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### Effect of Phenytoin and Vitamin C on Granulation Tissue Thickness and Total Lymphocyte Infiltration in Enterocutaneous Fistula: In Vivo Study

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#### ABSTRACT

**Background:** Enterocutaneous fistula (ECF) is still a problem in the surgical field because it causes high morbidity and mortality. The rate of closure of the ECF without surgical intervention in the evolving era of wound care and with parenteral nutrition has been reported to vary, with most studies showing closure rates in the 20 to 30% range. The administration of phenytoin and vitamin C is expected to be one of the modalities in the non-operative treatment of enterocutaneous fistulas. This study aims to assess the effect of phenytoin and vitamin C on granulation tissue thickness and the amount of lymphocyte infiltration in enterocutaneous fistulas. **Methods:** This study is an experimental study with a post-test only with a control group design. A total of 24 rats were divided into control groups, P1 (phenytoin+ vitamin c), P2 (phenytoin), and P3 (vitamin c). Histopathological assessment of granulation tissue thickness and lymphocyte infiltration was performed. Data analysis was performed using SPSS with univariate and bivariate methods. **Results:** The P1 group showed the thickest average thickness of granulation tissue compared to all groups. The P1 group showed a lower number of lymphocyte infiltration cells than all groups. **Conclusion:** The administration of phenytoin and vitamin C was effective in increasing the thickness of granulation tissue and reducing lymphocyte cells in enterocutaneous fistulas.

#### 1. Introduction

Enterocutaneous fistula (ECF) is defined as an unintentional abnormal connection of the digestive tract (GI) with the skin of the abdominal wall.<sup>1,2</sup> Morbidity due to ECF is estimated to be experienced by 90% of patients ranging from skin excoriation, and dehydration to sepsis. Enterocutaneous fistulas also cause many complications such as longer hospital stays, increased treatment costs, electrolyte imbalance, and sepsis. Reported mortality ranges from 6% - 33%, with the most common causes of death being malnutrition and sepsis.<sup>1-3</sup> The rate of closure of

the ECF without surgical intervention in the developed era of wound care and with parenteral nutrition has been reported to vary from 19 to 92%, with most studies showing closure rates in the range of 20 to 30%. 90% spontaneous fistula closure occurs in the first month after the resolution of sepsis, with an additional 10% closure in the second month, and will not close spontaneously after more than two months.<sup>4,5</sup> The decision to operate on ECF should not be taken arbitrarily because the recurrence rate after ECF surgical intervention is still high ranging from 13

to 34%.<sup>4</sup>

One of the most studied therapeutic modalities that has beneficial effects on wound healing is phenytoin. A study examining the beneficial effects of phenytoin in wound healing in epileptic patients with gingival hyperplasia concluded that phenytoin increases collagen deposition, which is necessary for wound healing. Since then, the effectiveness of phenytoin in wound healing has been reported in several clinical trial studies for various types of wounds.<sup>5</sup> The mechanism by which phenytoin accelerates wound healing is not known with certainty. Studies suggest that phenytoin may be involved in the healing process at several levels, including stimulating fibroblast proliferation, increasing granulation tissue formation, reducing collagenase activity (by reducing production or secretion or both), promoting deposition of collagen and other connective tissue components, reducing bacterial contamination, and reduce wound exudate. Phenytoin-treated open wound biopsies showed neovascularization, collagenization, and decreased polymorphonuclear cell and eosinophil infiltration.<sup>6-10</sup> Vitamin C is an important co-factor for several enzymatic reactions and has been shown to suppress pro-inflammatory processes and promote anti-inflammatory effects. Vitamin C is also closely involved in the metabolism and regulation of collagen. This study is an initial study that aims to determine the effect of phenytoin and vitamin C on inflammation and the resolution of enterocutaneous fistulas by assessing granulation tissue and lymphocyte infiltration in vivo.

