Effect of Papain Enzymes on the Density and Amount of Collagen in Scar Tissue in rats

Fauzan*, Herman Josef **

*General Surgery Resident of Brawijaya Faculty of Medicine, Malang, East Java, Indonesia
** Plastic Surgery Staff of Saiful Anwar General Hospital Malang, East Java, Indonesia

Corresponding Author: Fauzan

Abstract: Introduction: Papaya sap containing papain which is a proteolytic enzymes has the ability to increase collagen degradation so that it is expected to be used for the treatment of keloids.

Material and Method: This study was an experimental study with Randomized Controlled Trial Post Test Only Design with experimental animal. Scar was exposed with papain enzyme and the changes of density and amount of collagen were evaluated. 30 rats were divided randomly into 10 groups. In group P0, excision was performed after 3 months in scarring without prior injection of papain enzymes. In group P1, the papain enzyme was injected at a dose of 5 mg to scar once; in group P2, the papain enzyme was injected at a dose of 5 mg to scar twice with 7-day interval; in group P3, the papain enzyme was injected at a dose of 5 mg to scar 3 times with 7-day interval; in group P4, the papain enzyme was injected at a dose of 7.5 mg to scar once; in group P5, the papain enzyme was injected at a dose of 7.5 mg to scar twice with 7-day interval; in group P6, the papain enzyme was injected at a dose of 7.5 mg to scar 3 times with 7-day interval; in group P7, the papain enzyme was injected at a dose of 10 mg to scar once; in group P8, the papain enzyme was injected at a dose of 10 mg to scar twice with 7-day interval; in group P9, the papain enzyme was injected at a dose of 10 mg to scar 3 times with 7-day interval. Furthermore the result of scar tissue was excision then divided into 2 parts. Histopathological preparations with Masson's trichrome were prepared. The collagen density was determined by using Olyvia software. The hydroxyproline content was measured by total collagen assay kit using a spectrophotometer.

Result: The average collagen density was the control group P0 = 231.073; P1 = 216.987; P2 = 203.480; P3 = 200.973; P4 = 203.120; P5 = 201.343; P6 = 200.627; P7 = 199.257; P8 = 193.480; P9 = 181.020. There was a significant differences on the collagen density between the groups P0, P1, P2, P3, P4, P5, P6, P7, P8 and P9 (p<0.05). The average hydroxyproline levels was the control group. P0 = 9.752; P1 = 9.073; P2 = 7.472; P3 = 6.184; P4 = 6.596; P5 = 5.894; P6 = 5.709; P7 = 5.821; P8 = 5.480; P9 = 5.176.

Conclusion: Density and amount of Collagen was decreased by papain on scar tissue in rats.

Key Words: Papain, Collagen density, Hydroxyproline, Scar Tissue, Masson's trichrome staining, Total Collagen assay

Date of Submission: 06-12-2018  Date of acceptance: 22-12-2018

1. Introduction

Scarring is a natural defect (Perdanakusuma, 2006; Niessen, 1998). Scarring can affect everyone, the incidence rate is higher in dark skinned people. In developing countries, there are 100 million sufferers with scarring complaints every year. About 55 million cases of scarring occur due to elective surgical wounds and 25 million cases of scarring occur in trauma surgery cases. An abnormal scar is formed from excessive accumulation of collagen. In the wound healing process, collagen synthesis occurs by fibroblasts as an ingredient for the strength of the injured tissue integrity and the collagen degradation by the collagenase enzyme to control the amount of collagen so that it is not excessive, both of which are in balance. Accumulation of collagen in abnormal scarring occurs due to a disruption of the balance between the synthesis and degradation of collagen. Abnormal scarring causes itching, pain, discomfort, aesthetically bad and if it occurs in the joint area, then it can affect joint movement (Murray, 1994; Niessen, 1999).

There are many methods of scar treatment, but each method has its weaknesses. Excision surgery is the easiest and most common method with a 50% recurrence rate, which often reappears more severe and worse than the initial condition (Shaffer et al., 2002). Cryosurgery using liquid nitrogen requires 8-10x of treatment, only for small scars, the side effects cause hypopigmentation, postoperative pain and slow healing. Intralosional steroid injection can cause normal skin atrophy around the scar, hypopigmentation, telengectasia and systemic steroid side effects such as Cushing’s syndrome. Other methods such as 5-fluorouracil injection, methotrexate, immunotherapy with interferon, imiquimod cream, radiation, laser surgery, silicon gel, also have side effects and do not heal completely. Traditionally, there have been various methods to treat abnormal scars, such as...
rubbing with phyton fat, cucumber, garlic, mashed aloe leaves, papaya sap, and others, but the methods have not produced clear results (From & Assad, 2003).

