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Submission date: 18-Mar-2016 05:18PM (UTC+0800)
Submission ID: 647030617
File name: Fatma_Yasmin_AUp_15_Pebruari_2016.rtf (12.53M)
Word count: 3753
Character count: 19976

Research Report

The Decreasing of Fibroblasts and Fibroblast Growth Factor-2 Expressions as a Result of X-ray Irradiation on the Tooth Extraction Socket in *Rattus novergicus*

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ABSTRACT

Background: *Wound healing involves cellular, molecular, physiological, and biochemical processes as responses to tissue damage. For instance, when a failure during tooth extraction occurs, radiographic examination, X-rays, is required. X-rays as an enforcer diagnosis can damage DNA chain, resulting in cell death and inhibition of wound healing process.* ***Purpose:*** *This research aims to analyze fibroblasts cell number and fibroblast growth factor-2 (FGF-2) expressions during wound healing process after tooth extraction as a result of X-ray irradiation.* ***Methods:*** *There were three research groups, each consisting of ten rats. Incisor tooth extraction was performed on the left lower jaw, and then X-ray examination was conducted at certain irradiation doses, namely 0 mSv, 0.08 mSv, and 0.16 mSv. Those animals were sacrificed on day 3, and on day 7 after the extraction, histopathology and immunohistochemistry examinations were conducted to determine fibroblast cell number and FGF-2 expressions. Data obtained were then analyzed by one-way ANOVA and Tukey HSD tests.* ***Results:*** *The number of fibroblasts decreased significantly in the group with the irradiation dose of 0.16 mSv applied on day 7 after the extraction ($p < 0.05$). Similarly, the number of FGF-2 expressions decreased significantly in the group with the irradiation dose of 0.16 mSv applied on days 3 and 7 after the extraction ($p < 0.05$).* ***Conclusion:*** *X-ray irradiation at a dose of 0.16 mSv can inhibit the healing process of tooth extraction wound due to the decreasing of fibroblasts cell number and FGF-2 expressions.*

Keywords: *Wound healing; tooth extractions; X-rays; fibroblasts; fibroblast growth factor-2*

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INTRODUCTION

Wound healing process is a body response to tissue damage. Wound healing process of tooth extraction actually has the same principles with wound healing process in general. The wound healing process of tooth extraction is a complex pathophysiological process involving cell proliferation, cell migration, synthesis and deposition of extracellular matrix proteins, and tissue remodeling.[1] There are four phases in the process of wound healing, namely haemostasis, inflammatory phase, proliferation phase, and remodelling phase.[2,3,4]

The prevalence of tooth extraction in Indonesia in 2007 is quite high, namely 38.5%. The extraction action can cause complications, such as bleeding, infection, fracture, and dry socket.[5,6] Thus, evaluation needs to be conducted to determine further actions in case of failure or complications during extraction process. One of them is by conducting dental radiographic examination.[7]

Dental radiographic examination is an examination aimed to get a picture of tooth socket by using X-ray irradiation. Periapical radiographic can provide information about the location and size of roots left due to tooth fracture. However, this examination can cause negative effects, such as mucosal epithelial cell damage and slow wound healing process. Saputra[8] even states that X-ray irradiation at a dose of 0.08 mSv can increase apoptosis and necrosis of the oral mucosal epithelial cells.

Dose used in a periapical radiographic examination actually is 0.08 mSv. This dose, unfortunately, can increase apoptosis and necrosis of oral mucosal cells.[8] Nevertheless, periapical radiographic examination may be conducted once more when a failure occurred during the previous manufacturing process of radiograph. As a result, irradiation dose derived from radiographic examination will increasingly transfer to the body. X-ray irradiation can damage DNA chains, carbohydrates, proteins, and lipids, resulting in inhibition of cell cycle checkpoint, inactivation of cell proliferation, induction of apoptosis, and inhibition of cell cycle. [9,10,11] Cells and tissues that play a role in healing process of tooth extraction wound are high radiosensitive, so mucosal epithelial cells, inflammatory cells, epithelial cells, and fibroblasts will get the direct effects of X-ray irradiation. Consequently, the wound healing process will be inhibited.[9,12]

