

## Does Papain Enzyme Improve Collagen Degradation?

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### ABSTRACT

Papain as a protease cysteine enzyme has the potential to increase the collagen degradation process. This research determined the effect of papain enzyme in the enhancement of collagen degradation under the acidic conditions of abnormal scarring. This study was a *randomized controlled trial post-test only design* using *Rattus norvegicus* as an experimental animal. The rats were threatened by 5 mg, 10 mg, and 20 mg of papain doses at 12<sup>th</sup>, 13<sup>th</sup>, and 14<sup>th</sup> weeks. The abnormal scar tissue was excised at 15<sup>th</sup>. The pH was measured by using the pH meter. The MMP-1 and TIMP-1 activity were observed by the Enzyme-Linked Immunosorbent Assay (ELISA) method. A modified Vancouver Scar Scale Modification (VSSm) score used to measure the morphology of abnormal scar in rats. All data were analyzed by SPSS 25 software. The results showed there are significant differences ( $p < 0.05$ ) between the treatments of papain enzyme in each variable (pH, MMP-1, TIMP-1, Ratio of TIMP-1 with MMP-1, and VSS) and controls. The reduction of the VSSm score results in the enhancement of papain dosage, represent this morphologically repair the abnormal scar of *Rattus norvegicus*.

**Keywords:** Abnormal scar, MMP-1, papain, TIMP-1, VSSm

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### INTRODUCTION

Abnormal scarring is a scar that disrupts the wound healing process, especially in functional and aesthetic aspects (Seo *et al.* 2013; Lee *et al.* 2015; Andrews *et al.* 2016). The abnormal scarring consists of hypertrophic scars and keloids (Seo *et al.* 2013; Xue and Jackson 2015). Keloid characterized by tension, reddish color, and neovascularization on the surface at the beginning of development (Gauglitz *et al.* 2011). The pressure decreases, but the scar continues to develop. This scar can lead to itching and pain. In contrast, hypertrophic also characterized by the growth of protruding scar but does not exceed the wound border (Gauglitz *et al.* 2011; Nicholas *et al.* 2012). Till now, the mechanism of abnormal scarring is on the wound healing process (Xu *et al.* 2017). This fact shows that disruption in each phase of wound healing increase the chances of abnormal scarring. In the of abnormal scars can occur even in a state of healthy collagen synthesis. As the most abundant protein in animals (Shingleton, 1996; Shoulders and Raines 2009), collagen is related to the collagenases. The collagenases are the enzymes necessary to initiate collagen turnover in normal connective tissue turnover and in disease (Shingleton 1996). Decreased collagen degradation is a representation of the weak function of the collagenases (Perdanakusuma 2006). However, during this time, the focus of research on abnormal scars, especially on the role of collagen degradation by the collagenases, still poorly investigated. In the process of wound healing, a local acidosis occurs due to the increased production of lactic acid and the need for oxygen. This condition triggers proliferation by fibroblasts with the highest activity at pH 5 (Liu *et al.* 2002; Schneider *et al.* 2007). Meanwhile, the proteolytic activity of collagenase cannot work optimally. Collagenase is active under alkaline conditions (pH 7-8). Studies of chronic wounds explained the overworked of pH > 8 collagenase conditions cause damage to the extracellular matrix structure and resulting in delayed wound healing (Greener *et al.* 2005; Jones *et al.* 2015). Decreased collagenase activity in abnormal scars, also caused by excessive *Tissue Inhibitor Metalloproteinase*