Based on the research, papaya sap containing various types of beneficial substances including papain enzyme and chemopapain which are proteolytic and as antimicrobials (Starley et al., 1999), issexcellent for accelerating, dissolving dead skin cells and helping to cleanse necrotic tissue so as to reduce the time needed to heal tissue. As a proteolytic, papain in sap papaya can also increase the degradation of collagen so that it can be used for inexpensive and easy-to-obtain scar treatment. For the reasons mentioned above, a study was conducted to determine the effect of papaya sap on scar tissue (Risti, 2000; Hanafi, 2014).

In mature normal scar, there is a balance between collagen synthesis through prolyl hydroxylase activity and collagen degradation by collagenase. The process of collagen metabolism is the synthesis and degradation of abnormal collagen which is the biological core of the formation of scar tissue. Excessive collagen synthesis or degradation barriers result in accumulation of collagen. Increased collagen synthesis that is not followed by a sufficient increase in degradation activity, or a decrease in collagen degradation activity even in normal collagen synthesis will form hypertrophic scar tissue. This is the basis of every treatment by suppressing the rate of collagen synthesis and increasing the degradation of collagen. Degradation of collagen is mainly carried out by the collagenase enzyme, protease will completely destroy collagen denatured by collagenase. Protease also induces the secretion of collagenase by fibroblasts (Werb & Aggeler, 1978). The presence of proteases will make the collagenase enzyme to remain active even though there are serum proteins that inhibit its activity (Sakamoto et al., 1972). Papain is a proteolytic enzyme so that it has properties like general proteases.

II. Material And Method

Material

This study used the experimental animal: Ratus Norvegicus, aged 8-12 weeks with an average weight of 200-250 gr, male, with a total sample of 30 animals. The animal feeds were raw corn and raw meat, antiseptic solution: Savlon®, ketamine injection, 10% formalin for tissue fixation, papain enzyme extract of papaya sap: Papain Sigma®, culture medium: Dulbecco’s Modified Eagle’s Medium (DMEM), Phosphate Buffer Saline (PBS), Fetal Bovine Serum (FBS), QuickZyme Total Collagen Assay kit, material and regensia for staining Masson’s trichrom.

Tool

Experimental animal cage, basic/minor surgery sets, analytical balance/scales with Ohyo Brand type Jupiter S4-160D, CO2 Incubator Type MEP-INCI 2007, Laboratory Incubator/Binder Oven, Laminar Flow, Leica Microtome RM2245, Olympus BX-53 Light Microscope, Spectrophotometer with Awareness Brand.

Research Design

Experimental laboratory study was carried out using the Randomized Controled Trial Post Test Only Design design using rat as the experimental animal. Scar tissue was exposed to the papain enzyme extract of papaya sap. Changes in the density and amount of collagen in scar tissue were observed.

Research Procedure

30 rats as experimental animals were weighed, then put into cages and numbered from 1 to 30. Each experimental animal was anesthetized with Ketamine of 10-20 mg/kg BW/im. Furthermore, the experimental animals were placed on the operating table, their back was cleaned and shaved followed by antisepsis using Savlon®, then excision was performed in the mid-back of the experimental animal until as deep as an elliptical fascia of 20 mm long and 6 mm wide, then the surgical wound was immediately closed with tulle and sterile gauze, metamizole of 1 mg/kg BW/im was administered to the experimental animals three times a day for 2 days. After 3 months, the research sample was taken gradually. Next, the intervention was performed in the form of injecting the papain enzyme intrasessionally after the formation of scar tissue. There were 10 groups in this study consisting of 1 control group and 9 intervention groups administered with 3 different doses of the papainenzyme, namely 5 mg, 7.5 mg and 10 mg. The control group was not administered the papainenzyme. Papain enzyme was injected using a 1 cc syringe with 25G needle in the middle of the scar tissue once. In group P0, excision was performed after 3 months in scarring without prior injection of papain enzymes. In group P1, the papain enzyme was injected at a dose of 5 mg once. In group P2, the papain enzyme was injected at a dose of 5 mg twice with 7-day interval. In group P3, the papain enzyme was injected at a dose of 5 mg 3 times with 7-day interval. In group P4, the papain enzyme was injected at a dose of 7.5 mg once. In group P5, the papain enzyme was injected at a dose of 7.5 mg twice with 7-day interval. In group P6, the papain enzyme was injected at a dose of 7.5 mg 3 times with 7-day interval. In group P7, the papain enzyme was injected at a dose of 10 mg
once. In group P8, the papain enzyme was injected at a dose of 10 mg twice with 7-day interval. In group P9, the papain enzyme was injected at a dose of 10 mg 3 times with 7-day interval.