Some growth factors, furthermore, also play a role in wound healing process. fibroblast growth factor (FGF) is a growth factor that has the potential effect in the repair and regeneration of tissue. FGF can be identified as a protein that can stimulate proliferation and deposition of fibroblasts, formation of granulation tissue, formation of new blood vessels, as

well as reepithelialization and deposition of extracellular matrix proteins. Some important FGFs required in wound healing process are Fibroblast Growth Factor-2 (FGF-2), Fibroblast Growth Factor-7 (FGF-7) and Fibroblast Growth Factor-10 (FGF-10).[13,14]

However, the effects of X-ray irradiation on the healing process of tooth extraction wound determined by fibroblast cell number and FGF-2 expression still have not been revealed. The reason is that fibroblasts and FGF-2 play a role in three of the four phases of wound healing, i.e. from the inflammatory phase to the last phase of tissue remodelling. Fibroblast Growth Factor-2 are mainly produced by macrophages and endothelial cells from day 2 to 4, while the active proliferation of fibroblasts occurs from day 3 to 7 after tooth extraction.[3] Therefore, this research aims to analyze fibroblasts cell number and Fibroblast Growth Factor-2 (FGF-2) expressions on days 3 and 7 after the extraction as a result of X-ray irradiation with a dose of 0.08 mSv and 0.16 mSv.

MATERIALS AND METHODS

Thirty male Wistar rats aged 8-10 weeks old and weighed 180-200 g were randomly divided into three groups, namely the control group, the treatment group 1 and the treatment group 2. Each of the groups was consisted of ten animals. Those animals were adapted at the Laboratory of Biochemistry, Faculty of Medicine, Universitas Airlangga, Surabaya for 7 days. They were then put in cages placed in a room with quite airflow and light. The base of the cage was covered with husks thickened 2 cm and replaced every two days.

Those animals were intramuscularly induced with ketamine and diazepam. Their mandibular incisors were extracted and exposed with X-ray irradiation. Luxation and rotation were conducted on the teeth until the teeth were unstable. The cervical of the teeth was drilled (± 1 cm) as a marker of making tooth fracture. Those teeth were then fractured. X-ray irradiation exposure was then given to the fractured tooth with 0.08 mSv dose for the treatment group 1, and 0.16 mSv dose for the treatment group 2.

Those teeth in the treatment groups were then extracted after exposed to X-ray irradiation. Meanwhile, those teeth in the control group were extracted without irradiation. The mandible of those five experimental animals was cut under anaesthesia ether 10% on day 3, while the mandible of the other five animals was cut on day 7. Tissue fixation was conducted in NBF 10% and decalcified into EDTA 10% to remove calcium from bone tissue. After the bone tissue has been softened, several processes were conducted, namely

dehydration, clearing, impregnation, embedding in paraffin blocks, and cutting tissue. The results of these phases obtained were slide preparations placed on object glasses.

HPA preparations were used for observing the number of fibroblast cells stained with HE, while immunohistochemistry preparations were used for observing the FGF-2 expression using antiFGF-2 (*Santa Cruz, SC-79*) and kit Novolink, Novocastra, RE7230-K. The reading of the results was conducted by observing those preparations under the light microscope with a magnification of 400 times to see fibroblasts and 1,000 times to observe FGF-2. The entire examination used H600L Nikon microscope equipped with a DS Fi2 300 megapixel digital camera and Nikkon image system as image processing software. Data then were analyzed with Statistical Product and Service Solutions (SPSS) for Windows version 17.0. Finally, data were analyzed by one way Anova test followed by Tukey HSD test.