(TIMP) activity. TIMP played a role in inactivating MMP when collagen sufficiently degraded. In abnormal conditions, including abnormal scars, MMP and TIMP activities are impaired (Rohani and Parks 2015; Nguyen *et al.* 2016). Papain, a phytochemical obtained from papaya sap (*Carica papaya* Linn.) is concentrated in the skin of papaya fruit and is commonly applied in pharmaceutical, food, cosmetic, and textile industry (Stremnitzer *et al.* 2015). Papain classified as an enzyme from the protease group C1 family of cysteine proteases (Stremnitzer *et al.* 2015; Malek *et al.* 2016; Martinez *et al.* 2019). Some studies showed the papain enzymes generally work optimally in an alkaline atmosphere, such as collagenase with optimally pH 3-7.5 (Schneider *et al.* 2007; Manosroi *et al.* 2014). Researchers estimate that the enzyme papain can work optimally on keloids (Nafiu and Rahman 2015) that tend to have an acidic pH (Milošević *et al.* 2019). Human has enzyme cathepsin K has similarity in structure and function with the papain enzyme (Turk *et al.* 2012). The in-silico study showed the effect of a bond between cathepsin K with collagen type 2 and *matrix-metalloproteases* (MMP)-1, -8, -13 with a strength of about 30-50%, but this still unclear whether this enzyme worked as an inhibitor or a stimulus in the process of collagen degradation (Dejica *et al.* 2012). In-vitro studies on human fibroblast cells explained that the papain enzyme would increase the activity of proteolytic and gelatinase production to degrade excess collagen. In hypertrophic and keloid scarring shows its role by stimulating the activity of MMP (Manosroi *et al.* 2013). Papain enzyme has a role in the process of angiogenesis and collagen degradation but is still limited to in-vitro and in-silico studies. Research about the utilization of the papain enzyme in animal models of abnormal scarring is unclear. Therefore, this research aims to determine the effect of the papain enzyme in increased degradation of collagen with an acidic atmosphere in abnormal scars.

### MATERIALS AND METHODS

#### Preparation of Animal Model

This research was an experimental study using a Randomized Controlled Trial Post Test Only Design. Thirty

*Rattus norvegicus* brown Norway was used as an experimental animal. The rats divided into three groups: negative control (C-), positive control (C+), and three treatment subgroups. Each rat was weighed, then given an anesthetic using Ketamine 10-20 mg/kg W/intramuscular. The hair of the dorsal part was shaved off and given antiseptis using chlorhexidine cetrimide (Savlon®). The control groups were made a scar by making an incision in the skin of the dorsal part with 2 cm length and done a primary suture—the stitches taken on the 7<sup>th</sup> day. Meanwhile, the treatment groups were made an abnormal scar by excision of a circular skin with a diameter of 15 mm on the dorsal part to the depth of the panniculus carnosus (Figure 1). Panniculus carnosus

identified as a thin layer of skeletal muscle between the subcutaneous fat and the dermis. The remaining panniculus carnosus on the edge of the wound sutured with the dermis part to leaving a defect on the subcutaneous base. This procedure performed to avoid contraction of the panniculus carnosus at the base of the wound, which can inhibit the formation of abnormal scarring. The wound was closed using tulle and sterile gauze, then fixed with hypoallergic tape (micropore®). The rat was given a metamizole of 1 mg/kg BW/intramuscular three times a day for two days (Fauzan and Josef 2018).

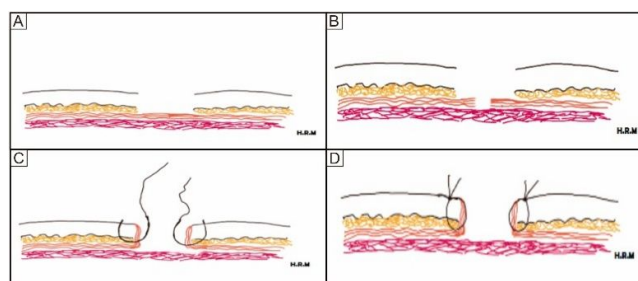


Figure 1: Induction of Abnormal Scarring in *Rattus norvegicus*. The skin tissue incised until a layer of Panniculus carnosus (orange) (A) found. The Panniculus carnosus layer is cut (B). The Panniculus carnosus layer was sutured together with the edge of the skin wound (C). The sutured done with a simple interrupted suture (D)

**Papain Treatment in Abnormal Scar Animal Model**

Treatment groups were divided into three sub-groups based on the injection of the papain enzyme, each with a dose of 5 mg, 10 mg, and 20 mg. The determination of dose based on the formula for the animal to human conversion (Human equivalent doses) according to body surface area. Doses in rat was: 5-10 mg/rat or 20-40 mg/kg Body Weight. The papain intervention conducted by injecting the papain enzyme (Worthington, USA) intralesional on the abnormal scar when reaching the final phase of wound healing (remodeling) in the 12<sup>th</sup> week. The injection used one cc 27 G needle syringe in the middle of the abnormal scar tissue—each dose given at 12<sup>th</sup>, 13<sup>th</sup>, and 14<sup>th</sup> weeks.

