

## Original Research Article

# Effect of papain enzyme administration in pH alteration, VEGF phosphorylation, and its impact on collagen degradation using a rat model of abnormal scarring

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Sent for review: 11 December 2020

Revised accepted: 23 April 2021

### Abstract

**Purpose:** To analyze the effect of enzyme papain administration, after its injection into abnormal scars of *Rattus norvegicus* rats, on pH alteration ( $\Delta$ pH) of scar tissue, vascular endothelial growth factor (VEGF) expression levels, and determine their impact on collagen degradation.

**Methods:** This study used *Rattus norvegicus* as an experimental animal. The pH measurement of the scar tissue was carried out before papain injection at 12th weeks while abnormal scar was established after papain injection at 15th weeks. Expression of vascular endothelial growth factor (VEGF) was examined by Western blot technique, while hydroxyproline levels were measured using QuickZyme Total Collagen Assay.

**Results:** Changes in  $\Delta$ pH, VEGF expression, and hydroxyproline levels in the treatment group were significant ( $p < 0.05$ ) compared to control. Path analysis showed a direct relationship between the parameters ( $p < 0.01$ ), except for the correlation between VEGF and hydroxyproline value ( $p = 0.23$ ).

**Conclusion:** Papain enzyme administration increased  $\Delta$ pH value of abnormal scar tissue. A high dose of papain also causes an increase of collagen degradation process, as a response to angiogenesis deflation.

**Keywords:** Angiogenesis, Collagen density, Hydroxyproline, Papain enzyme, VEGF

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## INTRODUCTION

Abnormal scarring consists of hypertrophic and keloid scars, resulting from the loss in balance of communication between cells. It's often difficult to distinguish between both kinds of scars because hypertrophic and keloid scars have

similar appearance. Histological comparison between keloids and hypertrophic scars usually uses hematoxylin & eosin (HE). In keloids, the thickening of collagen fibers is more arranged irregularly than hypertrophic scarring [1]. Various factors are responsible for the incidence of abnormal scarring, such as trauma or deep injury

through dermis, excessive tension, the activity of several hormones, race, skin color, and genetic factors.

In general, wound healing imbalance occurs in one of the three main phases to form the abnormal scar. These are inflammation, proliferation, and remodeling [2]. The increase in collagen synthesis activity can become exaggerated due to elongation and repetition of the inflammatory phase, which can cause abnormal scarring. Prolongation of inflammation will result in endothelial damage, due to the accumulation of pro-inflammatory cells and excessive fibroblast activity [3]. This phase, evidenced by an increase in NF- $\kappa$ B levels, activates vascular endothelial growth factor (VEGF) to leading to an angiogenesis process, granulation tissue formation [4], and activation of fibroblasts [5,6]. This mechanism generates abnormal scars.

Currently, many phytochemical agents have studied the therapeutic effect of modulating abnormal scars [6]. Papain is one of the phytochemicals obtained from papaya sap (*Carica papaya* Linn.), a K-protease family group of cysteine proteases [7]. Several studies have shown that papain can prevent inflammation through the inhibition of the NF- $\kappa$ B pathway, and inhibition of phosphorylation of protein kinase B (Akt), ERK-1/2, and p38-MAPK [8,9]. *In vitro* studies by Mohr and Desser on the human umbilical vein endothelial cells (HUVEC) culture showed papain enzyme promoted an anti-angiogenic effect on VEGF, which is activated through the downregulation of the MAPK signalling pathway (ERK-1) [10]. The elongation and repetition of angiogenesis in abnormal scars could be controlled, resulting in lowering collagen density.

Papain enzymes are optimally active in acidic environment with a pH range 3-7.5 [11]. Researchers estimate that the enzyme papain can work optimally in abnormal scars, increase the process of angiogenesis, and reduce collagen density. An *in vitro* study from Wihastyoko and Hanafi stated that the papain enzyme could reduce collagen density and increase hydroxyproline levels as a marker of collagen degradation activity [12]. This study aims to analyze the effect of enzyme papain administration after injected into abnormal scars of *Rattus norvegicus* rats against pH alteration ( $\Delta$ pH) of scar tissue, vascular endothelial growth factor (VEGF) expression levels, and determined their impact on collagen degradation.

## EXPERIMENTAL

### Sample preparation

This research was a Randomized Controlled Trial Post Test Only Design, with *Rattus norvegicus* Norway Brown rats as experimental animals. This study used twenty-five experimental animals, which were divided into two control groups (negative control or K- and positive control or K+), and three different doses of papain, consisting of 5 mg doses (P1), 10 mg doses (P2) and 20 mg doses (P3) (n = 5). Negative control group (K-) were induced with a normal scar. Positive control group (K+) and the treatment group (P groups) were induced with an abnormal scar. Before scar formation, the rats were anesthetized with ketamine 10-20 mg/kg by intramuscular route, weighed, and given antisepsis with a solution of chlorhexidine cetrimide (Savlon®).