## 2. Methods

This study is an experimental study with a post-test-only approach with a control group design. A total of 24 rats (*Rattus norvegicus*) Wistar strain was included in this study and met the inclusion criteria in the form of the male sex, weight between 150-200 grams, and of age 8-10 weeks first. The rats were acclimatized for 7 days, then divided into 4 groups (control, P1, P2, and P3) randomly, where each group consisted of 6 rats. Control group: Rats were induced

to have enterocutaneous and were only treated with moist gauze. Group P1 : Rats were induced for enterocutaneous and received a combination of phenytoin 10 mg/kg BW intragastric and vitamin C 90 mg/kg BW intragastric. Groups P2 and P3 each received only phenytoin 10 mg/kg BW and only vitamin C 90 mg/kg BW. The treatment was given for 7 days. This study has been approved by the Health Research Ethics Commission of the Faculty of Medicine, Universitas Diponegoro, with the number No. 67/EC/H/FK-UNDIP/VII 2020.

Induction of enterocutaneous fistula is performed by the first anesthetic rat using ketamine (dose 0.015 mg/gBW) intramuscularly and chlorate (0.0025 mg/gBW) subcutaneously. The rats were positioned so that they were lying on their backs, and the entire extremity was fixed with a bandage. Sepsis-antiseptic was performed on the rat's abdomen using 0.5% chlorhexidine then the sterile area was covered with a sterile drape. An incision is made in the lower left area with a length of 7 mm. You will see the caecum, which is located above the intestine in the left inferior abdominal region. The cecum is removed and then fixed with thread and needle. A blunt subcutaneous duct was made in the left inferior abdominal region. A 5 mm excision was performed at the end of the tunnel. The caecum was pulled into the abdominal muscle wall layer and fixed by suturing with PGA 5-0. Then the fistula was closed using moist gauze.

Rats were monitored daily for signs of distress and signs of infection. The fistula is covered with moist gauze sterile On the 7<sup>th</sup> day. The fistula tissue was terminated at the same time: The rats were anesthetized with a mixture of Ketamine-Xylazine (Ketamine dose 80 mg/kg BW; Xylazine dose 10 mg/kg BW) intraperitoneally, and the skin and subcutaneous tissue were cut with a scalpel with a size of 1 cm x 1 cm x 1 cm. Pieces of fistula tissue were placed in a buffered formalin solution (10% formalin solution in sodium acetate buffer until it reached a pH of 7.0). Then do fixation for 18-24 hours. After fixation, the tissue was then put into aquadest for 1 hour so that the fixation solution was lost. The tissue pieces were

then put in graded concentration alcohol, alcohol-xylol solution for 1 hour, and then pure xylol solution for 2 x 2 hours. The tissue was then put into a liquid paraffin solution for 2x2 hours, after which the tissue was embedded in solid paraffin, having a melting point of 56-58°C. The tissue was cut about 6 m and glued onto a glass object that had been previously smeared with polylysine. Glass object was then heated in an incubator at a temperature of 56-58°C until the paraffin melted.

The next step is to stain the tissue with hematoxylin-eosin. The tissue was put into xylol I and II preparations for 5 minutes each. Rehydrate using absolute alcohol for 3x2 minutes. The preparation was washed with running water for 2 minutes. The preparation was put in hematoxyline-eosin (Lilie-Mayer) for 5 minutes and rinsed with running water for 2 minutes. The preparation was differentiated with 0.6% HCl for 1-2 dips and rinsed with running water for 2 minutes. The preparation is immersed in a saturated lithium carbonate solution about 2-3 times and rinsed with running water for 2 minutes. If the color is not blue enough, the preparation can be put back into the H&E solution for 2 minutes and then rinsed with running water. The preparation was immersed in eosin for 3 minutes. Dehydrated using 70% alcohol for 3x3 minutes for each concentration. Clearing with xylol I and II, dripped 1-2 drops of entelan. Sediaan is covered with a cover glass. Furthermore, the histopathological assessment was

carried out by anatomical pathologists with the help of ImageJ software.