RESULTS

Fibroblasts observed were found in the apical third of the tooth socket. Fibroblast cells had certain criteria, such as large, flattened, and oval core cells covered with delicate nuclear membrane and purplish red branches. Figure 1 shows fibroblast cells. The fibroblast cells in the control group, the treatment groups 1 and 2 on day 3 looked equally solid as shown in Figure 1. Meanwhile, fibroblast cells in the control group and in the treatment group 1 on day 7 looked more solid than in the treatment group 2. The highest mean of the number of fibroblasts on day 3 and 7 was found in the control group, while the lowest mean of the number of fibroblasts on day 3 and 7 was found in the treatment group 2.

Kolmogorov-Smirnov and Levene tests were conducted on the data about the number of fibroblasts cells after X-ray irradiation on tooth extraction wound. The results showed that the distributions of data on days 3 and 7 were normal and homogeneous ($p > 0.05$). Therefore, one way ANOVA test was conducted. The results showed that there was no significant difference in the number of fibroblasts cells on day 3 between in the control group, in the treatment group 1 and in the treatment group 2 ($p > 0.05$). However, there was a significant difference in the number of fibroblasts cells on day 7 among the groups ($p < 0.05$). Consequently, Tukey HSD test was conducted to determine differences in the groups on day 7. The results showed that there was a significant difference between the control group and the treatment group 2, and between the treatment group 1 and the treatment group 2 ($p < 0.05$).

Positive of FGF-2 expressions were shown in brown color. FGF-2 expressions were shown in Figure 2. On day 3, the densest FGF-2 expression was found in the control group.

On day 7, however, FGF-2 expressions in the control group and in the treatment group 1 seemed equally solid. FGF-2 expression in the treatment group 2 on day 7 was rarely found. It indicates that FGF-2 expression in this group was low. The highest mean of FGF-2 expression on day-3 was found in the control group, while the lowest mean was found in the treatment group 2. On the other hand, FGF-2 expressions on day 7 in the control group were the same as in the treatment group 1, while the lowest one was found in the treatment group 2.

DISCUSSION

X-ray irradiation can ionize atoms or molecules of the body, especially water molecules composing 70% of the body's components. The ionization makes free radicals formed become not stable and destructive.[3,12] The sensitivity level of various cells and tissues to irradiation is actually very diverse. The sensitivity of irradiation is called as radio-sensitivity. Rapidly dividing cells are very sensitive to irradiation, or so-called high radiosensitive, such as blood vessel cells (endothelial cells), white blood cells, blood-forming cells, mucosal epithelial cells, as well as forming sperm cells and egg cells. Meanwhile, cells dividing when there is damage have mid-radiosensitive, such as fibroblast, salivary gland cells, liver parenchymal cells, kidney, and thyroid. Cells that require a long maturation process has a low radiosensitive, such as muscle-forming cells, bone-forming cells and nerve tissue-forming cells.[12]

The results of the research on the effect of X-ray on the extraction wound of the mandibular incisors of the Wistar rats showed that there was no significant difference in the number of fibroblasts between in the control group, in the treatment group 1 and in the treatment group 2 on day 3 ($p>0.05$). It indicates that the initial response of fibroblast cells was good. In other words, the body can neutralize free radical damage caused by X-ray irradiation exposure at doses of 0.08 mSv and 0.16 mSv on day 3. Thus, there was no difference in the number of fibroblasts significantly.

Meanwhile, the results of the research on day 7 showed that there was no significant difference in the number of fibroblasts between in the control group and in the treatment group 1 ($p>0.05$). It means that X-ray irradiation at a dose of 0.08 mSv on day 7 did not decrease the number of fibroblasts. In other words, the body up to day 7 can still neutralize free radicals produced by X-ray exposure. Therefore, the irradiation exposure at this dose cannot damage or increase fibroblast cell death. As a result, the number of fibroblasts in this group was no significantly different from in the group without X-ray irradiation.