**pH Measurement of Scar Tissue**

The pH measurements held 12<sup>th</sup> week before the injection of papain and after excision of the scar in the 15<sup>th</sup> week. Tissue pH was measured using a Lutron 201 pH meter. The pH tip calibrated in buffer pH 4.0 and 8.0 before used. The tip of the needle pH meter contacted with wound tissue for

1 minute. The pH measurement results will appear on display.

**Measurement of MMP-1 and TIMP-1 Activities in the Abnormal Scar Animal Model**

Tissues were homogenization first using RIPA buffer 10% (w/v). Samples were placed in a cool box 10-15 minutes; then, centrifugation is carried out for 10 minutes in 13,000 rpm × g, 4° C. Supernatants analyzed as soon as possible. The ELISA procedure performed using the ELISA Kit from Rat TIMP-1 ABBEXA cat. abx050215 for TIMP-1 measurement, and Elisa Kit from Rat MMP-1 Elisa Kit ABBEXA cat.abx255821 for MMP-1 measurement. All procedures followed the standard procedure.

**Measurement of the Vancouver Scar Scale Modification (VSSm)**

Vancouver Scar Scale Modification (VSSm) scores on rat scars were measured by observing the scars of each rat before its tissue taken. The researchers had modified the VSS Score according to Tables 1 and 2.

Table 1: Score and Parameter of Modification of the Vancouver Scar Scale (after modified by Pham *et al.* 2017)

Parameter	Sub-Parameter	Score
Consistency	Soft	0
	Chewy	1
	Tough	2
Color	Similar to Surrounding Skin	0
	Hypopigmentation	1
	Hyperpigmentation	2
High	Similar to Surrounding Skin	0
	<1 mm	1
	> 1 mm	2
Appearance	Normal as Surrounding Skin	0
	Shine	1
Hair Grow	Hair grows above the scar	1
	No hair grows	0

Table 2: Interpretation score of *Rattus norvegicus* (after modified by Pham *et al.* 2017)

Total Score	Interpretation
0 - 2	Good
3 - 5	Medium
6 - 8	Adverse

**Ethical Clearance**

This research was conducted on experimental animals and approved by the ethics committee of the Faculty of Medicine, Brawijaya University No. 168 / EC / KEPK-S3 / 05/2019.

**Statistical Analysis**

All of the data was analyzed using Microsoft excel and SPSS 25. The correlations of Papain, MMP-1, TIMP-1, and VSSm also conducted using WarpPLS 7 software.

**RESULTS AND DISCUSSION**

**Results**

Based on the paired sample t-test result, the pH alteration between negative and positive control groups showed a significant difference. Meanwhile, the pH comparison between treatment groups showed a significant difference in the with the negative control group ( $p < 0.05$ ), but no significant with positive groups. The pH value of papain doses 5 mg, and 10 mg had a slight increase, but both had significant difference with scars injected with dose 20 mg ( $P < 0.05$ )(Figure 2).

