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Increased Number of Fibroblasts and Neovascularization after Tooth Extraction in Wistar Rats with Moderate-Intensity Continuous Exercise

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Abstract

Rapid wound healing after tooth extraction is expected to occur. Continuous moderate intensity exercise can improve the tissue oxygenation, that is required by fibroblasts and neovascularization to form granulation tissue which is an important process in wound healing. This study aimed to examine the increased number of fibroblasts and neovascularization after tooth extraction in wistar rat after continuous exercise with moderate intensity.

Wistar rats were divided into 4 groups, control group and the other 3 is the treatment group. The treatment group was given moderate intensity continuous exercise for 6 weeks (swim test). In control group (K1), the number of fibroblast and neovascularization was observed five day after tooth extraction. In the treatment group (K2, K3, and K4), observation was conducted on day 3, 5, and 7 after tooth extraction.

The number of fibroblasts and neovascularization of the control group was less than the treatment group. The data show a significant difference in control group (K1) with treatment group (K4) seven days after tooth extraction.

There was increase in the number of fibroblasts and neovascularization in the group was given moderate intensity continuous exercise, and rapid wound healing process correlate with increased the number of fibroblasts and neovascularization.

Experimental article (J Int Dent Med Res 2018; 11(3): 840-845)

Keywords: Fibroblasts, Neovascularization, Wound healing, Moderate intensity continuous exercise.

Received date: 16 April 2018

Accept date: 21 May 2018

Introduction

Wound is an injury case that is often experienced by human beings, one of which is the treatments performed by the dentists. One of the most frequent treatments performed by a dentist is tooth extraction. Based on the Basic Health Research (RISKESDAS) 2013, 74.8% of the population experienced delays in handling of the caries teeth and should require extraction, the percentage of tooth extraction increases by the age.¹ Tooth extraction is a conduct that give rise to a wound in the tooth socket. Wounds can easily heal, yet sometimes, there are various complications that may delay the wound healing

process.² Disruptions in the wound healing process may lead to tissue wound healing process can not takes place optimally.^{2,3}

Wound healing of the skin is a complex process, since involving various responses to injury. Generally, wound healing shows the organism's response to physical damage of tissues or organs and efforts to restore homeostasis to achieve physiological tissue or organ stability. That is characterized by the formation of functional epithelium over the wounded area.⁴

Wound healing process involves the cellular and extracellular components that ultimately regenerate the injured tissue. It begins with a series of important processes of hemostasis, inflammation, cell proliferation and migration, angiogenesis, matrix synthesis, remodeling and wound contraction.^{2,5}

The inflammatory stage involves inflammatory cells, including neutrophils, monocytes, eosinophils, basophils, lymphocytes, and platelets. Neutrophil infiltration reaches the peak around the first 24 hours. Within 2-3 days,

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the population of inflamed cells will be shifted and dominated by monocytes, that later will differentiate into macrophages. Macrophages not only resume tissue and bacterial debris phagocytosis, but also secrete growth factors as well as cytokines such as TNF- α , Interleukin 1 (IL-1) and Interleukin 6 (IL-6). These growth factors and cytokines initiate angiogenesis, proliferation of fibroblasts, and ECM (Extra Cellular Matrix) synthesis, it affects wound healing.⁶⁻⁸

The proliferation phase, the most important stage in the tissue recovery process that undergoes inflammation is the formation of granulated tissue. Histologically the granulated tissue is characterized by the proliferation of new blood vessels (neovascularization) and fibroblasts. In this phase the role of fibroblasts is very important in the process of wound healing.⁹ Fibroblasts will produce Vascular Endothelial Growth Factor (VEGF) that stimulates angiogenesis resulting in increased proliferation of new blood vessels. Angiogenesis is associated with the formation of granulation tissue, as newly formed cellular complexes must be supplied with oxygen and nutrients. Increased proliferation of new blood vessels and fibroblasts signifies an increased formation of granulation tissue which is an important process in wound healing.^{2,10}

The last phase in the wound healing process is the phase of maturation (remodeling). This phase is the longest phase in the wound healing process, occurring on the 21st day until the first year.¹¹

Physical exercise is one important way to keep the body healthy and fresh. Many benefits are obtained by doing physical activity regularly, for instance, can increase life spirits, passion, reduce stress, reduce working fatigue and can improve body fitness.¹²