### Scar induction procedure

Normal scar induction was obtained by making an incision on the dorsal part of a 2 cm length surgical wound and doing a primary suture. Then the stitches were taken on the 7th day from when scar were made. For the abnormal scar induction, the dorsal skin was excised in a circular shape with a 15 mm diameter panniculus carnosus (PC) depth. Panniculus carnosus is a thin layer of striated muscle attached to the skin and fascia. This layer mostly located in mammals and body regions [13]. The remaining panniculus carnosus located on the edge of the wound was sewn with dermis around to avoid contraction, which could inhibit the induction of abnormal scars. The wound was covered with sterile tulle and gauze, and fixed with hypoallergic tape (micropore®). The rats were given metamizole 1 mg/kg three times a day for two days by the intramuscular route [14].

### Papain injection

The papain used was papain 25 mg (Worthington, USA). Three doses of papain was used: 5, 10, and 20 mg/rat. The papain was dissolved in carboxymethyl cellulose (CMC) solution and injected intra-lesionally into the scar once at 12<sup>th</sup>, 13<sup>th</sup>, and 14<sup>th</sup> weeks sequentially, and counted from the first week the scar was induced. Papain administration utilized a 1 mL 27 G needle syringe.

### Measurement of tissue pH

The pH of the scar tissue was measured on the 12<sup>th</sup> week before papain administration, and 15<sup>th</sup>

week before the excision of the scar, using the Lutron 201 pH meter. The pH glass membrane containing the electrode bulb was calibrated to pH 4.0 and 8.0 before it was used. The measurement of the scar tissue was obtained in 1 minute. The pH number appeared on display. The difference in pH before and after papain administration was written down as delta pH ( $\Delta$ pH).

### Tissue Sampling Procedure

Tissue collection was done in the 15th week. First, the rats were anaesthetized intramuscularly using ketamine 100 mg/kg. The hair on the rat dorsal was cleaned using a chlorhexidine cetrimide solution (Savlon®). The scar tissues were obtained and washed with a sterile phosphate buffer saline (PBS), wrapped in aluminium foil, and put into an icebox filled with ice gel. The samples were carried out for further analysis of VEGF and hydroxyproline in the laboratory.

### Assessment of VEGF

The sample tissues were homogenized with radio immuno-precipitation assay (RIPA) buffer 10% (w/v) and stored in a cool box for 10 - 15 min, and then centrifuged 13,000 rpm for 10 min. One microliter supernatant was taken to be examined for protein concentration using Nanodrop ND1000. The remaining sample with a concentration of 0.73 mg/mL was done by sodium dodecyl sulphate poly acrylamide gel electrophoresis (SDS-PAGE). The supernatant was mixed reducing sample buffer (RSB) with a ratio of 1: 1, and heated in boiling water for  $\pm$  10 min. Twenty  $\mu$ l was run at 100 volts for 90 min. The gel was taken, then stained with commasie brilliant blue for 3 h, and destained every 1.5 h. The SDS-PAGE gel was transferred by semidry transfer (Biorad), with the following arrangement: Filter paper, Nitrocellulose (NC) membrane (MACHEREY-NAGEL Porafil) 7 x 9 cm size, SDS-PAGE gel, and filter paper.

The gel was conducted for 2 h, 20 V, 300 mA. The NC membrane was removed and washed in distilled water. The membrane was then immersed in 5 % skim milk (Tropicana slim plain) in 4°C for 30 min, removed, and awaited to room temperature. The skim milk was discarded, and then the membrane was washed 3 times in 0.05 % TBS-Tween 20 (Merck), for 5 minutes. Anti VEGF monoclonal antibody (SANTA CRUZ, cat. Sc-7269) was dissolved with TBS-Tween 0.05% with a ratio of 1: 1000 in 5 ml, added into membrane, and incubated for 2 hours. Biotin anti-mouse antibody was dissolved into TBS in a

ratio of 1: 10000, with volume of 5 ml. Membrane was soaked for 60 minutes.

A Streptavidin-HRP (SA HRP) (Merck) was dissolved in 0.05% TBS-Tween solvent in a ratio of 1: 1000 in 5 ml. The membrane was placed in an incubator for 40 minutes. Substrate 3,3',5,5'-Tetramethylbenzidine (TMB) (KPL SureBlue™) 2 ml was added and incubated for 30 minutes until the band detected. The reaction was stopped with H<sub>2</sub>O.