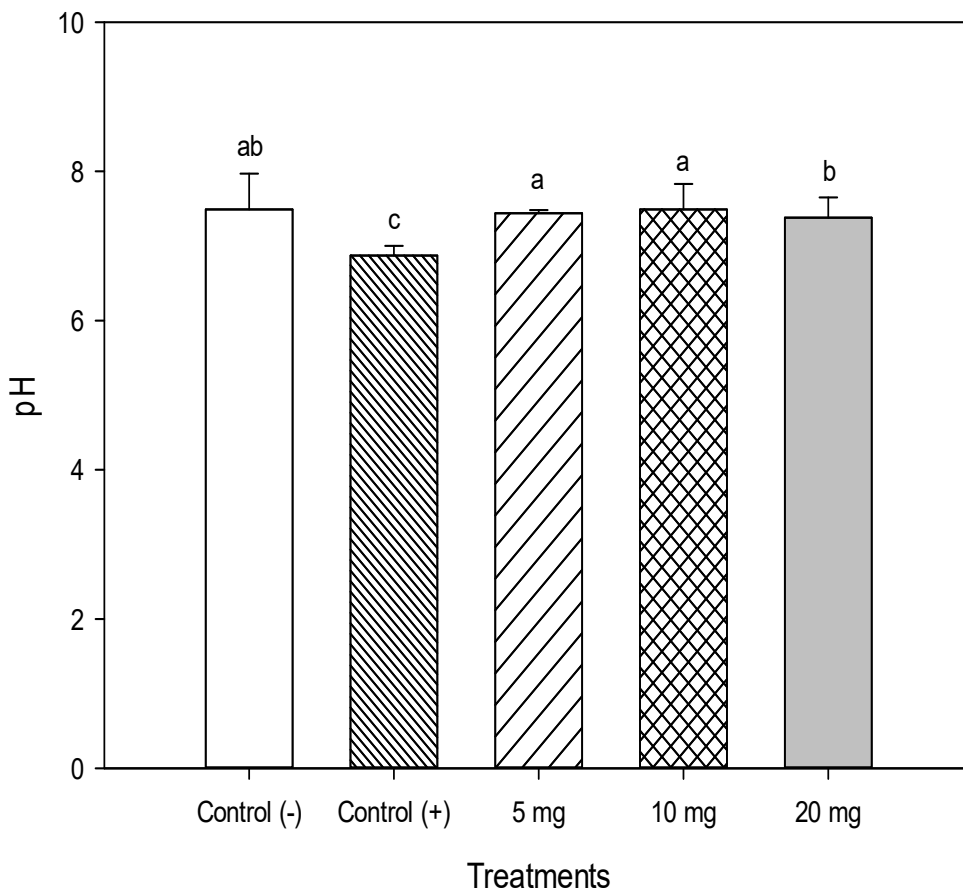


Figure 2: pH alteration between control groups (C- and C+) and treatment groups (5, 10, and 20 mgs)

The MMP-1 and TIMP-1 had a significant difference ( $p < 0.05$ ) between control and treatment groups. The enhancement of MMP-1 in each treatment (Figure 3) followed with a reduction of TIMP-1 value (Figure 4).

Meanwhile, the MMP-1 and TIMP-1 also obtained the ratio between MMP-1 and TIMP-1. The statistical result showed a significant difference ( $p < 0.05$ ) with a reduction of ratio value in each treatment (Figure 5).

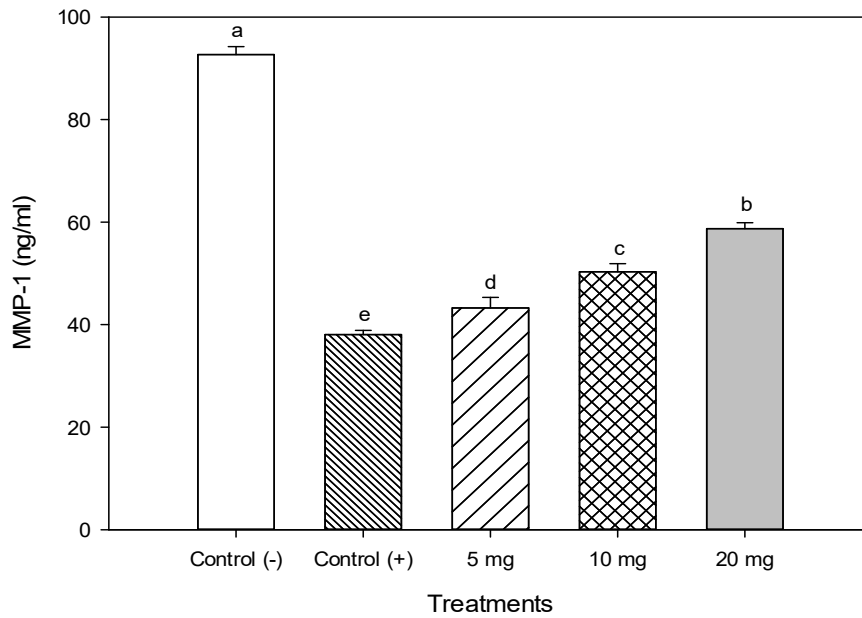


Figure 3: The MMP-1 result between control groups (C- and C+) and treatment groups (5, 10, and 20 mgs)

