The Peak of Cytochrome-c (Cyt-c) Gene Expression in Inflammatory Stage after Amputation of Digit Tip Mice (*Mus musculus*)

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Abstract: The complexity of the tissue regeneration process requires the activity of cells and energy. The energy obtained from cellular respiration processes. The cellular respiration involves the enzyme has a role transfer an electron. Cytochrome-c (Cyt-c) is an enzyme in the mitochondria inner membrane that has a role in cell respiration and stimulates apoptosis cells. We studied the Cyt-c gene expression in inflammatory phase of tissue regeneration of digit tip mice (*Mus musculus*) after amputation to analyze a role in tissue regeneration. The result of the Cyt-c gene expression reached a peak on day 3. It showed that the tissue regeneration process needs high energy and involves cell apoptosis to stimulate regeneration. The result of ANOVA homogeneity test of Cyt-c gene expression differentt significantly for each growth day ($p < 0.05$).

1 INTRODUCTION

The tissue regeneration process involves the forming of new cells and tissue. The damaged cells will undergo a process of apoptosis that the programmed of cell death (Gauron et al., 2013). Apoptosis cell is interested in the tissue regeneration process because it is related to the formation of new cells. The macrophages will be phagocytosed into the dead cells and stimulate extracellular matrix formation that plays a role in tissue regeneration (Reinke & Sorg, 2012).

The higher the apoptotic cell process occurs, the higher the extracellular matrix produced (Calve & Simon, n.d.). An apoptosis, cell process involving the enzymes and proteins, one of which is cytochrome c (Allen, 2011). This enzyme has a role in electron transfer from system III to system IV in mitochondria inner membrane and a role in cell apoptosis. The Cyt-c biogenesis process will increase the Cyt-c enzyme in cell apoptosis. A Cyt-c biogenesis process is a multiplication of the Cyt-c enzyme (Panigrahy et al., 2013).

The process of tissue regeneration occurs in four phases, the wound-healing, the blastema, the regeneration phase, and the materials phase (Krafts, 2010). The tissue was dominated by the white blood cells in the wound-healing phase. The macrophage phagocytes the antigen and phagocytes the apoptotic cells in the wound-healing phase. The wound-healing phase occurs in the first days after injury (Mescher, 2017).

In this study, we will analyze the Cyt-c gene expression in tissue regeneration of digit tip mice post-amputation. The study result to be the initial research for further analysis stimulates the regeneration process in organisms that have limited ability in tissue regeneration.

2 MATERIAL AND METHOD

2.1 Sample

The research ethics code for this study proposed by the Research Ethics Commission Esa Unggul University. The research sample was thirty male mice (*Mus musculus*) for Swiss Webster that eight weeks old and weighing twenty grams. The number of samples is adopted by the Federer formula. We got the mice from the research and development laboratory, the Ministry of Health of the Republic of Indonesia. The sample was maintained and treated by the laboratory assistant from the Health Ministry of the Republic of Indonesia. Mice were anesthetized by ketamine/xylazine at a dose of 0.5 gr/kg BW and

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amputated on the 3rd phalanges of digit tip mice. The tissue growth on day 0 (4 hours after amputation), day 1, day 3, and day 5 after the amputation was collected to analyze the sample for gene expression and histological analysis.

2.2 Histology Preparation

Histology preparations stained with hematoxylin-eosin (HE) strokes and histochemical: 10% formalin; 70% alcohol; 80% alcohol; 95 % alcohol; and 100% alcohol; xylol; paraffin block; hematoxylin-eosin; equates; the outward appearance of Van Gieson.

Software ImageJ I-46 has various features to analysis the semi-quantitative of the histological sample of HE staining. Image J software can use to calculate the number of cells and measure the length or area of cells and tissues. Image J software can be downloaded for free and used offline.

The length calculation is done by opening the histology image file first and setting the image scale using the scale set feature. The length calculation used in the line drawing feature is got automatically by using the measure feature.

2.3 Analysis of Gene Expression

The primary DNA of the Cyt gene and the 18S gene, as a reference gene, were amplified by qPCR procedure. Desain primer Cyt-c gene Forward ATTCCTTCATGTCGGACGAG and Reverse ACTGAGAAGCCCCCTCAAAT.

The qPCR method amplification DNA through the stages is DNA synthesis, reverse transcriptase, amplification with 40 cycles at an annealing temperature of 55^0C. Finally, the stage is the melting curve stage. Negative controls operated by free water as a substitute for RNA to get the incorrect positive results. The results of qRT-PCR obtained the value of efficiency and Cycle Threshold (CT) of DNA amplification. Analysis of gene expression assessed by relative qualification by the value of mRNA expression quantification relatively by the Livak method.

2.4 Statistical Analysis

Statistical analysis of the normality test used the Kolmogorov Smirnov test. The data distributed not normally, so analytical analysis using a non-parametric test. The homogeneity test performed using the ANOVA test and the correlation test by the Spearman test.