### Determination of hydroxyproline

Hydroxyproline measurement was carried out to determine the value of density-dependence of collagen degradation. Samples were first homogenized using RIPA buffer (10w/v) and stored for 10 - 15 min. Sample was then centrifuged 13,000 rpm for 10 min. A 50  $\mu$ L of supernatant was taken, then the hydroxyproline level in the sample in the medium was measured using the QuickZyme Total Collagen Assay kit following the standard procedure.

### Ethical clearance

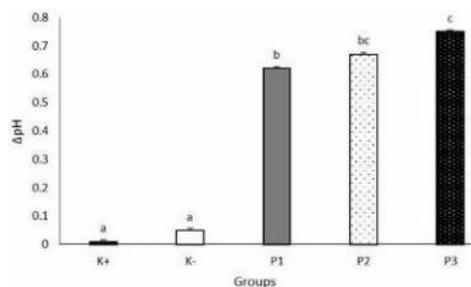
This research was approved by the ethics committee of the Faculty of Medicine, Brawijaya University (approval no. 168/EC/KEPK-S3/05/2019). The procedure adopted in the animal studies followed international guidelines [15].

### Statistical analysis

Data were analyzed using Microsoft Excel and SPSS 25 software. Correlation of papain doses, delta pH, VEGF, and hydroxyproline value was assessed using ANOVA, followed by Tukey's test; statistical significance was fixed at  $p < 0.05$ . Path analysis was performed with WarpPLS 7 software and statistical significance was set  $p < 0.01$ .

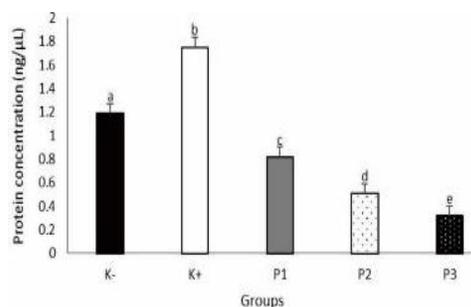
## RESULTS

The  $\Delta$ pH, VEGF, and hydroxyproline value had mean with significant result ( $p < 0.05$ ). No significant differences showed in  $\Delta$ pH between the control (K) groups, but significant elevation was expressed when compared to papain treatment (P groups) ( $p < 0.05$ ) (Figure 1). Abnormal scar injected with 5 mg doses of papain (P1) had a lowest  $\Delta$ pH value compared to other doses.

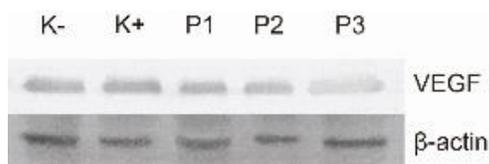


**Figure 1:** Comparison of ΔpH in the negative control group (K-), positive control (K +), papain dose 5 mg (P1), papain dose 10 mg (P2), and papain dose 20 mg (P3)

Treatment with papain doses 5 mg, 10 mg, and 20 mg respectively reduced the VEGF expression (Figure 2) and band levels showed a significant difference ( $p < 0.05$ ). This result demonstrated the administration of papain influenced stimulation of angiogenesis through VEGF suppression.

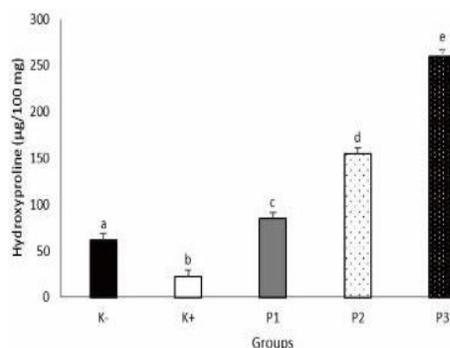


**Figure 2:** Protein quantification showed papain suppressed the apoptosis expression via VEGF regulation (negative control group (K-), positive control (K +), papain dose 5 mg (P1), papain dose 10 mg (P2), and papain dose 20 mg (P3))



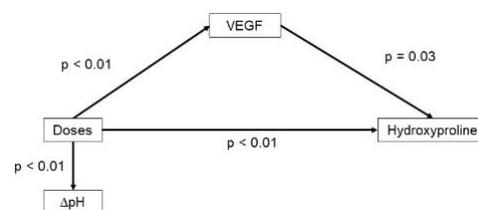
**Figure 3:** Comparison of VEGF band expression by western blotting view after abnormal scar was treated by papain (Negative control (K-), positive control (K+), papain dose 5 mg (P1), papain dose 10 mg (P2), and papain dose 20 mg (P3))

Hydroxyproline value as indicator of collagen density levels indicated significantly increased results ( $p < 0.05$ ) after papain treatment was administered (Figure 4). It suggested that the papain doses consecutively produced high expression of collagen density-dependence.