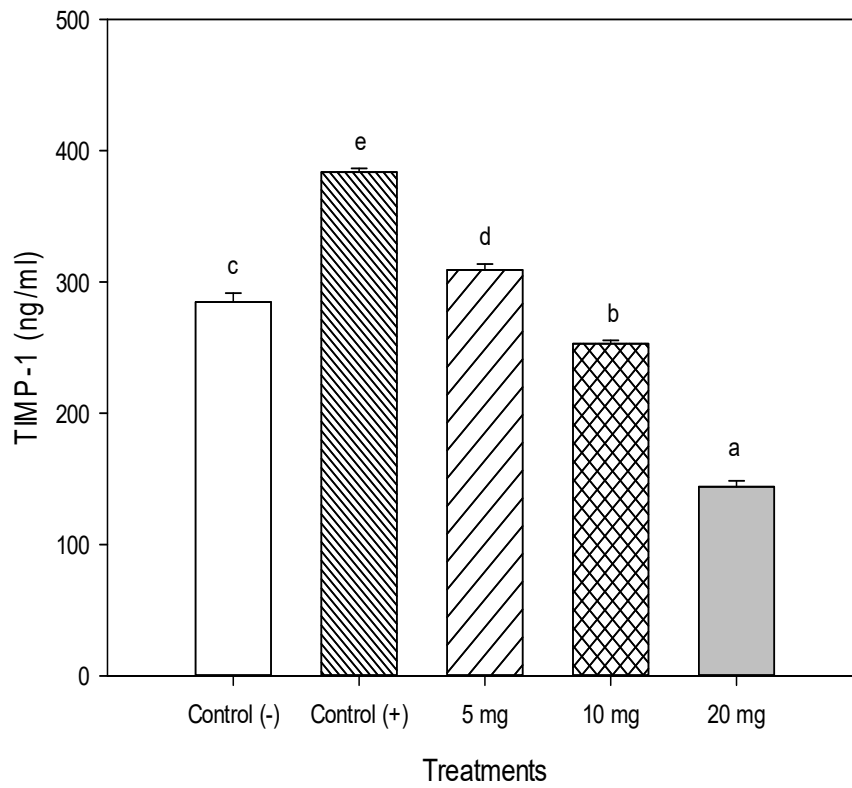


Figure 4: The TIMP-1 result between control groups (C- and C+) and treatment groups (5, 10, and 20 mgs)

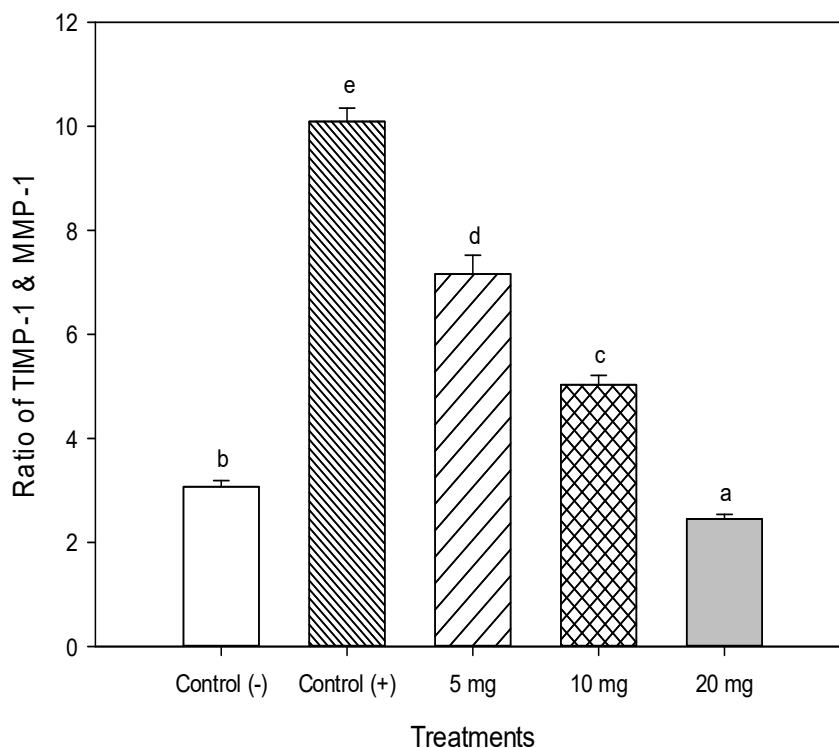


Figure 5: The ratio of TIMP-1 results between control groups (C- and C+) and treatment groups (5, 10, and 20 mgs)