Physical exercise with moderate intensity can increase the amount of VO₂ max in the body so that oxygen (O₂) is distributed throughout the body more, resulting in good tissue oxygenation. The oxygenation state of the injured tissue is a major determinant of wound healing.^{13,14} Tooth extraction will lead to a wide-open wound, most will experience healing in the absence of complications and the mucosa will be closed quickly.¹⁵ The occurrence of injury will cause the condition of the area around the wound into hypoxia due to disruption of the blood vessels. Acute hypoxia conditions can trigger cytokine synthesis and growth factors such as PDGF,

TGF- β , VEGF, and TNF- α . In chronic hypoxic conditions will have a negative impact on the body so that enough O₂ is needed to avoid damage to the area and the wound healing process can run properly.³

Researchers used mammals of wistar rats (*Rattus norvegicus*) because they had several advantages, such as histological structure and arrangement of rat mucosal tissue is similar to mucosal tissue in humans and the properties of rats are easy to maintain, easy to breed, relatively healthy, cheap so easy to get them, and have physiological similar to human.^{16,17}

Meanwhile, studies of the effects of moderate intensity exercise on increased fibroblasts and neovascularization in wistar rats have not been performed. Based on the above, the researchers wanted to do further research on the effect of moderate intensity exercise on the increase of fibroblasts and neovascularization in wistar rats (*Rattus norvegicus*) after tooth extraction.

This research was conducted in August-October 2017 at the Biochemistry Laboratory of Faculty of Medicine, Universitas Airlangga.

Materials and methods

This research was conducted in accordance to ethical clearance certificate no 214/HRECCFODM/X/2017, that is declared by ethical committee at Faculty of Dental Medicine Universitas Airlangga. This research took place in Biochemistry Laboratory of Medical Faculty of Airlangga University, using male Wistar rats (*Rattus norvegicus*) (28) that were divided into 4 groups: control group (K1) and treatment group (K2, K3, and K4).

Prior to tooth extraction in rats, the rat was first adapted for 1 week. The purpose of the first adaptation is to maintain the condition of the body to remain constant in different environmental conditions. In addition, adaptation is needed because adaptation is the way in which organisms overcome the environmental stresses to survive.¹⁸

The control group did not do any physical exercise while the treatment group did continuous intensity physical exercise (swim test). The selected physical exercise is the moderate intensity of 50% of the maximum swimming capacity (MSC). To determine MSC, mice were given a 3% load of weight and put in a

water bath. The initial time when a mouse starts to swim until it looks tired (air bubbles appear) is the time of MSC.

The treatment group (K2, K3, K4) was given continuous physical exercise (swim test) with medium intensity and given 3% load for 6 weeks. After the treatment has been completed, then all the rats in pull the mandibular incisors by using pliers. Then the irrigation tooth socket uses aquades.

The preparation of histometric preparations begins by cutting the rat's left lower jaw for a tooth socket after tooth extraction by including normal tissue and then fixed to maintain cell structure and components using formalin buffers. Then proceed with tissue process technique with paraffin method and coloring to facilitate the identification of the components in the network using light microscope.¹⁹

The control group (K1) was taken mandibular mandibles on the fifth day after tooth extraction and made preparations of tissue with Hematoxylin Eosin (HE) staining (Merck Chemical, Germany), after which the number of fibroblasts and neovascularization cells was calculated. Treatment groups (K2, K3, and K4) were taken mandibular mandibles on the third, fifth and seventh day after tooth extraction and the preparation of tissue preparation by HE was performed, after that, the cell count of fibroblasts and neovascularization was performed. Calculating the number of fibroblasts and neovascularization cells using a light microscope with 400x enlargement in a 5x viewing field (Figure 1). The data obtained were analyzed using *OnewayAnalysis of Variance* (ANOVA).

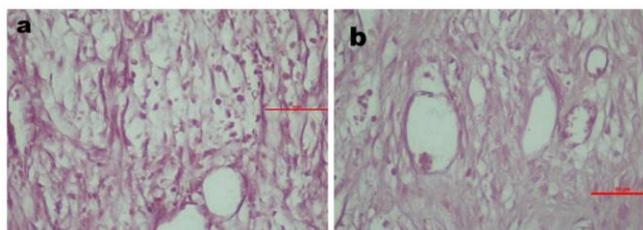


Figure 1. Fibroblast cells and neovascularization in the mandibular incisors sockets of wistar rats with HE staining 400x microscopy in the control group. (a). Cell fibroblasts (b). Neovascularization.

