Expression of FGF-2 and Fibronectin in Citrus limon Fruit Peel Malang Essential Oil Gel Treated Traumatic Ulcer in Diabetic Wistar Rats (Rattus novergicus)

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ABSTRACT:
Diabetes Mellitus is a metabolic disease that delayed traumatic ulcer healing process. Citrus limon contains fumaric acid, d-limonene and citral that can accelerate the healing of traumatic ulcer. The aims of this study were to investigate the effect of 0.78% Citrus limon essential oil topical application can increase Fibroblast Growth Factor-2 (FGF-2) and Fibronectin expression. This study was true experimental analytic study with random sampling methods. The study was conducted on 30 male Wistar rats (Rattus novergicus) induced with DM by injecting 50 mg/kg of Streptozotocin, intraperitoneally, and a traumatic ulcer on their lower lip mucosa. These were divided into six groups; three each for control and treatment groups. Each control and treatment group consisted of five rats. The control groups treated with CMC 5% gel and treatment groups were administered with Citrus limon peel essential oil gel. The expression of FGF-2 and Fibronectin was observed on Days 5, 7 and 9. Furthermore, mice sacrificed and the lower lip labial mucosa tissue of mice has been taken to make the Histopathology Anatomy preparation by means of immunohistochemical examination with monoclonal antibodies anti-FGF2 and anti-Fibronectin. ANOVA analysis was performed to analyze the difference between group (p<0.05). The increased FGF-2 and Fibronectin expression in the treatment group as compared to that of the control group. There were significant differences (p<0.05) of both FGF2 and Fibronectin expression between the two groups. The citrus limon peel essential oil gel increased the FGF-2 and Fibronectin expression during the healing process of traumatic ulcers on the oral mucosa of diabetes afflicted Wistar rats (Rattus novergicus).

KEYWORDS: Diabetes Mellitus, Citrus limon Essential Oil Gel, Traumatic ulcer, Fibroblast Growth Factor-2, Fibronectin, Herbal Medicine.

INTRODUCTION:
Diabetes mellitus (DM) is a metabolic disease with hyperglycemia condition because an impairment towards insulin secretion, the work of insulin, or combination1,2. According to a few studies there is an increase on the prevalence of DM patients worldwide3,4. DM patients in Indonesia as many as 384 million5. Ulcer is the most common disease that could be found in the oral cavity mucosa6,7. Ulcer is the condition where there is a loss of epithelial layers until more than the basal membrane and affects the lamina propria. Traumatic ulcer is an ulcer caused by physical, chemical, and also thermal trauma. There are a few complications that could happen in DM patient such as an extended ulcer healing time. There is an increase of complication incidences such as infection and wound dehiscence when ulcer that does not heal completely8,9,10.

Several Growth Factors (GFs) play an important role in the wound healing process of the ulcer. Fibroblast Growth Factor (FGF) is a growth factor that has a potential effect towards the fixing and regeneration of tissues. There are many important FGFs in the healing process such as FGF-2, FGF-7, and FGF-1011,12,13,14.
known from the FGF-2 expression, the amount of fibroblast and capillary vessels. The expression of FGF-2 could be known immunohistochemically as a variable to be studied because the fibroblasts chemotactic GF could stimulate angiogenesis and synthesize Fibronectin. Fibronectin at the end produces new blood vessels thus accelerates wound healing. The aim of this study to investigate the topical application of 0.78% Citrus limon essential oil can increase FGF-2 and Fibronectin expression of traumatic ulcers on the oral mucosa of diabetes afflicted Wistar rats (Rattus norvegicus).

MATERIAL AND METHODS:
Ethical Clearance:
This study received an ethical clearance approval letter for animal subjects from the Ethics Research Committee Faculty of Dental Medicine, Universitas Airlangga Surabaya, East Java, Indonesia with number 065/HRECC.FODM/VI/2017.

Citrus Limon Essential Oil Gel Preparation:
Citrus limon used was taken from BALIJRESTO of Malang city, East Java, Indonesia. The peel of Citrus limon is taken to obtain the essential oil with the steam distillation method. The essential oil is dissolved with 3% CMC (Bioworld, China) thus it is obtained the result of the essential oil gel containing citrus limon with the 0.78% concentration.