All of the groups with papain treatment, in VSSm value showed a significant difference ( $P < 0.05$ ) compared to the positive control group (Figure 6), but not significant between the treatment group. The rat with a 20 mg dose

of papain had a slight lowest value among the treatment groups. This result indicated the 20 mg papain can repair the abnormal scar of rats compared to other doses (Figures 7 and 8).

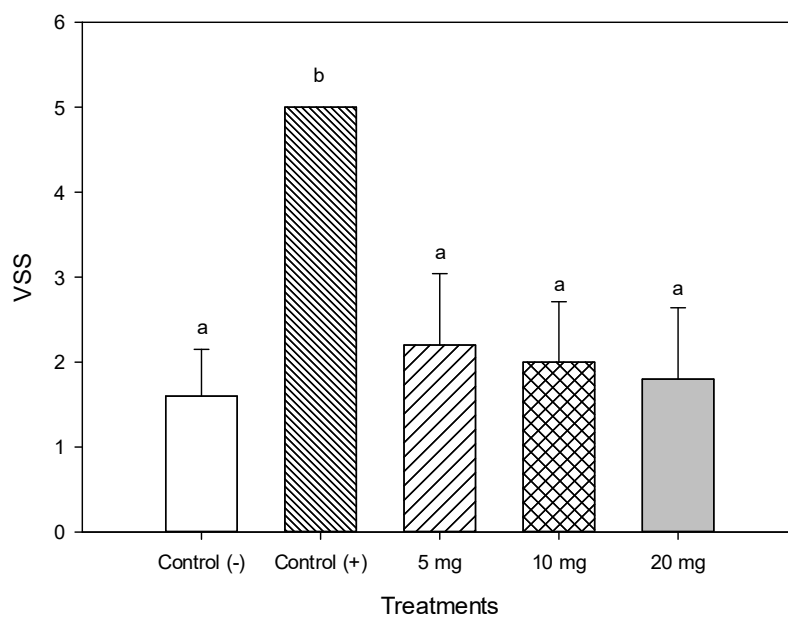


Figure 6: The VSSm result between control groups (C- and C+) and treatment groups (5, 10, and 20 mgs)

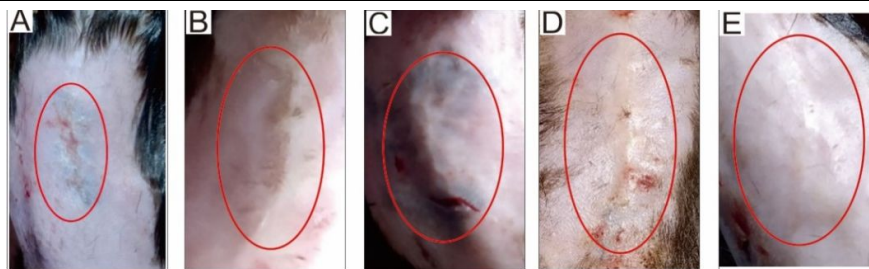


Figure 7: The VSSm result, (A) negative control (C-); (B) positive control (C+), (C) papain treatment 5 mg, (D) papain treatment 10 mg, and (E) papain treatment 20 mg.

The path analysis using warpPLS showed a significant direct relationship ( $P < 0.01$ ) between papain doses to pH, TIMP-1, ratio TIMP-1, and MMP-1. The papain doses and MMP-1 had no significant relationship ( $P = 0.28$ ). However, the significant undirect relationship ( $P < 0.01$ ) happened between papain doses and MMP-1 through TIMP-1. This result showed the papain doses of rats could affect the

MMP-1 value. The TIMP-1, MMP-1, ratio of TIMP-1, and MMP-1 also significantly affect the alteration of the VSSm score ( $P < 0.01$ ) (Figure 8). It demonstrated the papain could influence the alteration of pH, collagen degradation activity through MMP-1, TIMP-1, and repair the abnormal scar morphologically through assessment result of the VSS modification score.

