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Effect of Histatin-1 Proteins to Fibroblasts Growth Factor-2 (FGF2) Concentrate and Fibroblasts Cell Migration on Human Gingival Fibroblast (HGF) Culture

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KEYWORDS

Histatin-1;
Fibroblast growth factor 2;
Human Gingival Fibroblast (HGF).

Abstract The purpose of this study is to determine the effect of histatin-1 protein increasing the concentration of fibroblast growth factor (FGF2) and fibroblast cell migration in human gingival fibroblast (HGF) culture. The HGF cell culture used was obtained from odontectomy patients and was the result of the 4th passage. Cell culture was divided into 4 groups: HGF control group (without treatment) and treatment groups with 5µM, 10µM, and 20µM concentration of histatin-1 respectively. The amount of fibroblast growth factor 2 (FGF2) was quantified by using ELISA method. In vitro fibroblast cells migration was measured using scratch/wound healing assay method. FGF2 concentration and HGF cell migration were measured at hour 0, 3, 11, 24, and 48. The results of the One Way Anova statistical test difference between FGF 2 (ΔFGF2) at hour 24 showed a value of P = 0.042 (P≤0.05) and HGF cell migration at hour 11 showed a P value = 0.003 (P≤0.05). The conclusion of this study is that Histatin-1 Protein is able to increase the concentration of Fibroblast Growth Factor 2 (FGF2) and the migration of Human Gingival Fibroblast cells.

Introduction

Nearly 37% of dental extraction, especially the third molar, are followed by a post–perative complication called alveolar osteitis (Khoswanto, 2010). Alveolar osteitis is commonly known as dry socket. Dry socket could be caused by the disruption in wound healing where fibroblast cells are not perfectly formed in the socket area of teeth (Kolokythas *et al*., 2010).

Histatin is one of the proteins found in human saliva. Based on the chemical structure of amino acids, histatin consists of several variations, namely histatin-1, histatin-2, histatin-3 and histatin-5. Histatin-1 and 2 are degradation products of HTN1 genes Histatin-3 is a degradation product of HTN3 genes. Histatin-1 and 2 play roles to accelerate the process of wound healing by increasing cell proliferation and migration (Oudhoff *et al*., 2008). Histatin-3 and 5 have long been known as antimicrobial and antifungal proteins (Boink *et al*., 2016). Histatin-1 is proven to increase the in vitro migration of epithelial cells. Histatin protein-1 can increase the wound healing process through bonds with g-protein-coupled receptors (GPCR) through the extracellular signal-related kinases (ERK) 1/2 mitogen-activated protein kinases (MAPK) pathway (Oudhoff *et al.*, 2009).

Fibroblast growth factor is a polypeptide that functions as a homeostatic factor in

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response to injury. Fibroblast growth factor-2 or basic FGF (bFGF) is functioned to help the formation of granulation tissue, re-epithelialization and remodeling. The previous in vitro studies have shown FGF2 could increase the cell migration (Barientos *et al*., 2014)

Material and Method

Human gingival fibroblast (HGF) cell culture

Culture cells of human gingival fibroblast (HGF) used in this study is taken from human gingivae with age range from 20 - 30 years old. The gingiva used were gingiva in patients with odontectomy which meet the specified inclusion criteria. All sampling activities must obtain informed consent where the patients had to sign a written agreement and meet the criteria determined by Ethics Committee of Faculty of Medicine of University of Brawijaya (No.60/EC/KEPK-S2/03/2018). Cell culture was carried out in Faculty of Medicine of University of Brawijaya's biomedical laboratory. Medium transfer consisted of HBSS and antibiotics (penicillin streptomycin amphotericin 1.25%). Gingiva were washed with serum free medium (SFM) which is composed of DMEM F12, nabic, ascorbic acid, DI water and antibiotics (penicillin streptomycin amphotericyn 1.25%). The gingivae wer inserted into a culture medium (complete medium) made from free medium serum with 10% FBS added. Gingiva were cut into small pieces by approximately 1 x 1 mm using a surgical scissors, and then it was put in a centrifuge for 8 minutes at a speed of 800 rpm. The culture was incubated in an incubator at 37°C with 5% CO2 content. Cell culture was washed and the culture medium was replaced 3 times a week. Cell culture harvesting was carried out after the cell culture reached 90% confluence. Cell culture used was the cell culture from the 4th passage. Cell culture method in this study accordance with Saczko *et al*. (2008)'s method but with modifications.

Cell Migration Measurement

HGF cell culture was observed with an inverted microscope and photographed to see changes in cell-free zone of the cell culture in hour 0, 3, 11, 24 and 48.