3 RESULTS

3.1 The Growth of Digit Tip Mice (*Mus musculus*)

After amputation, the digit tip mice grow to replace the lost tissue (figure 1). On day 0 and day 1, therein common is no visible growth of tissue. We suspected that it is the inflammatory tissue. From day 3 to day 5, the tissue appeared to grow faster in the wound area. We suspect that the cell was more actively dividing. Growing tissue remains the collecting dividing cells that form the new tissue.

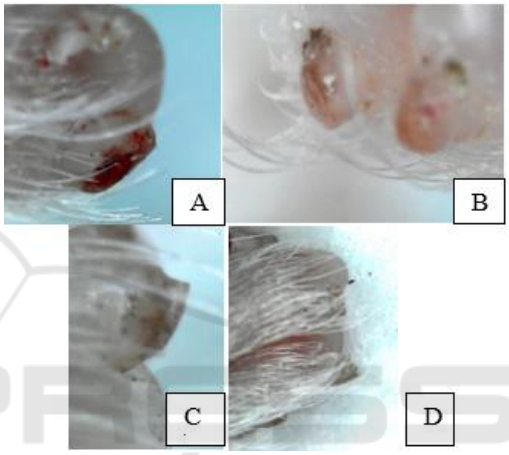


Figure 1: Growth of digit tip mice (*Mus musculus*) from day 0 to day 5 after amputation (A) Digit tip on day 0 (4 hours after amputation) (B) 1 day after amputation (C) 3 day after amputation (D) 5 day after amputation.

The growth curve of the digit tip mice after amputation shown in fig 2. The growth curve grows faster, significantly from day 0 (4 hours after amputation) until day 5 after amputation. The growth indicates that some cells divided actively.

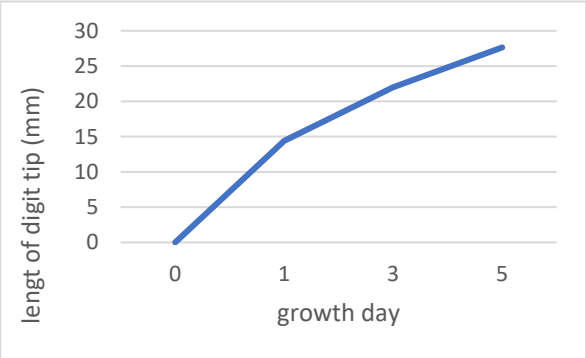


Figure 2: Growth curve of a digit tip mice (*Mus musculus*) from day 0 (4 hours after amputation) until day 5. Curve lines increase rapidly from day 0 to day 5.

3.2 The Result of Histology Staining

The histological analysis of digit tip mice (*Mus musculus*) shows the intense activity of cells in Figure 3. On day 0 (4 hours after amputation) and day 1, the wound area tissue dominated by white blood cells. In some of the wound area, there are red blood cells. On day 5, stem cells divided and differentiated to form the new tissue.

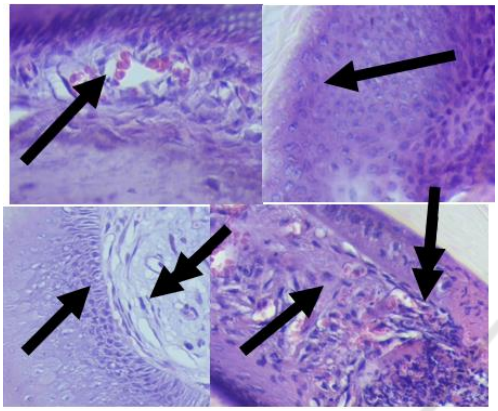


Figure 3: The Tissue histology of digit tip mice (*Mus musculus*) (A) Tissue digit tip mice on day 0 shows the presence of red blood cells (single arrow) that spread in the tissue due to injury when amputation (B) day 1, white blood cells that have many cell nuclei (single arrow) spread in the area wound (C) osteoblast cells (single arrow) that are actively dividing, fibroblasts like cells appear to start spreading in fat tissue (D) cells like fibroblasts (single arrows) division results begin to increase, the appearance of new blood vessels (double arrows) (enlargement 400 x).

3.3 mRNA Gene Expression

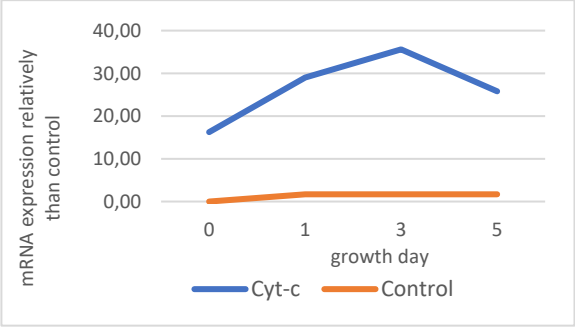


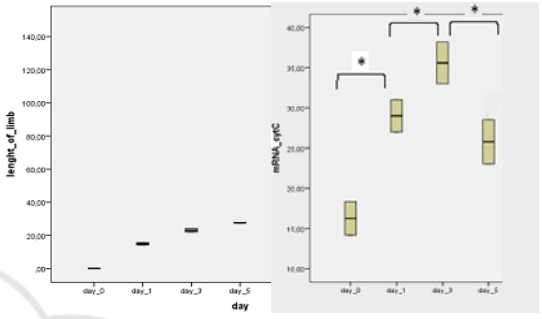
Figure 4: Cyt gene mRNA expression relative to control.