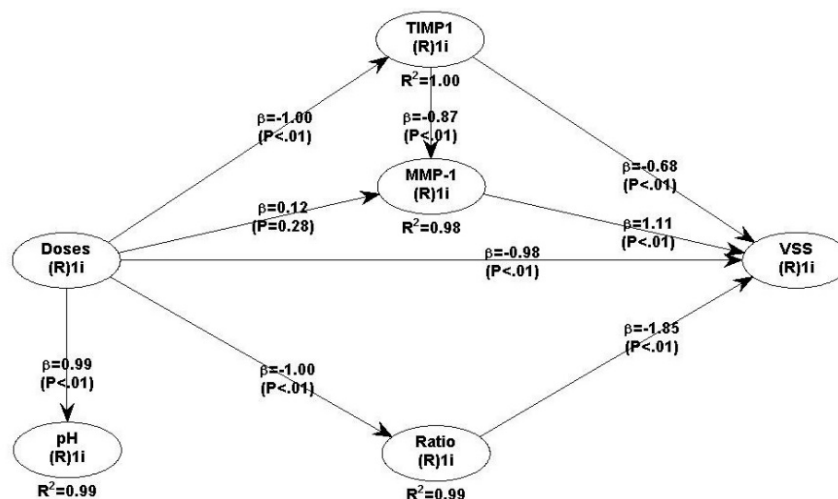


Figure 8: Path analysis of papain doses, pH, TIMP-1, MMP-1, ratio TIMP-1 and MMP-1 (ratio), and VSS

**DISCUSSION**

The result of papain in pH showed an increased value on treatment groups compared with the control. The papain with dose 5 mg had a slight difference with dose 10 mg, but both are quite different from dose 20 mg. This result indicated the papain could increase the level of pH in scar (Figure 2). The range of pH in this research is 6.9-7.6. The mechanism of papain can increase collagen degradation is by a direct mechanism (Figure 8). Researchers estimated that the enzyme papain could work optimally in abnormal scars that tend to have an acidic pH. Papain enzyme works optimally at pH 3-7.5 (Schneider *et al.* 2007; Manosroi *et al.* 2013).

The Papain enzyme belongs to the family K-cysteine endopeptidase protease enzyme (Cotabarren *et al.* 2007; Martinez *et al.* 2019). Cysteine group protease consists of Cys-His-Asn on its active side. Histidine residues appear on the active site as proton donors, which increases the nucleophilicity of cysteine residues (Laskar and Chatterjee

2009; Verma *et al.* 2016). This function concludes that the papain works to degrade collagen by attaching directly to its active site to decompose the target protein of collagen. Protease enzymes generally work optimally in an alkaline atmosphere, such as collagenase. Collagenase is active under alkaline conditions (pH 7-8) (Greener *et al.* 2005; Jones *et al.* 2015). However, the proteolytic activity of collagenase does not work optimally under acidic conditions (acidic pH).

Related to type I collagen fibrils degradation, superficial degradation of collagen fibrils can be promoted by papain. The application of papain-gel causes the softening of carious dentin due to the inability of the enzyme to attack the hydroxyapatite-coated collagen (Bertassoni and Marshall, 2009; Santos *et al.*, 2015). At pH 5.8-7.0 and at temperature 50-57°C papain can work optimally when casein is used as the substrate (Cotabarren *et al.*, 2007). The mechanism of papain in degrading collagen at acidic pH can be related to autocatalysis, where this activation