FGF2 Concentration Measurement

HGF cell culture was moved to a 24-well plate and was incubated for 48 hours until it reached 80% confluence. Culture medium in the well plate was disposed and the HGF cell culture was washed with serum free medium (SFM). Each well plate was scratched using tweezers and filled with the culture medium (complete medium) and histatin-1 protein according to each treatment namely: 5µM of histatin-1, 10 µM of histatin-1, 20 µM of histatin-1 and the control group (culture medium and HGF. Then, the cell culture was placed in an incubator with temperature was set to 37° C with 5% of CO2 content. HGF cells were observed at hour 0, 3, 11, 24, and 48. The culture medium was taken as much as 100µL in every observation period and stored at -80°C and would be further used in an ELISA test.

Results and Discussion

Cell Culture Characteristics of Human Gingival Fibroblast

The fibroblast cells were planted in a round-form culture media. Three hours after planting in the culture media, the cell began to stick to the culture flask. Day 4th, the cells extended in spindle-like shapes and convex edges which showed the features of fibroblast cells. Morphological features of fibroblasts are elongated cells, oval-shaped core and form a linear or bundle-like formation. Fibroblast cells' growth in gingiva's explant area is starting from the edge of the explant. On day 7, the fibroblast cells formed colonies. There was a tendency for fibroblast cells to grow near cell colonies and then form new colonies. On day 14, fibroblast cells reached around 80% confluence. Fibroblast cells started to have a growth spurt on day 4 until day 14 (Figure 1)

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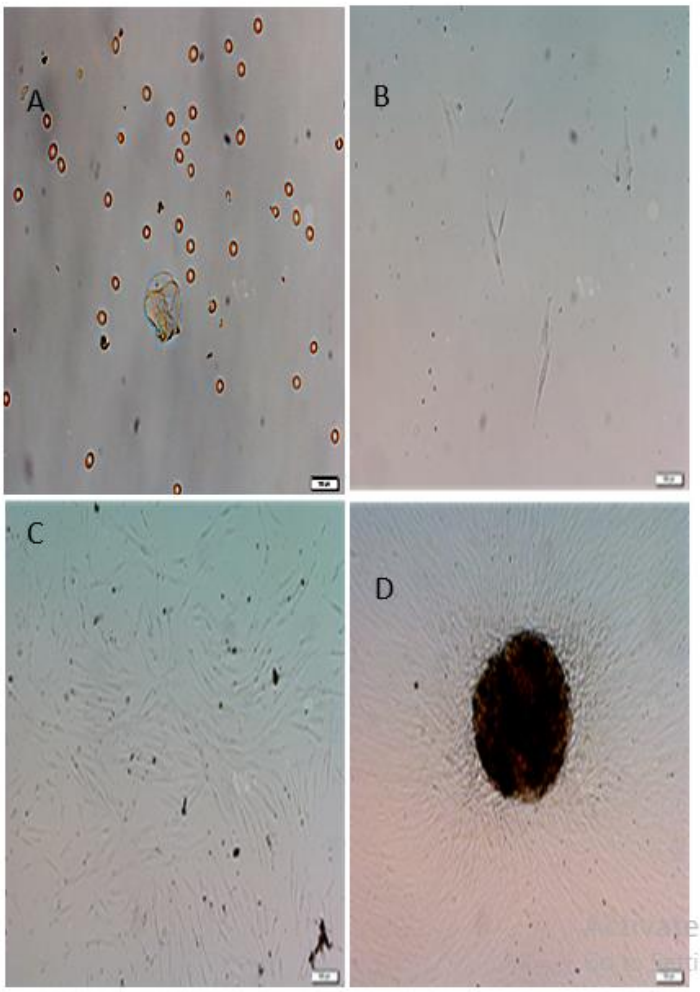

Figure 1 A) Fibroblast cells on day 0 after planted in round-shape cell culture medium B. Day 4, the cells are in spindle form. C. Day 7, the cells formed colonies D. Day 14

HGF Cell Migration

Cell migration test was carried out by observing each well plate at hour 0, 3, 11, 24 and 48. Cells were observed on an inverted microscope and photographed according to the results seen under the microscope. To ensure the photo taken is in the same area for each observation, each well plate lid was marked. Cell migration test result is presented in percentages and measured using the formula below:

$$\% \text{ migration} = [(A0 - A1)/ A0] \times 100$$

A0 is the initial distance of cell-free zone after a scratch is created using tweezers, A1 is the final distance of the cell-free zone at each time of observation. The cell-free zone edge border is determined by connecting at least 3 cells on the edge of the cell-free zone (Khrisnawamy, 2014). It can be seen in Figure 2 and 3.