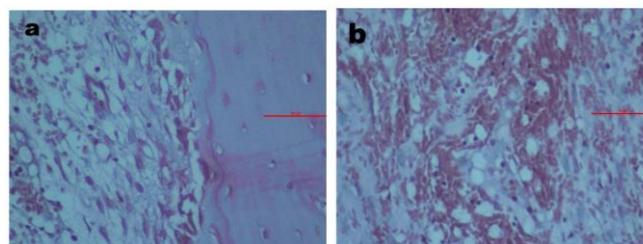


Figure 2. Fibroblasts and neovascularization cells in mandibular incisors sockets of wistar rats with HE staining, 400x microscopy in the post-tooth extraction treatment group. (a). Cell fibroblasts (b). Neovascularization. The images show an increase in the number of fibroblast cells and neovascularization in the treatment group.

Results

To confirm the hypothesis that moderate intensity continuous exercise (swim test) can increase the number of fibroblasts and neovascularization in wistar rats (*Rattus Norvegicus*) after tooth extraction, a descriptive statistical analysis was performed by normality test using Kolmogorov Smirnov test and homogeneity test of variance by using Levene's test. Based on the results of normality test by using Kolmogorov Smirnov test on cells fibroblasts and neovascularization obtained Sig value. above 0.05 for the control and treatment group which means that each group has normal distributed data.

The result of homogeneity test by using Levene's test on fibroblast cells obtained Sig value. 0.353 so that the data is the same or homogeneous. While the results of homogeneity test by using Levene's test on neovascularization obtained Sig value. 0.004 so that the data is not homogeneous. The end result of testing on fibroblast cells is normal and homogeneous so that further statistical tests performed are difference test using *Oneway Analysis of Variance* (ANOVA) test. At the end of the test the angiogenesis is normal but not homogeneous so that the appropriate difference test is the Kruskal-Wallis test.

The results of this study showed that the number of fibroblasts and neovascularization cells in the treatment group (K2, K3, and K4) was increased compared with the control group (K1).

The result of calculating the amount of fibroblasts obtained the results on average number of cell that showed in Table 1. From the calculation results, the amount of fibroblasts in

Group	Mean	SD	N
K1	11.43	1.27	7
K2	10.86	0.69	7
K3	14.57	2.37	7
K4	16.14	2.97	7

treated rats had a higher number than in normal or untreated rats. The parametric statistic test using Oneway Anova (Table 1) on fibroblasts is $P=0.155$ ($P>0.05$) meaning that there is difference but not significant between each group, either control group or treatment group. To find which intergroups differed was followed by a LSD post hoc test (Table 2).

The results of counting the number of neovascularization results obtained on the average number of cells could be seen at Table 3. From the results of the calculation, the number of neovascularization in treated rats had a greater number than in normal or untreated rats. This suggests that the amount of neovascularization increases with the given treatment.

Group	Mean	SD	N
K1	21.71	2.13	7
K2	23.14	1.07	7
K3	22.43	1.51	7
K4	23.86	2.12	7

Table 1. Average Number of Fibroblast Cells. Description: Control group (K1) and treatment group (K2, K3, and K4).

Group	F1	F2	F3	F4
F1	-	0.143	0.457	0.032*
F2	0.143	-	0.457	0.457
F3	0.457	0.457	-	0.143
F4	0.032*	0.457	0.143	-

Table 2. Different Fibroblast Test Results with LSD post hoc. The Significant Differences Are Indicated by Asterix Symbol.

The Kruskal-Wallis test was performed to determine the significant differences between the number of post-extraction teeth neovascularization in wistar rats (*Rattus norvegicus*) control group and treatment group that had been treated in the form of physical exercise. The result of non parametric statistic test using Kruskal-Wallis on neovascularization is $P=0.004$ ($P<0.05$) which means that there are significant differences

between each group, both control group and treatment group. To find out which among the different groups was continued with the Mann-Whitney test (Table 4).

Table 3. Average Number of Neovascularization. Description: Control Group (K1) and Treatment Group (K2, K3, and K4).