Animal subject preparation:
The subjects consisted of 30 male Wistar rats (Rattus norvegicus) 200-250 gr body mass, aged 2-3 months, adapted for seven days, provided with standard rat food constituting 10% of their body weight and drinking water at a temperature of 22 ± 2°C. Wistar rats were injected intraperitoneally with a single 50 mg / kg BW dose of streptozotocin (STZ) (Bioworld, China). After three days, rats with random blood glucose ≥ 126 mg / dl or fasting blood glucose ≥ 126 mg / dl were used as subjects in further procedures. Ulceration of the labial mucosa of subjects’ lower lips was induced by thermal injury through application of a ball burnisher tip animal models were injected with 0.05-0.1ml/10g body Intramuscular (IM) rodent anesthesia (ketamine, xylazine, acepromazine and sterile isotonic saline) (Sigma Aldrich, US). After 24 hours, the formation of ulcers could be observed.

The Propolis Citrus Limon Gel Extract Administration:
30 male Wistar rats suffering from DM with induced ulcers on the lower labial mucosa were divided into 3 control groups (Days 5, 7, and 9) each consisting of five rats treated with 5% CMC gel and 3 treatment groups (Days 5, 7, and 9) with the same number of rats, were administered gel Citrus Limon Peel Essential Oil topically. Observations of FGF-2 and Fibronectin expression were conducted on Days 5, 7, and 9.

The Effect of Citrus Limon Peel Essential Oil Gel FGF-2 and Fibronectin Expression on the Healing Process of Traumatic Ulcer:
The rats acclimated and the lower labial mucosa tissue was prepared for immunohistochemical imaging using monoclonal antibody (anti FGF-2) with antigen reaction (FGF-2) and monoclonal antibody (anti-Fibronectin) (Sigma Aldrich, US). with antigen reaction (Fibronectin) reacted with Diaminobenzidine (DAB) substrate (Sigma Aldrich, US). FGF-2 expressing endothelial cells appear brown under 1000x magnification and fibroblast cells expressing Fibronectin appeared brown under electron microscope (Olympus, Japan) at 1000x magnification in 5 different fields examined by two expertise and cells were counted.

Data Analysis:
The obtained data was then tested for its normality using One-Sample Kolmogorov-Smirnov test to know whether the data obtained distributed normally an Levene test was performed to know data homogeneity variance (P>0.05). One Way ANOVA was conducted to understand the significant difference between control group and treatment group (p<0.05).

This experiment demonstrated increased FGF-2 and Fibronectin expression on treatment group with application of Citrus Limon Peel Essential Oil Gel compared to the control group. Immunohistochemistry examination showed FGF-2 and Fibronectin expression have significant difference between treatment and control group that can be seen on Figure 1 and Figure 2.

![Fig. 1. Immunohistochemistry examination showed FGF-2 expression (1000x magnification). Positive reaction showed brown color on cytoplasm indicating reaction between antigen (FGF-2) and monoclonal antibody (anti FGF-2) (Red Arrow). (A) FGF-2 expression in endothelial cells of control group on Day 5. (B) FGF-2 expression in endothelial cells of treatment group on Day 5. (C) FGF-2 expression in endothelial cells of control group on Day 7. (D) FGF-2 expression in endothelial cells of control group on Day 7. (E) FGF-2 expression in endothelial cells of control group on Day 9. (F) FGF-2 expression in endothelial cells of treatment group on Day 9.](image-url)
Fig. 2. Immunohistochemistry examination showed Fibronectin expression (1000x magnification). Positive reaction showed brown color on cytoplasm indicating reaction between antigen (Fibronectin) and monoclonal antibody (anti Fibronectin) (Red Arrow). (A) Fibronectin expression in fibroblast cells of control group on Day 5. (B) Fibronectin expression in fibroblast cells of treatment group on Day 5. (C) Fibronectin expression in fibroblast cells of control group on Day 7. (D) Fibronectin expression in fibroblast cells of treatment group on Day 7. (E) Fibronectin expression in fibroblast cells of control group on Day 9. (F) Fibronectin expression in fibroblast cells of treatment group on Day 9.

There were also significant differences in the mean FGF-2 and Fibronectin expression that can be seen on Figure 3 and Figure 4. The One-Way Analysis of Variance (ANOVA) showed significant differences (p<0.05) in FGF-2 and Fibronectin expression between the groups (Table 1).