mechanism is common in the activation of protease cysteine from the zymogen form under the influence of changes in pH (acidic pH). The change in pH triggers the termination of the interaction between the form of the prodomain and the mature (active) domain, which finally reveals the active site of the enzyme (where it interacts with the target protein). Verma *et al.* (2016) reported the auto-catalysis (auto-activation) model of falcipain-2 (FP2), a protease cysteine of *Plasmodium falciparum* that plays a role in hemoglobin degradation, parasite egress, and surface proteins that make FP2 a target for malaria therapy (Shenai and Rosenthal 2002). The capsid adenovirus-associated virus (AAV) has more than one active site of protease and is sensitive to the induction of acidic pH, where at pH 5.5, it significantly activates capsid autocleavage in several amino acids (Salganik *et al.* 2012). Collagen degradation also controlled by several proteolytic enzymes of the matrix metalloproteinase (MMP) secreted by macrophages, epidermal cells, endothelial cells, and fibroblasts. Interstitial collagenase or MMP-1 is the kind of principal proteinases capable of degrading type III collagen, which is a significant component in the first wound matrix (Klein and Bischoff 2011; Nguyen *et al.* 2016). During the tissue remodeling process, the synthesis of MMP-1 controlled by TIMP-1 produced by skin fibroblasts (Leonardi *et al.* 2003; Liu *et al.* 2016). In abnormal scar, the MMP-1 level is excessively decreased and followed by enhancement of TIMP-1 levels (Aoki *et al.* 2014). TIMP-1 is MMP-1 inhibitors with an essential role in controlling the MMP-1 regulation. Normally, TIMP-1 inhibits the MMP-1 activity by bounding with an active form of MMP-1 receptor. It can cause the active MMP-1 to turn into pro-MMP-1 (inactive) (Brew *et al.* 2000; Baker *et al.* 2002; Visse and Nagase 2003; Gauglitz *et al.* 2011). Decreasing MMP-1 leads the matrix extracellular to become excessive and causes abnormal scar, like hypertrophic or keloid (Brew *et al.* 2000; Visse and Nagase 2003). In Figures 2 and 3 showed the papain could increase the MMP-1 and decrease the TIMP-1. This mechanism can produce the matrix extracellular more degraded, and the abnormal scar has improvement. Meanwhile, Figure 8 showed the papain could affect the TIMP-1 directly, but not for MMP-1. This result demonstrated that papain affected the MMP-1 result indirectly through the regulation TIMP-1.

The result of TIMP-1 and MMP-1 also supported by the result of the TIMP-1/MMP-1 ratio. Figure 5 showed that the ratio of TIMP-1/MMP-1 decreased in the treatment group compared with positive control. This result indicated the papain affects the TIMP-1/MMP-1 ratio. The TIMP/MMP ratio is critical pathogenesis that causes abnormalities in collagen degradation. An imbalance between MMP and TIMP in abnormal keloid scar has observed (Ulrich *et al.*, 2010). This study analyzes scar tissue and serum by comparing the concentrations of MMP-2, MMP-9, MMP-1, TIMP-1, and TIMP-2. This research resulting in a decrease in MMP: TIMP ratio found in combustio traumatic injuries. The study of MMP-TIMP deserves attention as a non-operative keloid therapy by not only decreasing TIMP activity but also increasing MMP activity.

The negative control (C-) group of TIMP-1 levels measured 3x higher than the measured MMP-1 level, and in the positive control (C+) group, the TIMP-1 level is 10x higher than the MMP-1. The assumption of collagenase inhibition activity (TIMP-1) causes low MMP-1 activity. It showed

that collagen degradation in the normal scar group is higher than the abnormal scar group. This result demonstrated that the papain reduced the TIMP-1 inhibitory activity against MMP-1 and increased the collagen degradation activity. It was still not known whether papain inhibits TIMP-1 directly via cross-linked or through other intermediate proteins. Further research needs to carry out more about the relationship between the Papain enzyme as the enzyme protease cysteine with TIMP as a metalloproteinase enzyme inhibitor.

The result of VSSm in this research showed a slightly different score in the treatment group compared with positive control groups (Figure 6). It concluded that the papain could affect the score result of VSS in the abnormal scar. Morphologically, the abnormal scar improved along with the enhancement of papain dose (Figure 7). In path analysis, the TIMP-1, MMP-1, and the ratio of TIMP-1 and MMP-1 had a directly significant relationship with VSSm (Figure 8). It assumed that the degradation of collagen affects the score result of VSSm in the abnormal scar.

Papain enzyme can increase collagen degradation by increasing MMP-1 levels and decreasing TIMP-1 following a decreasing TIMP-1/MMP-1 ratio. The decreased TIMP-1/MMP-1 ratio defined the function of collagenase or MMP-1 increased because its inhibitor, TIMP-1, was suppressed by the papain. The enhancement of pH influenced by papain also affected the collagen degradation process and helped the improvement of the abnormal scar of rats.

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