Group	K1	K2	K3	K4
K1	-	0,418*	0,039	0,008
K2	0,418*	-	0,019	0,005
K3	0,039	0,019	-	0,519*
K4	0,008	0,005	0,519*	-

Table 4. Neovascularization Difference with Mann-Whitney Test. The Significant Differences are Indicated by Asterix Symbol.

Discussion

The results showed that the average number of fibroblast cells on the fifth day after tooth extraction in the control group (K1) without treatment was 21.71. In the treatment group (K2) showed the average number of cells of fibroblasts of 23.14 and the treatment group (K3) was 22.43 cells. The highest number of fibroblast cells was found in the 7th day treatment group after the retraction (K4), which was 23.86 cells. These results indicate that there is a difference between the control group (K1) without treatment with the treatment group (K2, K3, and K4). The number of fibroblast cells in the treatment group was more than the normal group.

The results of the average number of neovascularization in the control group (K1) was 11.43 cells. In the treatment group (K3) as many as 14.57 cells. The highest number of neovascularization was found in the 7th day treatment group post-retraction (K4), which was 16.14 cells. In the treatment group (K2) showed the average yield of neovascularization amounted to 10.86. The average number is smaller than the control group. This is happened because at the time of cutting the sample of preparations to be made preparations, the object to be observed is covered by bone so that the observation does not give maximum results. These results indicate that there is an increase in the number of neovascularization in the fifth treatment group (K3) and the seventh day (K4). The number of neovascularization in the

treatment group was higher than the control group. However, in the third day treatment group (K2) the results decreased.

Increasing the number of fibroblast cells and neovascularization in the treatment group was influenced by physical exercise given 3 times per week for 6 weeks on a regular basis, thus increasing the oxygen consumption which will affect the proliferation phase. $VO_2\text{max}$ is the maximum amount of oxygen that can be consumed during intense physical activity. Physical exercise is expected to increase the supply of oxygen into the body, so as to increase the $VO_2\text{max}$ in the body, and oxygenation in the tissues increased.^{19,20}

Instantaneous injuries occur in the flow of blood vessels in the injured area, and inflammatory cells become very active and require a lot of oxygen for metabolic processes to be able to phagocytes microorganisms and necrotic tissue. This is what makes the conditions in the area around the wound into hypoxia. The condition of hypoxia is a state when the concentration of oxygen in the cell is very low. The condition of hypoxia can not be left for long. The process of wound healing in chronic wounds still requires a sufficient amount of tissue oxygenation.^{21,22} Oxygenation of the tissues can be increased by doing aerobic exercise continuously so that $VO_2\text{max}$ in the body increases.

In the inflammatory phase NADPH-linked oxygenase produces large amounts of oxidants through the consumption of large amounts of oxygen. Wound healing can occur in the presence of the enzyme, because oxidants are needed to prevent infection. Recent research shows that almost all cells in the environment around the wound are closely related to certain enzymes that convert O_2 into reactive oxygen species (ROS), free radicals and H_2O_2 . ROS acts as a mobile messenger in performing several important processes that support wound healing. Thus, O_2 acts as a nutrient for wounds and also acts as an antibiotic, and O_2 therapy is an alternative choice in wound healing.^{23, 24}

From the results of normal and homogeneous tests, Oneway Analysis of Variance (ANOVA) test is conducted to determine whether there is a significant difference between groups. The parametric statistic test by using Oneway Anova on fibroblas is $P=0.155$ ($P>0.05$) which means that there are differences but not significant between each group, either control

group or treatment group. The last test on neovascularization with non-parametric statistic test using Kruskal-Wallis was obtained $p = 0,004$ ($P<0.05$) meaning that there was significant difference between each group, both control group and treatment group.

This study resulted that there was a significant difference between control group wistar rats (K1) without treatment and wistar rats treated with continuous intensity physical exercise in the wound healing process due to tooth extraction. The results of this study also in accordance with previous research which proves that the process of wound healing post-extraction can be improved by doing moderate intensity exercise.

Conclusions

Continuous moderate intensity exercise can increase the number of fibroblasts and neovascularization in Wistar rats (*Rattus norvegicus*) after tooth extraction.

Declaration of Interest

The authors report no conflict of interest and the article is not funded or supported by any research grant.

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