (mm)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std. Deviation</td>
</tr>
<tr>
<td>Control (n=9)</td>
<td>2.24</td>
<td>1.26</td>
</tr>
<tr>
<td>Trial (n=9)</td>
<td>3.57</td>
<td>1.85</td>
</tr>
</tbody>
</table>

*Statistically significant if p-value less than 0.05

Figure 3. VEGF-C expression on IHC evaluation under 40 times magnification of light microscope from a specimen taken two months after flap surgery from control mice K2B (A) and trial mice P9B (B).

Table 3. The mean difference of VEGF-C expression between the control and trial groups.

<table>
<thead>
<tr>
<th>VEGF-C expression</th>
<th>Control group (n=9)</th>
<th>Trial group (n=9)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.23</td>
<td>0.14</td>
<td>1.47</td>
</tr>
<tr>
<td>Two months after surgery</td>
<td>1.13</td>
<td>0.04</td>
<td>1.92</td>
</tr>
<tr>
<td>Delta</td>
<td>-0.10</td>
<td>0.14</td>
<td>0.45</td>
</tr>
</tbody>
</table>

*Statistically significant if p-value less than 0.05; VEGF-C: Vascular Endothelial Growth Factor-C; SD: standard deviation

Table 4. The mean difference of CD68 expression between the control and trial groups.

<table>
<thead>
<tr>
<th>CD68 expression</th>
<th>Control group (n=9)</th>
<th>Trial group (n=9)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>Baseline</td>
<td>1.26</td>
<td>0.24</td>
<td>1.49</td>
</tr>
<tr>
<td>Two months after surgery</td>
<td>1.36</td>
<td>0.18</td>
<td>1.76</td>
</tr>
<tr>
<td>Delta</td>
<td>0.09</td>
<td>0.33</td>
<td>0.26</td>
</tr>
</tbody>
</table>
lymphedema-related protein expression with a more advanced technique like quantitative analysis by PCR ELISA or immunofluorescence.

CONCLUSION
The addition of a flap after inguinal dissection improved lymphatic flow, as shown by the methylene blue visualization, increased expression of VEGF-C and CD68, and fibrotic tissue development on the flap region.

CONFLICT OF INTEREST
All authors declare no conflict of interest in this study.

ETHICAL CONSIDERATION
The study had been approved by Faculty of Medicine, Universitas Indonesia, Ciptomangunkusumo General Hospital Ethical Committee with ethical approval No. 761/UN2.F1/ETIK/2017.

FUNDING
There is no financial support for conducting and publishing this research.

AUTHOR CONTRIBUTION
SS and DK were responsible for the study concept and design, participating in the experimental studies, and data acquisition. AD and IG were responsible for literature searching, study design and concepts, data and statistical analysis. IG and DA did manuscript preparation. All authors participated in editing and reviewing the manuscript.

REFERENCES