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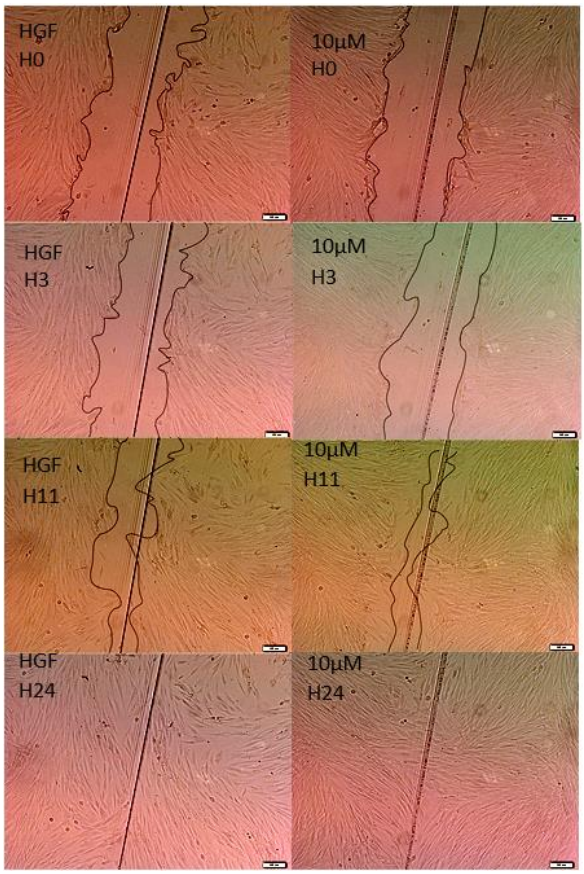

Figure 2 Cell migration test results of the control group and 10µM histatin-1 (HTN1) treatment group at hour 0, 3, 11 and 24. The results of HGF cell migration test showed the distance between the edges of the cell-free zone on 10µM treatment group at hour 11 was smaller than the control group (HGF). The cell-free zone was covered for 100% at hour 24 on both treatment and control groups.

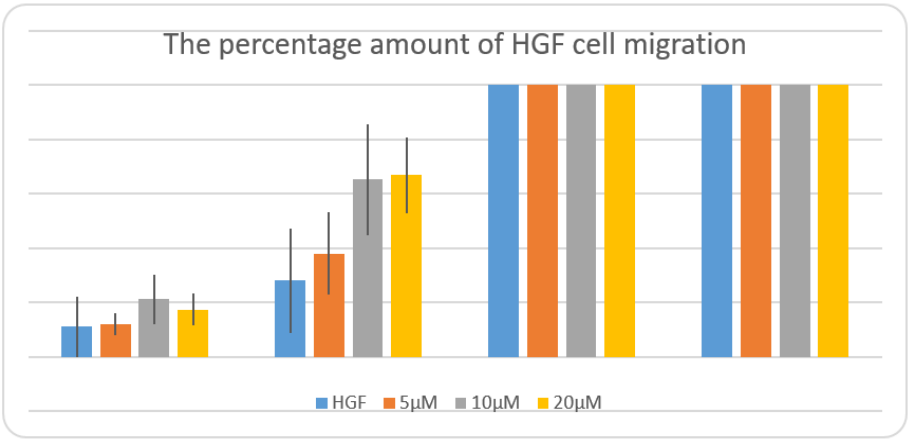

Figure 3 The percentage amount of HGF±SD cell migration at hour 0, 3, 11, 24 and 48

The highest value of HGF cell migration at hour 3 is shown by 10µM treatment group (21.22%±9.13%) and the lowest is shown by the control group (11.34% ±11.07%). At hour 11, the highest HGF cell migration is shown by 20µM of histatin treatment group (66.85%±13.93) and the lowest value of HGF cell migration is shown by the control group (28.30±19.31). The value of HGF cell migration in all treatment groups and control groups at hour 24 and 48 showed 100% migration.

