Effect Phenytoin Therapy to Fibroblasts and Angiogenesis of Enterocutaneous Fistula in Wistar Rat

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Abstract

Background: Comprehensive wound care in enterocutaneous fistula (ECF) is the therapeutic of choice currently, which may increase of closure rate without surgery from 19 to 92%. Phenytoin has been reported have anticolagenase effect on wound healing is hoped to improve the ECF closure.

Aim: The study was aimed to demonstrate the effect of phenytoin on closure of enterocutaneous fistula seen from the number of fibroblasts and angiogenesis

Methods: This study was "Randomized Controlled trial with post test only group design" on 18 male wistar rats with ECF, that were divided randomly into three groups: group(K) control, P1(topical phenytoin), P2(oral phenytoin). After 7 days of treatment, they were terminated and histopathological examinations were performed to do fibroblast cell counting and the amount of angiogenesis by Hematoxilin Eosin staining.

Results: Mean of the number of fibroblastin groups K, P1 and P2 were 69.50 ± 10.07, 155.50 ± 13.50 and 182.16 ± 11.85 respectively (One way Anova *P*=0.001) and mean of the number of angiogenesis in groups K, P1, and P2 were 95.66 ± 9.72, 178.66 ± 11.75, and 205,16 ± 9.74 respectively (One way Anova *P*=0.001). Post Hoc Test LSD showed thatthe number of fibroblast of group P1 vs K(*P*=0.001), P2 vs K(*P*=0.001), P1 vs P2(*P*=0.004) and the number of angiogenesis of group P1 vs K(*P*=0.001), P2 vs K(*P*=0.001), P2 vs P1(*P*=0.002).

Conclusion: The therapy of topical and oral phenytoin increased the number of fibroblasts and angiogenesis in Wistar rat with ECF. Out come of oral phenytoin therapy better than topical.

Key words: *Enterocutaneous fistulas, phenytoin, fibroblasts, angiogenesis*

Introduction

Enterocutaneous fistula (ECF) is an abnormal communication between the intra-abdominal digestive tract and the skin.[1,2] The ECFoccurs spontaneously on 30% due to malignancy, sepsis, radiation or inflammatory diseases of the intestine, whereas due to postoperative complications of more than 75% .[2, 3]ECF patients are faced with conditions of increased morbidity and mortality due to it complications. Comprehensive wound care accompanied with parenteral nutrition (TPN) is currently the treatment of choice, with closure of fistulas without surgery increasing from 19% to 92%.[5-7]

After the ECF occurs, therapy must be immediately carried out with optimal patient stabilization and non-operative therapy which is conventional therapy.[1]

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Prognostic factors that allow spontaneous closure of fistulas are influenced by several factors, namely ECF that occurs due to surgery, transferrin value> 200 mg/dl , fistulasize of <2 cm, absence of obstructive, inflammatory, and intestinal infections.[5] Presence of components in the form of foreign bodies, radiation, inflammation, infection, inflammatory bowel disease, epithelization of the fistula tract, neoplasms, distal obstructions, and steroids (FRIENDS) are indication for surgical intervention.[4,8] In conventional therapy of ECF wound care plays an important role in the process of spontaneous closure, so that the administration of therapy to the wound in order to speed up closure is very necessary. The healing of passage in fistulas consists of various processes, including cell migration and the formation of new extracellular matrices, one of which is increased of fibrosis.

Presently, wound care at ECF also has many methods that can be done with moist dressing until the treatment using negative pressure wound therapies (NPWT) or one of which is often known as vacuum assisted closure (VAC).[5] The use of VAC is currently reported to improve the quality of life for patients EFC, which the skin around the EFC wound is protected.[5] The implementation of this method has a high cost and expertise of trained stoma nurses, so that there is still a need for an EFC wound care method that can more easily be developed.

Phenytoin drug has long been known as an anti-seizure drug is currently reported from several studies showing it had a therapeutic effect on wound healing. This has been supported by many studies and studies that reported since decades.[10] Several studies have shown the advantages of therapeutic effect of the administration of phenytoin on healing burns, trauma wounds, venous static ulcers.[11,12]

The mechanism of phenytoin in wound healing at this time is still uncertain, but in vitro research, it is known that there are several mechanisms that can support wound healing. The mechanism consists stimulation of proliferation of fibroblasts, increasing angiogenesis, increasing the formation of granulation tissue, glucocorticoid antagonists, decreasing collagenase activity, increasing collagen deposits, decreasing exudate in wounds and also finding antibacterial effects.[13-15]In other studies phenytoin was found to have a mechanism to reduce MMP-1 and 9 which prove the anti-collagenase effect of phenetoin.[16] This mechanism is the basis for the application of the case in ECF, with an increase in the number of fibroblasts and angiogenesis will enable the closure of the fistula canal.