![Fig. 3. The mean comparison of FGF-2 expression each group.](image)

![Fig. 4. The mean comparison of Fibronectin expression each group.](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>p-value</th>
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<td>C1 and T1</td>
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<tr>
<td>C2 and T2</td>
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<tr>
<td>C3 and T3</td>
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* Significant p<0.05.

Patient with DM there are a lot of physiological factors that are impaired such as the altered of growth factor production, angiogenic response, macrophage function, the collagen formation, the granulation tissue amount, fibroblasts migration and proliferation, and the balance between Extracellular Matrix (ECM) component and remodelling by the Matrix Metalloproteinase (MMPs)\textsuperscript{20,21}. The wistar rat was chosen because there is a similarity between the structure of rat oral mucosa and the human’s oral mucosa that basically formed by epithelial tissues and connective tissues. The male wistar rat was used to prevent hormonal factor that can influences or alter the experiment variables\textsuperscript{22}.

The wistar rat was induced by STZ with the dosage of 150 mg/kg body weight of the rat. STZ works by formed the free radical that could damage the cell membrane, protein, and Deoxyribonucleic Acid (DNA). The free radical altered in insulin production by the Pancreas’ Beta Langerhans cell. STZ invades the Pancreas’ Beta Langerhans cell through Glucose Transporter 2 (GLUT 2) through the nitrous urea chain. STZ was a Nitric Oxide (NO) donor that could increase the xanthin oxidase activity reduced oxygen. NO has an effect toward blocked the Krebs cycle. The limitation of ATP production in the mitochondria will cause the nucleotide decreased in the β cell. STZ has a high success rate to induce DM than alloxan, uric acid, dehydroascorbate acid and dialurate acid\textsuperscript{23,24,25}.

The normal fasting blood glucose rate in the rat was 3.95 ± 1.31 or equivalent to ±116 mg/dL. In this study, the measurement the fasting blood glucose level in all STZ induced rats showed a blood glucose level above 126 mg/dL. The result in accordance with WHO classification for DM condition in humans which is the fasting blood glucose level is above 126 mg/dL\textsuperscript{25,26}. Traumatic ulcer was formed on the rat’s lower labial mucosa by thermal trauma using heated burnisher for 60 seconds and applied without pressure in order not to perforated. The labial mucosa was selected by the consideration it is easy to create and apply the citrus limon essential oil\textsuperscript{27,28,29}.

The active component of the Citrus Limon fruit Peel Essential Oil was assessed using Gas Chromatography-Mass Spectometry (GCMS) test. GCMS test showed 31.78% fumarat acid, 17.38% d-limonene, 13.55% citral, 8.51% L-Linalool, and other compounds in a low concentration. In this study, the application of the Citrus Limon fruit Peel Essential Oil was done 3 times daily.
The previous study of d-limonene topically it is shown that it will reach the highest concentration in the tissue 3-6 hours after application. The d-limonene on the essential Citrus Limon fruit Peel Essential Oil besides that there is citral component. Citral component was known as toxic characteristic. Citral component has the half-life of 8 hours in a human’s body.\textsuperscript{30,31}

In this study, the application of Citrus Limon fruit Peel Essential Oil Gel on the oral mucosa traumatic ulcer was done third times daily or every 6-8 hours. The 0.78% Citrus Limon fruit Peel Essential Oil Gel was used to accelerate the traumatic ulcer healing. The FGF-2 expression increased in treatment group. The increased expression of FGF-2 was influenced by the d-limonene. D-limonene has the ability as an antioxidant that could reduce the free radical, furthermore d-limonene could prevent apoptosis and cell function alteration.\textsuperscript{32,33} The D-limonene as anti-inflammatory agent could increase number and the stability of Th17/Tregs. D-limonene could stimulate fibronectin expression in the healing process of traumatic ulcers on the oral mucosa of diabetes afflicted Wistar rats (\textit{Rattus norvegicus}).

Fibronectin expression is higher on the treatment group compared to the control group. The result was in line with the increased expression of FGF-2. FGF-2 is the growth factor that has important role as chemotactic and mitogenic fibroblasts to stimulate angiogenesis and synthesizing Fibronectin.\textsuperscript{32,33,34} In this present study that can be concluded the citrus limon peel oil gel increased the FGF-2 and Fibronectin expression during the healing process of traumatic ulcers on the oral mucosa of diabetes afflicted Wistar rats (\textit{Rattus novergicus}).

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CONFLICT OF INTEREST:
The authors declare no conflict of interest.

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