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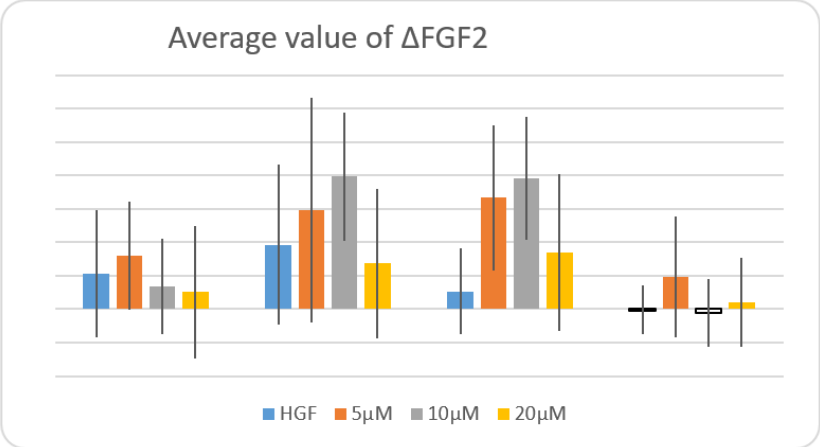

Figure 4 Average value of ΔFGF2±SD in each treatment groups and control group at hour 3, 11, 24 and 48.

The results of the One Way Anova statistical test of HGF cell migration at hour 3 showed P value = 0.125 (P>0.05) which means there is no significant difference between treatment groups and control groups at hour 3. One Way Anova test for HGF cell migration at hour 11 showed P value = 0.003 (P≤0.05), this shows that there is significant difference in HGF cell migration between the treatment and control groups. The follow-up Tukey post-hoc test showed that control group and the 10µM treatment group have the most significant difference.

The Kruskal Wallis test for HGF cell migration at hour 3 showed a P-value of 0.057 (P>0.05), which means that there is no significant effect on cell migration at hour 3.

FGF2 Concentration

This research used 23 samples divided into four groups, namely: The first group is in the control group consisted of culture medium and fibroblast cells (HGF). Another three groups worked as treatment groups. Treatment groups are consisted of three different concentration of histatin (HTN1) protein, namely 5 µM, 10 µM dan 20 µM. The result of ELISA test for the four groups is as indicated in Figure 4.

The highest average value of ΔFGF2 at hour 3 is shown by histatin-1 5µM (79.87±80.66) treatment group and the lowest is shown by 20µM (25.41±99.08) treatment group. The highest average value of ΔFGF2 at hour 11 is shown by 10µM (198.60±63.30) treatment group the lowest is shown by 20µM (68.30±112.21) treatment group. The highest average value of ΔFGF2 at hour 24 is shown by 10µM (195.28±91.93) treatment group meanwhile, the lowest average value of ΔFGF2 is shown by the HGF (26.36±64.17) control group. An observation at hour 48 showed that the highest average value of ΔFGF2 is found in the 5 µM (48.25±90.22) treatment group.

The results of the One Way Anova statistical test of ΔFGF2 at hour 3 showed P value = 0.713 (P>0.05) which means there is no significant difference between groups at hour 3. The results of the One Way Anova statistical test of ΔFGF2 at hour 24 showed P value = 0.042 (P≤0.05) which means there is a significant difference between treatment groups and control groups. A follow-up Tukey test was carried out to find out which groups would show significant difference. At hour 24, the results showed that control group (HGF) and 10µM histatin-1 treatment group have significant difference. So, it can be concluded that the exposure of 10µM histatin-1 is able to increase the expression of FGF2 in HGF cell cultures. The results of One Way Anova of ΔFGF2 at hour 48 showed P value = 0.498 (P>0.05). Because P value is greater than 0.05, there is no significant difference of ΔFGF2 between control group and

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treatment groups at hour 48. The Kruskal Wall is test was carried out as a non-parametric test of One Way Anova. The data of ΔFGF2 at hour 11 was abnormally distributed in 20μM treatment group, the P-value was 0.033 (P≤0.05) which means it is not significant.

Histatin is one of the proteins found in human saliva. Histatin protein-1 can increase the wound healing process by inducted cell migration process (Oudhoff *et al*., 2008; Shah *et al*., 2017). The results of this research showed a significant difference of ΔFGF2 between treatment groups exposed to histatin-1 and control group (HGF) at hour 24 with value of P = 0.042 (P≤0.05). In the previous study, it is found that the histatin with concentration ranges from 5 μM - 50 μM can induce cell migration without causing cell death. This is in line with other studies that show that histatin-1 can improve wound healing through increased cell migration.