After the data is collected, data cleaning, coding, and tabulation are carried out. All results were assessed by means  $\pm$  standard deviation accompanied by a normality test (Shapiro Wilk) and data homogeneity test (Levene Statistic). The test used in this study was one-way Anova, followed by a post-hoc test to assess differences between groups. The results are said to be meaningful if  $p \leq 0.05$ . Data analysis was performed using SPSS version 25 for Windows.

### 3. Results

The thickness of granulation tissue was in treatment group 1 (P1), which was given a combination therapy of phenytoin and vitamin C, which was  $24,843 \pm 6.9 \mu\text{m}$ , and the smallest was in control, which was  $6.342 \pm 1.76 \mu\text{m}$ . Based on the One way ANOVA test, it can be seen that the  $p < 0.05$ , which means the difference is significant. In the Post-Hoc test, there were differences in granulation tissue thickness among the group given combination therapy with oral phenytoin and oral vitamin C, oral phenytoin, and oral vitamin C had a significant difference compared to the control group in each group ( $p < 0.05$ ). There was a significant difference in the group given a combination of oral phenytoin and oral vitamin C compared to the group given oral phenytoin or oral vitamin C only ( $p < 0.05$ ).

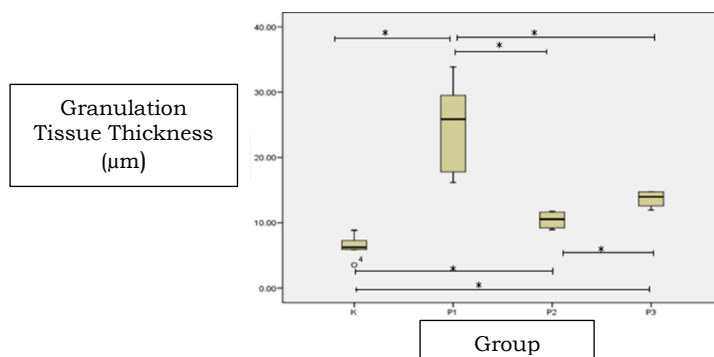


Figure 1. Boxplot graph of granulation tissue thickness of each group. K: fistula wound treatment with moist gauze, P1: fistula wound treatment with a combination of oral phenytoin and oral vitamin C, P2: fistula wound treatment with oral phenytoin (P2), P3: fistula wound treatment with oral vitamin C (P3). There was a statistically significant difference (One Way ANOVA  $P < 0.05$ ). And also found a significant difference when compared in each group (Post Hoc test); \* significant  $P < 0.05$ .