Furthermore, histopathological preparations were made by staining Masson’s trichrome. Prepared preparations were observed using the OLYMPUS BX 52 series light microscope equipped with an Olympus DP-72 digital camera with 400x magnification in one field of view, the location of collagen observation was in the central area of the preparation. Next, the images were analyzed to determine collagen density semiquantitatively by using OlyVia computer software to assess the intensity of blue reflecting collagen density. To determine the amount of collagen, hydroxyproline levels in the medium were measured using QuickZyme Total Colloagen Assay with a spectrophotometer. The value of hydroxyproline levels in a tissue or medium showed the total amount of collagen in the tissue or medium. Data of examination results in the study were then statistically analyzed using one-way ANOVA. Data analysis was performed using the SPSS software.

### III. Result And Discussion

This study was an experimental laboratory using the Randomized Controlled Trial Post Test Only Design using rat as experimental animal. The formed scar tissue was exposed to the papain enzyme, then changes in the density and amount of collagen in the scar tissue were observed. There were 10 groups in this study. In group P0, excision was performed after 3 months in scarring without prior injection of papain enzymes. In group P1, the papain enzyme was injected at a dose of 5 mg once. In group P2, the papain enzyme was injected at a dose of 5 mg twice with 7-day interval. In group P3, the papain enzyme was injected at a dose of 5 mg 3 times with 7-day interval. In group P4, the papain enzyme was injected at a dose of 7.5 mg once. In group P5, the papain enzyme was injected at a dose of 7.5 mg twice with 7-day interval. In group P6, the papain enzyme was injected at a dose of 7.5 mg 3 times with 7-day interval. In group P7, the papain enzyme was injected at a dose of 10 mg once. In group P8, the papain enzyme was injected at a dose of 10 mg twice with 7-day interval. In group P9, the papain enzyme was injected at a dose of 10 mg 3 times with 7-day interval.

#### Data of Amount and Density of Collagen

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>N</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Mean</th>
<th>Std. Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of Collagen</td>
<td>P0</td>
<td>1</td>
<td>9.901871</td>
<td>9.848923</td>
<td>9.50653</td>
<td>9.752</td>
<td>0.215</td>
</tr>
<tr>
<td></td>
<td>P1</td>
<td>2</td>
<td>9.273562</td>
<td>9.195905</td>
<td>8.748676</td>
<td>9.073</td>
<td>0.283</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>3</td>
<td>7.67808</td>
<td>7.483939</td>
<td>7.254501</td>
<td>7.472</td>
<td>0.212</td>
</tr>
<tr>
<td></td>
<td>P3</td>
<td>4</td>
<td>6.474409</td>
<td>6.082598</td>
<td>5.994352</td>
<td>6.184</td>
<td>0.256</td>
</tr>
<tr>
<td></td>
<td>P4</td>
<td>5</td>
<td>6.732086</td>
<td>6.576774</td>
<td>6.477939</td>
<td>6.596</td>
<td>0.128</td>
</tr>
<tr>
<td></td>
<td>P5</td>
<td>6</td>
<td>5.973173</td>
<td>5.759054</td>
<td>5.948465</td>
<td>5.894</td>
<td>0.117</td>
</tr>
<tr>
<td></td>
<td>P6</td>
<td>7</td>
<td>5.879033</td>
<td>5.550794</td>
<td>5.697847</td>
<td>5.709</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>P7</td>
<td>8</td>
<td>5.937875</td>
<td>5.720226</td>
<td>5.803742</td>
<td>5.821</td>
<td>0.110</td>
</tr>
<tr>
<td>Collagen Density</td>
<td>P8</td>
<td>9</td>
<td>5.63784</td>
<td>5.500176</td>
<td>5.302506</td>
<td>5.480</td>
<td>0.169</td>
</tr>
<tr>
<td></td>
<td>P9</td>
<td>10</td>
<td>5.353439</td>
<td>5.118955</td>
<td>5.073067</td>
<td>5.176</td>
<td>0.140</td>
</tr>
</tbody>
</table>

Parameter Test Result Analysis

Before carrying out testing using ANOVA, the data obtained for each treatment were analyzed for the homogeneity of variance using the homogeneity of variance test (Levene’s test) with the aim to determine the data have the same variance.