The results showed that there were significant differences between the treatment group 2 and the control group, as well as between the treatment group 1 and the treatment group 2 on day 7 ($p<0.05$). It indicates that X-ray irradiation at a dose of 0.16 mSv on day 7 decreased the number of fibroblasts since X-rays can generate quite high free radicals that can damage the chains of DNA, proteins, carbohydrates, and fibroblast cell lipid, while the body is still not able to repair the damage. The body needs time to regulate damage resulting in inhibition of fibroblast proliferation.[11]

Free radicals, furthermore, cause damage to DNA, such as change and loss of bases, breakage of hydrogen bond among chains, cross-linking, and breaking strands of DNA, both single-strand break (SSB) and double-strand break (DSB). Deoxyribonucleic acid and ribonucleic acid (RNA) are the building blocks of genes and chromosomes controlling all the metabolic processes in the body. DNA damage will cause a deviation in the metabolic process controlled by the defective gene. Disruption to the DNA then can be seen through cell death or genetic mutation. X-rays also interfere with the function of mitochondria of the cells, resulting in oxidation of carbohydrates, lipids and cell proteins, thereby disrupting the cycle of energy in the cells. Damage can also be in the form of protein denaturation and coagulation. Consequently, hydrogen bonds and disulphide bonds can be disconnected, so damaging secondary and tertiary structures that result in changes in protein activities.[11,15]

In addition, free radicals that are not neutralized by the body can lead to inactivation of cell proliferation, induction of cell apoptosis, checkpoint of cell cycle, as well as inhibiting cell cycle.[10,11] Therefore, the number of fibroblasts in tooth extraction wound after exposed to X-ray irradiation at a dose of 0. 16 mSv was significantly different from the group without irradiation and the group with X-ray irradiation at a dose of 0.08 mSv on day 7.

Saputra (2012), similarly, states that X-ray irradiation at doses of 0.08 mSv, 0.16 mSv and 0.24 mSv can decrease the number of mucosa epithelial cells of the Wistar rats on day 10 after exposure. The apoptosis of epithelial cells occurs because of the activation of the p53 protein occurred due to free radicals. The activation of p53 causes the induction of the cyclin-dependent kinase (CDK), thus retaining cell cycle on growth-1-synthesis (G1-S) phase and slowing down the repairing process of the damaged DNA before replication and mitosis progresses. In other words, apoptosis in epithelial cells will increase as the dose of X-ray irradiation increase.[8]

X-ray irradiation can decrease the number of inflammatory cells, epithelial cells, endothelial cells, and fibroblasts proliferating from day 3 to day 9 after an injury. The

decreasing of cell proliferation then can make granulation tissue formed be less. The imbalance of proliferation and the increasing of apoptosis can disturb the proliferative phase of wound healing process, so inhibit the whole wound healing process. The results also stated that on day 9, the size of the granulation tissue area in Wistar rats during the normal wound healing process is at 80.32 ± 2.11% area, so much bigger than in the wound irradiated with 1 mGy, which is 45.67 ± 0.86% area. Those results support the results of this research since the area of granulation tissue in the treatment group 2 on day 7 was smaller than the area of granulation tissue in the control group and in the treatment group 1.[9]

The results of this research showed that there was significant difference in the number of FGF-2 expressions on days 3 and 7 between in the control group and in the treatment group 2, and between in the treatment groups 1 and 2 ($p<0.05$). This indicates the body is able to repair the damage caused by irradiation exposure at a dose of 0.08 mSv. In other words, the irradiation at this dose does not give any effect on the regulation of FGF-2 in the wound healing process, but decreases FGF-2 expressions.