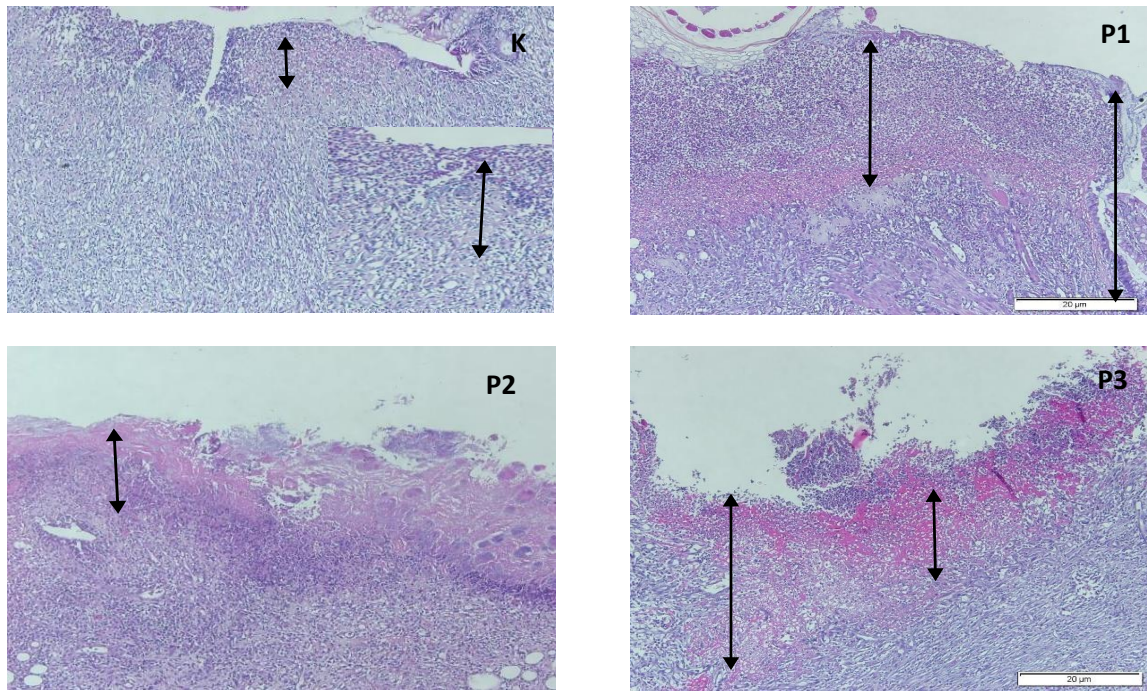


Figure 2. Granulation tissue appearance on histological preparations of enterocutaneous fistula tissue with 100x magnification in each group. (K: Control group, P1: Group given phenytoin and vitamin C, P2: Group given phenytoin, P3: Group given vitamin C).

There is a difference in lymphocyte infiltration where the lowest number is in treatment group 1 (P1) given combination therapy phenytoin, and vitamin C is  $160.2 \pm 10.4$  cells. The highest lymphocyte infiltration was found in the control group who received wound care with gauze, which was  $320,167 \pm 50.4$  cells. Based on the One way ANOVA test, it can be seen that the  $p < 0.05$ , which means the difference is significant. In the Post-Hoc test, there were

differences in the amount of lymphocyte infiltration from the group given combination therapy with oral phenytoin and oral vitamin C, oral phenytoin and oral vitamin C had a significant difference compared to the control group in each group ( $p < 0.05$ ), and there was a significant difference in the group given a combination of oral phenytoin and oral vitamin C compared to the group given oral phenytoin or oral vitamin C ( $p < 0.05$ ).

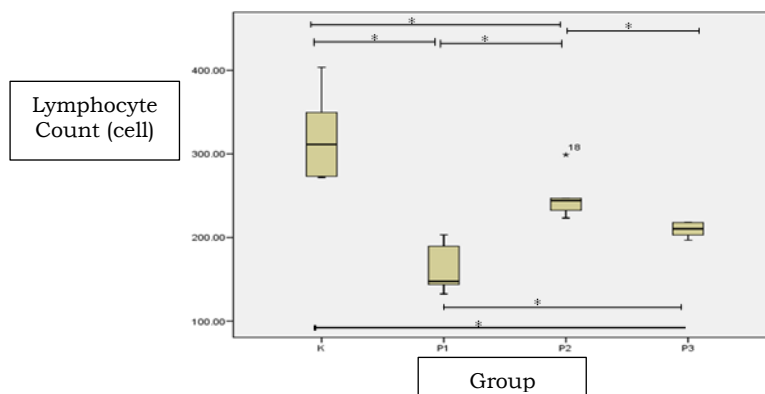


Figure 3. Boxplot graph of enterocutaneous fistula lymphocytic infiltration of each group. K: fistula wound treatment with moist gauze, P1: fistula wound treatment with a combination of oral phenytoin and oral vitamin C, P2: fistula wound treatment with oral phenytoin (P2), P3: fistula wound treatment with oral vitamin C (P3) There was a significant difference statistically (One Way ANOVA  $P < 0.05$ ), and also found a significant difference when compared to each group (Post Hoc test); \*significant ( $P < 0.05$ ).