Histatin can activate cells through bonds with G-protein-coupled receptors which then activate the extracellular signal-related kinases (ERK1/2) pathway (Oudhoff et al, 2009). Extracellular signal-related kinases (ERK1/2) is one of the cascades in the mitogen activated protein kinase (MAPK) signaling pathway that plays a role in cell proliferation and migration (Sun, Yu et al, 2015). Oudhoff et al conducted a study to determine the signaling pathways that mediate histatin to induce cell proliferation and migration. The result showed that histatin does not induce proliferation and cell migration in samples exposed in inhibitor U0126 which is an ERK 1/2 inhibitor. In the meantime, the other samples exposed to SB203580 inhibitors (p38MAPK inhibitors) and AG1478 (EGRF inhibitors) do not affect cell proliferation and migration. This shows that histatin is able to induce cell proliferation and migration through the signaling pathway of extracellular signal-related kinases (ERK1/2).

In this study, the concentration of 10μM is the most effective in increasing the concentration of FGF2. When the concentration of histatin-1 is increased, the number of ligand bonds and receptors (GPCR) will also increase. Unfortunately, the increasing number of intracellular ligand-receptor bonds resulted in a longer endocytosis process. After the ligand is degraded by the cells, the receptors will be recycled again to return to the cell membrane. But the number of bonds between ligand and receptors at higher doses of histatin-1 results in less effective recycling of receptors towards cell membranes (Oudhoff *et al*., 2009). This resulted in doses greater than 10μM for not being effective in increasing the concentration of FGF2. The average value of ΔFGF2 concentration was gradually increased starting from hour 3 until hour 11. The highest peak effect on control group and 10 μM treatment groups was seen at hour 11 Average value of ΔFGF2 expression was gradually decreased starting from hour 24 until hour 48. Treatment groups of 5 μM and 20 μM Histatin-1 showed an increase in average value of ΔFGF2 expression at hour 3, 11 and 24 where it reached the peak effect at hour 24. Treatment groups of 5 μM and 20 μM Histatin showed a decrease in average value of ΔFGF2 expression at hour 48. This shows that histatin-1 proteins does not caused an increase in FGF2 concentration continuously. FGF-2 expression started to decrease gradually at hour 24 (10μM treatment) and hour 48 (5 μM and 20 μM treatments) after there is an increase in FGF-2 expression. Excessive expression of fibroblast growth factor (FGF) can lead to the development and metastasis of neoplasms in humans (Korch, 2009).

The results of the human gingival fibroblast cell migration test at hour 3 showed the highest average value of HGF cell migration is found in the 10μM histatin treatment group with an average cell migration value by 21.22%. The

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results of the Kruskal Wallis test between groups at hour 3 showed no significant effect of HGF cell migration (P = 0.057). At hour 11, the highest average value of cell migration was shown by the 20 µM treatment group which reached 66.85%. In the 10µM treatment group, the value of HGF cell migration in hour 11 was 65.10%. The results of the One Way Anova test between groups at hour 11 showed a significant difference in HGF cell migration (P = 0.003). The cell migration in all treatment groups and control groups reached 100% at hour 24 and 48. This shows that the peak of cell migration occurs before the 24th hour.

Histatin-1 with a 10µM concentration is able to increase the cell migration (Shah *et al*., 2017). This is in line with the result of this study that with a concentration of 10µM histatin-1, there is a significant difference in ΔFGF2 and significant HGF cell migration. In addition, the 20µM treatment group also showed a significant difference in HGF cell migration but does not induce a significant difference in ΔFGF2 concentration value.

Before performing a scratch assay, a serum starving was carried out for 6 - 12 hours. Serum starving is carried out by decreasing the percentage of serum (FBS) to 0.5% in a culture medium. Serum starving is designated to reduce cell proliferation ability caused by serum. But this study did not carried out the serum starving first. The reason being is because this study used HGF cell in the passage or the fourth sub-culture. Passage process or sub-culture involved enzymatic reactions could be resulted in an aging process for fibroblast cells. Aging process cause a decrease in the cell's ability to proliferate. In this study, HGF cells were confluent within 24 hours of the first phase. In the third passage, HGF cells took 7 days to become confluent. This shows that the passage process/sub-culture process cause the ability of HGF cells to proliferate to decrease. Human gingival fibroblasts used for cell migration test are the result of the fourth passage and there was no serum starving carried out prior to the migration test (Nguyen, 2018). In Fronza *et al.* (2009)'s research, the culture medium used in the scratch assay for fibroblast cell culture was 10% DMEM and 10% fetal calf serum, or in other words it was carried out without serum starving procedure. This shows that the observation of cell migration can be performed on cells with decreased proliferation ability without serum starving.

Conclusion

Histatin-1 protein is able to increase the concentration of Fibroblast Growth Factor 2 (FGF2) and the migration of Human Gingival Fibroblast cells.

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