In addition, the treatment group 2 had the lowest number of FGF-2 expression, both on day 3 and day 7. This condition is caused by the decreasing of FGF-2 produced by macrophages and endothelial cells. Endothelial cells that have a high radiosensitive character will get a direct effect when exposed to X-ray irradiation, such as inhibition of proliferation and increasing of apoptosis. The decreasing of the number and function of endothelial cells then can result in the decreasing of FGF-2 expression.[12]

Macrophages are the results of monocyte differentiation in tissues with chronic inflammation. Macrophages play an important role in the immune system. Monocytes, on the other hand, are inflammatory cells that are hypersensitive or high radiosensitive. As a result, monocytes can be damaged when exposed to X-ray irradiation. X-rays lead to oxidative stress and trigger reactive oxygen species (ROS) in the body. ROS can easily penetrate walls and monocyte components, and can also damage monocyte DNA. Consequently, the number and function of macrophages derived from monocyte differentiation decrease.[15]

Monocytes exposed to X-ray irradiation, furthermore, experience DSB induction and DNA base modification. The DNA damage is the originator of cell death due to DNA damage response (DDR) controlled by ATR-Chk1-ATM-Chk2-p53 pathway, leading to response of Fas and activation of caspase-3, caspase-7, and caspase-8. Apoptosis is an activity caused by the execution of DDR, and can result in the death of monocytes 3 hours after the irradiation exposure.[15,16]

During wound healing process, macrophages and endothelial cells are the major producers of FGF-2, which has the role of stimulating fibroblast proliferation and regenerating blood vessels as a supply of oxygen and nutrients to the cells. The decreasing of the number and function of macrophages and endothelial cells then will lead to the decreasing of FGF-2 and fibroblast proliferation, the formation of new blood vessel, the deposition and synthesis of extracellular matrix, thus inhibiting the whole healing process.[1,13,14]

The conclusion of the study was X-ray irradiation could delay wound healing. X-ray irradiation on tooth extraction socket at a dose of 0.08 mSv cannot decrease the number of fibroblast cells on days 3 and 7 after the extraction. X-ray irradiation on tooth extraction socket at a dose of 0.16 mSv, on the other hand, cannot decrease the number of fibroblast cells on day 3, but can lower the number of fibroblasts on day 7 after the extraction. X-ray irradiation on tooth extraction socket at a dose of 0.08 mSv cannot also decrease FGF-2 expressions on days 3 and 7 after the extraction. Finally, X-ray irradiation on the tooth extraction socket at a dose of 0.16 mSv can decrease FGF-2 expressions on days 3 and 7 after the extraction.

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Table 1. The mean and standard deviation of the number of fibroblast cells and FGF-2 expressions on days 3 and 7

	Number of fibroblast cells		FGF-2 expressions	
Group	Day 3	Day 7	Day 3	Day 7
Control	355.4 ± 109.07	435.4 ± 68.332	8.6 ± 1.673	7.0 ± 0.707
Treatment 1	269.6 ± 69.547	374.2 ± 66.792	6.8 ± 1.789	7.0 ± 1.581
Treatment 2	214.4 ± 74.942	203.8 ± 59.912	3.2 ± 0.837	1.8 ± 0.837