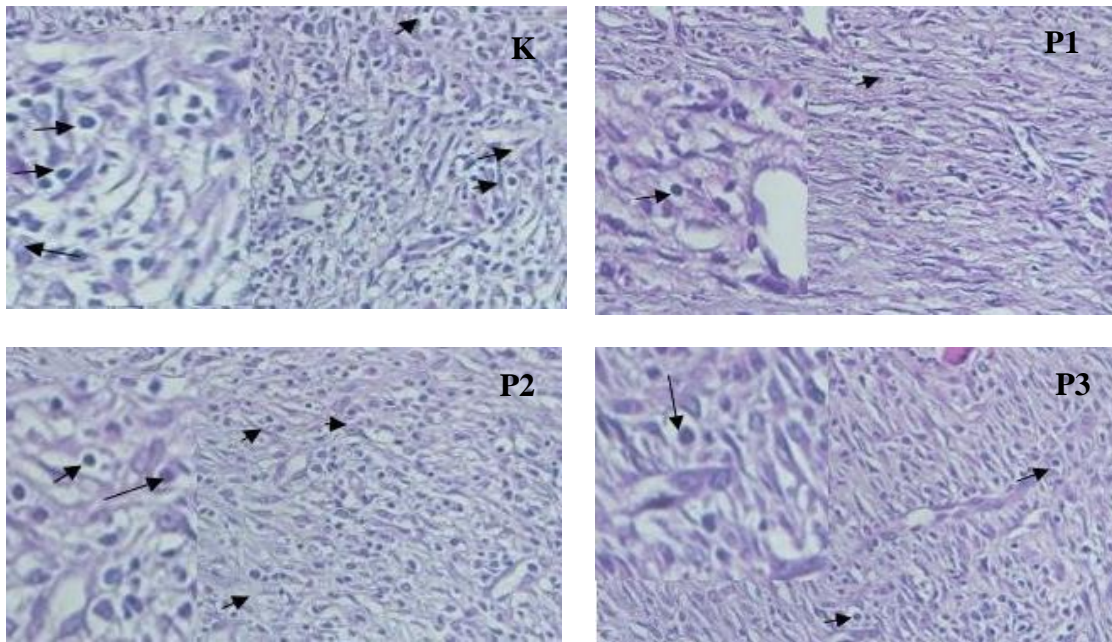


Figure 4. Microscopic view of lymphocytic infiltration of enterocutaneous fistulas after therapy. (K: Control group, P1: Group given phenytoin and vitamin C, P2: Group given phenytoin, P3: Group given vitamin C).

#### 4. Discussion

Progress from the use of phenytoin, which was initially used as antiepileptic therapy until the effect of gingival hyperplasia was discovered. Which provides opportunities for the development of phenytoin in tissue repair and healing.<sup>11</sup> The results of this study indicate that the administration of phenytoin can increase the thickness of granulation tissue. This the potential for phenytoin to trigger the activity of transforming growth factor-beta 1 (TGF-beta1), followed by the activation of the SMA alpha protein, fibroblast activation, and fibrocyte activation. Furthermore, the fibrocytes will produce collagen, which plays a role in the initiation of granulation tissue. Another study showed that giving phenytoin to excision wounds of rats showed that phenytoin accelerated wound healing and increased the hydroxyproline content of the wound.<sup>12</sup> Another study of 60 patients with diabetic ulcers showed that administration of phenytoin 100 mg was able to significantly accelerate the healing of diabetic ulcer wounds compared to controls without phenytoin.<sup>13</sup> Another study assessed the effect of phenytoin on colonic anastomosis healing in rats, where the anastomotic bursting pressure value and

hydroxyproline levels were also higher with phenytoin administration compared to controls without phenytoin.<sup>14</sup>