#### Homogeneity Test

<table>
<thead>
<tr>
<th>Test of Homogeneity of Variances</th>
<th>Levene Statistic</th>
<th>df1</th>
<th>df2</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount of Collagen</td>
<td>1.011</td>
<td>9</td>
<td>20</td>
<td>.464</td>
</tr>
<tr>
<td>Collagen Density</td>
<td>1.974</td>
<td>9</td>
<td>20</td>
<td>.999</td>
</tr>
</tbody>
</table>

DOI: 10.9790/0853-1712074753  www.ijosrjournals.org  49 | Page
The test results showed that the value of Levene’s test for the amount of collagen was 1.011 with a significance value of 0.464, while the value of Levene’s test for collagen density was 1.974 with a significance value of 0.099. Where all parameters have a sig value greater than alpha 0.05 because p value > 0.05, then Ho is accepted and it can be concluded that the data had homogeneous variances.

In addition to the homogeneity of variance tests, the normality of the data was also tested to find out whether the data have a normal distribution or not by using Kolmogorov-Smirnoff test.

Normality Test

The results of normality test in Table 5.3 show that the value of Kolmogorov-Smirnoff test with a significance value (p) for the amount of collagen is 0.088, while the value for collagen density is 0.055. P value > 0.05, then Ho is accepted and it can be concluded that the data are normally distributed. Thus, the testing using ANOVA can be continued because both assumptions have been fulfilled.

One Way ANOVA Test

One way ANOVA test aims to determine whether there are significant differences between treatments. To test whether there are significant differences between one treatment to another, then the analysis is carried out using Kruskall-Wallis test.

Variance Analysis

Based on the analysis results of One Way ANOVA in the table above, it is found that F statistic for the amount of collagen is 0.000, while F statistic for collagen density is 0.000. Because the amount of collagen and collagen density parameters have p value < 0.05, then Ho is rejected, meaning that there is a significant difference between treatments at a 5% confidence level.

To find out the difference between treatments, a further test was performed using Tukey’s test with the test results in the following Table:

1. Amount of Collagen

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average</th>
<th>Notation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P9</td>
<td>5.136</td>
<td>A</td>
</tr>
<tr>
<td>P8</td>
<td>5.480</td>
<td>Ab</td>
</tr>
<tr>
<td>P6</td>
<td>5.709</td>
<td>Abc</td>
</tr>
<tr>
<td>P7</td>
<td>5.821</td>
<td>Bc</td>
</tr>
<tr>
<td>P5</td>
<td>5.894</td>
<td>Bc</td>
</tr>
<tr>
<td>P3</td>
<td>6.184</td>
<td>Cd</td>
</tr>
<tr>
<td>F4</td>
<td>6.596</td>
<td>D</td>
</tr>
<tr>
<td>F2</td>
<td>7.472</td>
<td>E</td>
</tr>
<tr>
<td>P1</td>
<td>9.073</td>
<td>F</td>
</tr>
<tr>
<td>P0</td>
<td>9.752</td>
<td>G</td>
</tr>
</tbody>
</table>
Based on the Table above, it is obtained the results of difference test between treatments. The treatment is said to provide a significant difference if it has the same notation. The treatment $P_0$ is able to provide highest difference in the amount of collagen compared to the other treatments.

**Collagen Density**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average</th>
<th>Notation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$P_9$</td>
<td>181.020</td>
<td>A</td>
</tr>
<tr>
<td>$P_8$</td>
<td>193.480</td>
<td>B</td>
</tr>
<tr>
<td>$P_7$</td>
<td>199.257</td>
<td>Bc</td>
</tr>
<tr>
<td>$P_6$</td>
<td>200.627</td>
<td>C</td>
</tr>
<tr>
<td>$P_5$</td>
<td>201.343</td>
<td>C</td>
</tr>
<tr>
<td>$P_4$</td>
<td>203.120</td>
<td>C</td>
</tr>
<tr>
<td>$P_3$</td>
<td>203.480</td>
<td>C</td>
</tr>
<tr>
<td>$P_2$</td>
<td>216.987</td>
<td>D</td>
</tr>
<tr>
<td>$P_1$</td>
<td>231.073</td>
<td>E</td>
</tr>
</tbody>
</table>

Based on the table above, it is obtained the results of difference test between treatments. The treatment is said to provide a significant difference if it has the same notation. The treatment $P_0$ has the most different notation among the other treatments, so that treatment $P_0$ is able to provide the highest difference in collagen density compared to the other treatments. The lowest collagen density is found in the treatment $P_9$.