**Figure 4:** Hydroxyproline levels in negative control group (K-), positive control (K +), papain dose 5 mg (P1), papain dose 10 mg (P2), and papain dose 20 mg (P3)

Path analysis result showed papain treatment directly affected the value of ΔpH, VEGF, and hydroxyproline levels ( $p < 0.01$ ). However, exposed VEGF expression to hydroxyproline levels generated no significant relationship with  $p$  value = 0.03.



**Figure 4:** Path analysis results showing papain treatment affected the changes in ΔpH, VEGF, and hydroxyproline levels

## DISCUSSION

Wound healing is a process that repairs the tissue layer from a damage that occurred in the body. This process involves a phase of collagen synthesis that requires the formation of new blood vessels or neovascularization for the supply of oxygen and other functional cells to the wound site [4,16]. Neovascularization can generally be through vasculogenesis or angiogenesis. Vasculogenesis is the process of forming new or de novo blood vessels, triggered by endothelial progenitor cells (EPC). Vasculogenesis occurs in adult human tissues in response to ischemia. Meanwhile, new blood vessels can also be formed from pre-existing vessels, and this is called angiogenesis. This process requires endothelial cells proliferating, migrating, and differentiating into new blood vessel structures. Both vasculogenesis and angiogenesis can be triggered by VEGF [17,18].

This study showed a reduction of VEGF expression after the injection of the Papain enzyme, accompanied by the enhancement of hydroxyproline levels. This result explained the papain enzyme's inhibition of VEGF phosphorylation during the wound healing remodelling process, resulting in the elevation of collagen degradation activity, evidenced by increasing hydroxyproline levels. Hydroxyproline is a direct marker of collagen measurement in tissues. The amount of hydroxyproline is an index of collagen degradation [19]. The papain enzyme itself had a strong anti-angiogenic effect on VEGF, activated through down-regulation of the MAPK signalling pathway in in-vitro studies conducted by Mohr and Desser using HUVEC culture. It was also concluded that the anti-angiogenic effect could be caused by the cytotoxicity of papain enzyme in endothelial cells [10]. Consequently, stimulated endothelial cells may be damaged or lysis. The papain enzymes were also disturbing the ligand to bond in primary VEGF receptor that mediates angiogenesis, such as VEGFR2.

This study also resulted in an increased tissue pH after the administration of papain enzyme. The acidic atmosphere in the tissues reduced the response of VEGF-mediated endothelial cells. In the study of Faes *et al*, the acidity of the tumor tissue reduced in-vitro VEGF-mediated endothelial cell response, and decreased anti-angiogenic efficacy of anti-VEGF therapy [20]. The Administration of sunitinib with sodium bicarbonate increased its efficacy by decreasing the number of new blood vessels. Giving sodium bicarbonate increased the pH of the tumor tissue to become alkaline. This study was similar to recent study of papain treatment increase level in pH tissue.

Angiogenesis in normal wound healing occurs in the inflammatory and proliferative phases. In abnormal scars, angiogenesis occurs in the remodelling phase, where the collagen formed should be degraded to achieve normal scar balance. In this research, the measurement of VEGF was suppressed in the remodelling phase of rat tissue. VEGF can increase scar formation indirectly, based on its ability to stimulate angiogenesis, and expand the number of inflammatory cells and the activity of dermal fibroblasts [17].

Abnormal scarring VEGF plays a role in every phase of wound healing, not only in the inflammatory and fibroplasia phase. In the phase of scarring and remodelling, VEGF still plays a role. This prolongation of VEGF activity makes it difficult to control abnormal scars. Further study

needs to be explored on papain treatment in relationship with other cells, or anti-angiogenic factors such as Sprouty2, pigment epithelium-derived factor, and CXCR3 ligands, for example, IFN $\gamma$ -inducible protein-10 (CXCL10).

## CONCLUSION

The administration of papain enzyme increases hydroxyproline levels, as a marker of density-dependent collagen degradation, through a decrease in VEGF phosphorylation activity in tissues with acidic pH. These results indicate that papain has a potential for use as a repairing agent for abnormal scar.

## DECLARATIONS

### *Acknowledgement*

We thank the Faculty of Medicine, Brawijaya University for their support for the study.

### *Conflict of interest*

No conflict of interest is associated with this research.

### *Contribution of authors*

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Herman Yosef Limpat Wihastyoko designed the work, drafted the acquisition, analysis, and interpreted the data; Setyawati Soeharto drafted the acquisition and revised the manuscript for publication; Edi Widjajanto interpreted the data and approved the final manuscript to be published, Kusworini Handono revised and gave the final approval of the version of manuscript; Bambang Pardjianto aggregated all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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