Vitamin C is a macronutrient that is needed by the body. Vitamin C is rich in ascorbic acid, which has various potentials related to the inflammatory response. An ascorbic acid is a flavonoid group that is rich in anti-inflammatory potential. Anti-inflammatory agents actively play a role in suppressing chronic inflammatory processes and disrupting tissue repair processes. Anti-inflammatory agents play a role in suppressing various cell activities and pro-inflammatory cytokines. In this study, it was seen that the administration of vitamin c was able to suppress the inflammatory process, which was characterized by a decrease in infiltration in lymphocyte cells anti-inflammatory activity will trigger the action of alpha SMA activation, fibroblast activation, and fibrocyte activation, which will lead to tissue repair through collagen production. Several studies have also shown that the administration of vitamin c was able to repair wound tissue repair faster and better than the group that did not receive vitamin C.<sup>15-17</sup>

## 5. Conclusion

The administration of phenytoin and vitamin C was effective in increasing the thickness of granulation tissue and reducing the infiltration of lymphocytes in enterocutaneous fistulas in rats.

## 6. References

1. Ortiz LA, Zhang B, McCarthy MW, Kaafarani HM, Fagenholz P, et al. Treatment of enterocutaneous fistulas, then and now. *Nutrition in Clinical Practice*. 2017 ;32(4):508-15.
2. Bhama AR. Evaluation and management of enterocutaneous fistula. *Diseases of the colon & rectum*. 2019; 62(8):906-10.
3. Dumas R, Moore S, Sims C. Enterocutaneous fistula: Evidence-based Management. *Clin Surg*. 2017; 2:1435.
4. Gribovskaja-Rupp I, Melton GB. Enterocutaneous fistula: proven strategies and updates. *Clinics in colon and rectal surgery*. 2016; 29(2):130.
5. Lloyd D, Gabe S, Windsor A. Nutrition and management of enterocutaneous fistulas. *British Journal of Surgery*. 2006; 93(9):1045-55.
6. Lynch AC, Delaney CP, Senagore AJ, Connor JT, Remzi FH, et al. Clinical outcome and predictive factors of recurrence after enterocutaneous fistula surgery. *Annals of surgery*. 2004; 240(5):825.
7. Hoedema RE, Suryadevara S. Enterostomal therapy and wound care of the enterocutaneous fistula patient. *Clinics in colon and rectal surgery*. 2010; 23(3):161.
8. Misky A, Hotouras A, Ribas Y, Ramar S, Bhan C. A systematic literature review on the use of vacuum assisted closure for enterocutaneous fistulas. *Colorectal Disease*. 2016; 18(9):846-51.
9. Wainstein DE, Fernandez E, Gonzalez D, Chara O, Berkowski D. Treatment of high-output enterocutaneous fistulas with a vacuum-compaction device. A ten-year experience. *World journal of surgery*. 2008; 32(3):430-5.
10. Shakeri F, Tebyanian H, Karami A, Babavalian H, Tahmasbi MH. Effect of topical phenytoin on wound healing. *Trauma Monthly*. 2016; 22(5):35488.
11. Bhatia A, Prakash S. Topical phenytoin for wound healing. *Dermatology Online Journal*. 2004; 10(1):342-50.
12. Jaber S, Rihy Z, Joseph R, Al-Khayat M. Does phenytoin improve the healing of gastrointestinal fistulas? *Case Reports in Gastroenterology*. 2011; 5(1):52-5.
13. Moores J. Vitamin C: a wound healing perspective. *British journal of community nursing*. 2013; 18(Sup12):6-11.
14. Mohammed BM, Fisher BJ, Kraskauskas D, Ward S, Wayne JS, et al. Vitamin C promotes wound healing through novel pleiotropic mechanisms. *International wound journal*. 2016; 13(4):572-84.
15. Singh S, Young A, McNaught CE. The physiology of wound healing. *Surgery (Oxford)*. 2017; 35(9):473-7.
16. Thiruvoth FM, Mohapatra DP, Sivakumar DK, Chittoria RK, Nandhagopal V. Current concepts in the physiology of adult wound healing. *Plast Aesthetic Res*. 2015; 2(5):250-6.
17. Pereira CAZ, de A Alchorne Ado. Assessment of the effect of phenytoin on cutaneous healing from excision of melanocytic nevi on the face and on the back. *BMC dermatology*. 2010; 10(1):7.