Jaber reported in 2013 a case series of studies conducted with intravenous phenytoin giving a positive effect on the healing of gastrointestinal fistulas. The positive effects of phenytoin shown through systemic stimulation are significant because of the side effects that can occur in long-term administration.[9] Teo, in vitro studies show safety in topical phenytoin administration and therapeutic effect on wound healing.[17] So that research into the effects of phenytoin on ECF healing needs to be further developed by topical administration and oral administration which has not been done at this time.

Materials and Method

Subject

This research is an experimental study with a "Randomized Controlled Trial with Post Test Only Group Design" design. The study used Wistarrat*RattusNorvegicus* strain which will be divided equally into three random groups, 1 control group and 2 intervention groups. All groups were made to have the presence of ECF by surgical procedures. One group will be a control group treated with ECF wounds treated with moist gauze which will be terminated on the 7th day, while other subjects according to the group division will be treated with topical and oral phenytoin wounds which will be terminated on the 7th day.

Experimental animals are Wistarrat strain*(RattusNorvegicus)* . Inclusion criteria were 8-10 weeks old who performed the procedure for making ECF, body weight ± 150-200 grams after acclimation

for a week in individual cages and no visible anatomical abnormalities. While the exclusion criteria were rat appeared to be sick (inactive movements)during the treatment of ECF. Rats that lost> 10% and died at the time of the study were included in the drop out criteria. In this study, the number of samples used 6 ratsin each group. The sample was 18 rats. Each rat was then labeled number 1-18. The division of groups is done randomly by drawing lots.

The research and data collection were carried out for 3 months from April-June 2019. The topical phenytoin production site was conducted at the Chemistry Laboratory of theSekolah Tinggi Ilmu Farmasi (STIFAR) Yayasan Farmasi Semarang. Procedures for making enterocutaneous fistula conditions, treatment of rats, tissue retrieval, the process of makingpreparations and HE staining were carried out at the Laboratory of the BioSains Institute of Brawijaya University, Malang.

The independent variables are :

1. Topical phenytoin

2. Oral phenytoin

3. Without the administration of phenytoin

The dependent variables are:

1. Number of fibroblasts in histopathological preparations

2. Number of total angiogenesis in histopathological preparations

Experimental design

Rats were acclimatized in the laboratory for one week in individual cages with periods of 12 hours of light and 12 hours of darkness. Rats were fed and drank ad libitum. Providing food with feeds whose nutritional value composition has been standardized. After acclimatization, the rats were carried out the procedure of making enterocutaneous fistulas.

Animals were anesthetized intraperitoneally with 80 mg/kg ketamine (Pantex Holland, Duizel, Netherlands) and 10 mg/kg xylazine (Pantex Holland) diluted in PBS. The cecum was accessed through a standard 7-mm stab incision on the lower left side of the abdomen, sparing the colon upon exposure, and a 5-mm enterotomy was performed and sutured to the abdominal wall to create an enterocutaneous fistula. To allow spontaneous closure of the fistula, the opening in the cecum wall was secured to the borders of the surgical wound without maturation. At this point, the animals were randomly allocated into one of three groups:

1. Control group (CG)—the enterocutaneous fistula wound that was treated with moist gauze and terminated on the 7th day,

2. Topical phenytoin group (TPG)— the enterocutaneous fistula wound that was treated with 10% phenytoin ointment and terminated on the 7th day

3.Oral phenytoin group (OPG)—the enterocutaneous fistula wound that was treated with oral phenytoin and terminated on the 7th day. Oral phenytoin given with a maximum human dose (300 mg/day) is converted into a rat dose (200gr) through a conversion table. Based on the conversion, the dosage is 0.03 mg/grBB given once a day. Drug administration is made by dissolving phenytoin with aquabidest and given orally with a sonde.

Statistical Analysis

After the data is collected, editing, coding, tabulation and entry are carried out. Data analysis includes descriptive analysis and hypothesis testing. In descriptive analysis the dependent variable is presented in the form of a mean table, SD, median and box plot graph. The data in this study were normally distributed after the data normality test with the Saphiro-Wilk test both on the number of fibroblasts and the number of angiogenesis in each treatment group. Data analysis continued with the hypothesis test used was the One Way Anova test followed by a Post-Hoc Test to determine differences between groups.