The results of gene expression quantitative relatively than controls produce diverse gene expression at each stage of tissue regeneration (Figure 3). In the inflammatory phase, the Cyt gene expression relatively higher than controls. Cyt gene expression increased and reached a peak on day 3 after amputation.

3.4 Statistical Analysis

3.4.1 Homogeneity Test

Homogeneity test results on the length of the digit tip of mice on each growth day showed a significant difference in tissue growth ($p < 0.05$) using the ANOVA test. There was no difference in the growth of digit tip tissue growth between day 0 to day 5. The difference in the expression of the Cyt gene mRNA was significantly different ($p < 0.05$) with the ANOVA test.



A B

Figure 5: Significantly different ANOVA homogeneity test ($p < 0.05$) (A) there is no different growth of digit tip mice (*Mus musculus*) from day 0 until day 5 (B) Cyt gene mRNA expression that is different between day 0 and day 1, between day 1 and day 3, between day 3 and day 5.

3.4.2 Correlation Test

Spearman correlation test results indicated there is no correlation between the expression of Cyt gene mRNA and the length of tip mice digit growth. Spearman correlation test results showed $p > 0.05$.

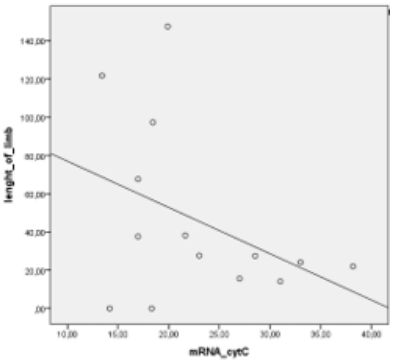


Figure 6: Spearman's correlation test between the growth length of digit tip mice with the mRNA expression of the Cyt gene ($p > 0.05$).

4 DISCUSSION

The growth of the digit tip mice (*Mus musculus*) tissue indicates a relatively rapid growth in the inflammatory phase. The results of the histological analysis showed the proliferation and differentiation of cell activity. White blood and fibroblast-like cells appear on day 5 Increaser than day 1. According to Mechner, in inflammation phase occurs the process of cleansing the tissue in the wound area from bacterial infection and cells undergoing apoptosis by white blood cells (Mescher, 2017).

This activity of cells requires high energy that it will trigger cellular respiration in mitochondria to produce energy (ATP) (Duguez, Féasson, Denis, & Freyssenet, 2002). Cytochrome-c (Cyt-c) is a protein in the inner membrane of mitochondria that play a role in capturing electrons in the respiration chain and acts as a deterrent and inhibits oxidative stress (Allen, 2011). The activity of cells requires high energy that will trigger cellular respiration in mitochondria to produce energy like ATP (Pelicano et al., 2003). Cytochrome-c (Cyt-c) protein is in the inner membrane mitochondria has a role in capturing of electrons, acting as a deterrent, and inhibiting oxidative stress. The other role of Cyt-c enzyme, it acts as an agent in the process of cell apoptosis (Allen, 2011); (Wright et al., 2007). The BCL-2 protein gives the signal to Cyt-c and stimulates the caspase enzyme in the process of apoptosis occurs (Allen, 2011).

In tissue regeneration of digit tip mice, the expression of Cyt-c gene is relatively high and reaches its peak in the inflammatory phase. We suspect that this expression is thought to be due to the role of Cyt-c in cellular respiration and the process of cell apoptosis.

Awarding to Osuma, there was an increase in energy demand that increased during the tissue regeneration process (Osuma, Riggs, Gibb, & Hill, 2018). The analysis of Cyt-c gene expression demonstrated that an increase in gene expression in the inflammatory phase accompanied by an increase in cell differentiation and proliferation. It's suspected that in this phase there was an increase in energy demands and increasing cell apoptosis process. Awarding to Bergmann and Steller, the process of cell apoptosis will stimulate the proliferation and differentiation of stem cells and progenitor cells in tissues (Akhmetshina et al., 2012). The histological analysis of digit tip mice tissue regeneration showed an increase in cell proliferation and differentiation after the severe of the Cyt-c gene expression. In the tissue, there is a proliferation of basal lamina cells, white blood cells, fibroblast-like cell cells, and the cells of dermis tissue. Activation of proliferation and differentiation of progenitor cells in the inflammatory phase produces the new blood cells and the layer of epidermis and dermis so that the wound area begins to cover.

The study results can be used as a reference for the next study about the stimulation of adult tissue regeneration that has limited ability in tissue regeneration. To stimulate adult tissue regeneration, we must try stimulating the expression of genes that play a role in overcoming inflammation, genes that play a role in providing energy, and genes that play a role in the process of proliferation, differentiation, cell migration, and tissue morphogenesis.

5 CONCLUSIONS

Cyt-c gene expression occurs in the inflammatory phase that stimulates the activity of cell proliferation and differentiation.

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