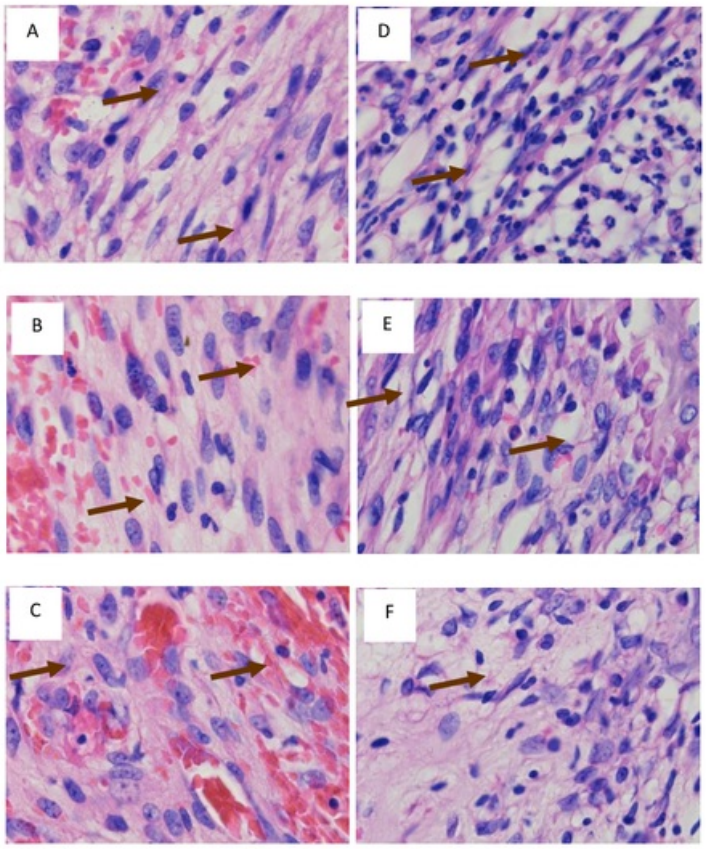

Figure 1. Fibroblasts as shown with arrows. Fibroblasts, in the control group on day 3 (A), in the treatment group 1 on day 3 (B), and the treatment group 2 on day 3 (C), looked equally solid. Fibroblasts, in the control group on day 7 (D) and in the treatment group 1 on day 7 (E) looked more solid than the treatment group 2 on day 7 (F).

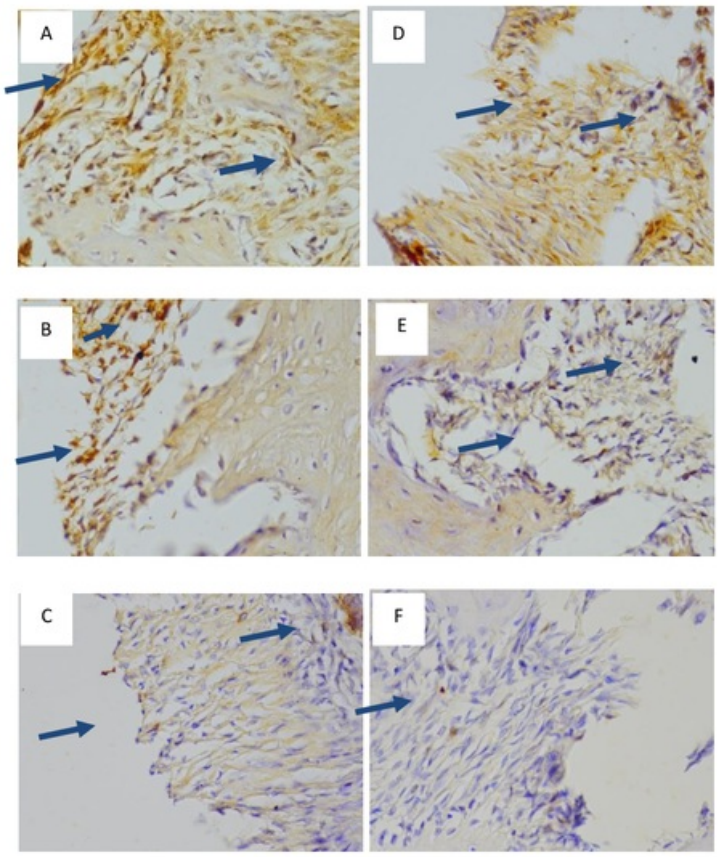

Figure 2. FGF-2 Expression as shown with arrows. FGF-2 expression, in the control group on day 3 (A) looked more solid than the treatment group 1 on day 3 (B) and the treatment group 2 on day 3 (C). FGF-2 expression, in the control group on day 7 (D) and the treatment group 1 on day 7 (E) looked equally solid. FGF-2 expression in the treatment group 2 on day 7 (F) is less observed.

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