**Correlation Analysis**

**Pearson Correlation**

Pearson Correlation is used in analyzing the relationship between two variables. The data is not only from one source, but the source can be more than one. In this study, there were two data sources, namely the amount of collagen and collagen density variables.

In the correlation test, there are two hypotheses to be taken, as follows:
1. $H_0$: There is no relationship (correlation) between Variable X (Amount of Collagen) and Variable Y (Collagen Density).
2. $H_1$: There is a relationship (correlation) between Variable X (Amount of Collagen) and Variable Y (Collagen Density).

The criteria for drawing conclusions on the correlation test between variable X and variable Y are as follows:
1. If the Sig. value (2-tailed) is greater than alpha of 5% (0.05), then $H_0$ is accepted, so that it can be concluded that there is no relationship (correlation) between Variable X (Amount of Collagen) and Variable Y (CollagenDensity).
2. If the Sig. value (2-tailed) is smaller than alpha of 5% (0.05), then $H_0$ is rejected and $H_1$ is accepted, so that it can be concluded that there is a relationship (correlation) between Variable X (Amount of Collagen) and Variable Y (Collagen Density).

<table>
<thead>
<tr>
<th>Coefficient Interval</th>
<th>Relationship Level</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00 – 0.199</td>
<td>Very Low</td>
</tr>
<tr>
<td>0.20 – 0.399</td>
<td>Low</td>
</tr>
<tr>
<td>0.40 – 0.599</td>
<td>Moderate</td>
</tr>
<tr>
<td>0.60 – 0.799</td>
<td>Strong</td>
</tr>
<tr>
<td>0.80 – 1.000</td>
<td>Very Strong</td>
</tr>
</tbody>
</table>

The calculation results of Pearson correlation using the assistance of SPSS version 16.00 software can be seen in Table 5.7.

**Relationship Between Variables**

<table>
<thead>
<tr>
<th>Variables Between Amount of Collagen and Collagen Density</th>
<th>Spearman Correlation</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.914</td>
<td>0.000</td>
</tr>
</tbody>
</table>

In the test result, it can be seen that the value of Spearman correlation coefficient is positive of 0.914, meaning that if the variable X (Amount of Collagen) is higher, then the variable Y (Collagen Density) will increase. The resulting correlation coefficient shows the extent of relationship between variable X (Amount of Collagen) and variable Y (Collagen Density) with $r$ value (correlation coefficient) of 0.914. This correlation value indicates that the relationship between Amount of Collagen and Collagen Density is in very strong...
Effect of Papain Enzymes on the Density and Amount of Collagen in Scar Tissue in rats

The results of the one-way ANOVA analysis, it was found that F value for collagen density was 0.000 (95.025). The collagen density parameter had p value <0.05, meaning that there is a significant difference in effect between treatments at a confidence level of 5%. To find out which group has a different average collagen density than the other groups, Mann Whitney test was performed. Treatment P0 has the most different notation (e) between the other treatments so that treatment P0 is able to provide the highest difference in collagen density compared to the other treatments. The lowest collagen density is found in treatment P9.

The assessment of hydroxyproline levels is carried out to determine the presence of degraded collagen in scar tissue. The value of hydroxyproline levels in a sample reflects the total level of collagen. The value of hydroxyproline levels in a sample that was the highest was found in treatment P9. Treatment P0 has the most different notation (g) between the other treatments, so that treatment P0 is able to provide the highest difference in the amount of collagen compared to the other treatments.

Reference
[6] Blackburn Cosman
[22] Hanafi, Pengaruh enzim papain terhadap kepadatan kolagen pada kultur jaringan keloid, FKUB, Malang, 2014
Effect of Papain Enzymes on the Density and Amount of Collagen in Scar Tissue in rats


[37] Miller EJ and Gay S. Collagen structure and function dalam Wound Healing. 2002


[61] Viegas V.M.T et al., Keloid explants culture: a model for keloid fibroblasts isolation and cultivation based on the biological differences of its specific region. Int Wound Journal. 2010


DOI: 10.9790/0853-1712074753 www.iosters.org 53 | Page