Research Ethics

The animal experiments were performed in

8-10-weekold Wistar rat strain*(RattusNorvegicus)* in which an enterocutaneous cecal fistula was created. The study was submitted and approved by the Diponegoro University Faculty of Medicine Research Ethics Commission No.78/EC/H /KPEK/FK-UNDIP/V/2019. Eighteen animals were used in the experiments..

Results

The study was conducted on 18 Wistar rats, which were divided into 3 groups; i.e. groups of rats who treated with fistula wound treatment with moist gauze (C), topical phenytoin (P1) and oral phenytoin (P2), each group consisted of 6 rats, and until the end of the study were healthy and not included in the dropout criteria.

Characteristics of Research Samples

The average calculation result of 18 rats body weight, on the 7th day of acclimatization where that day is also the first day of the treatment process. This can be seen in table.

Number of Fibroblasts

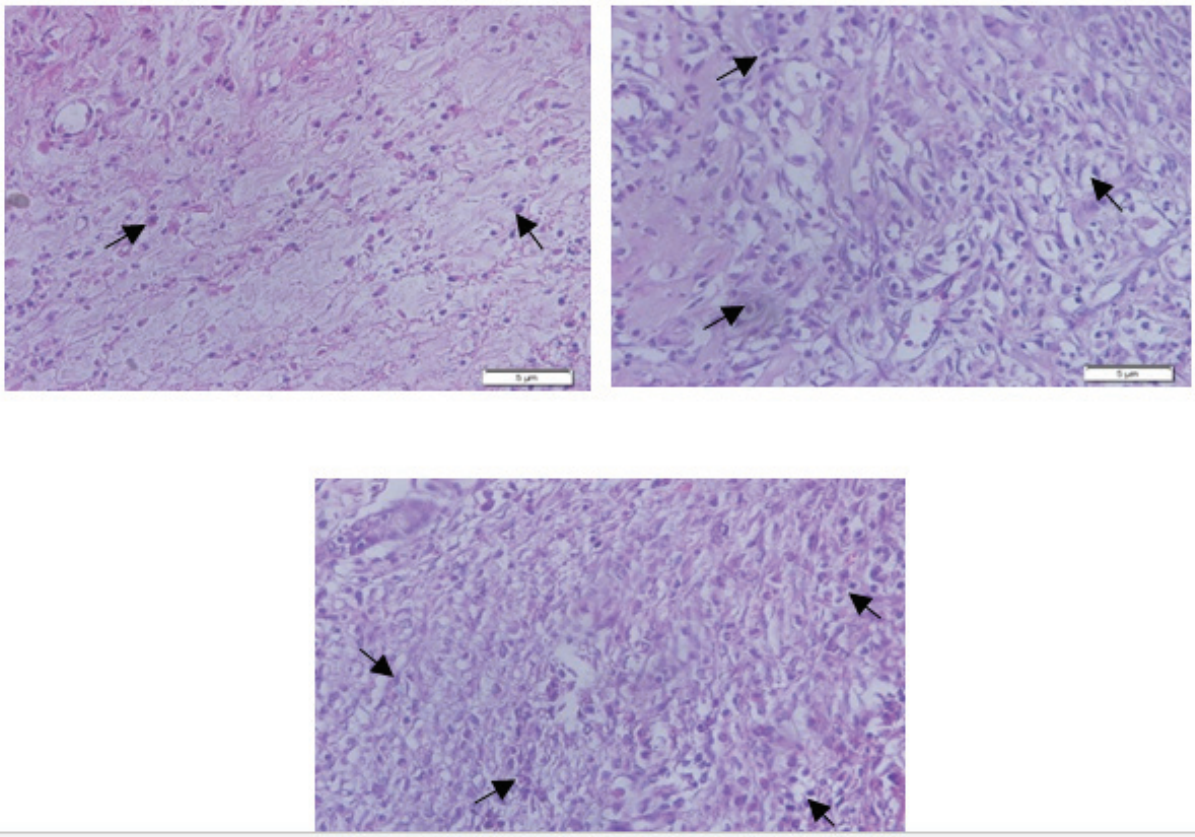

Figure 1. Histopathological picture of the number of fibroblasts with hematoxylin-eosin staining with 400x magnification. a.Without treatment, b.Topical phenytoin, c.Oral phenytoin; arrows indicate fibroblast cells.

Table 1. Descriptive tables and normality of body weight data (Grams)

Groups	Body weight (Grams)		p	Homogenity
	Mean ± SD	Median (min – max)		
C	180,17 ± 5,629	179 (175 – 190)	0,576	0,866**
P1	183,50 ± 5,010	183 (177 – 190)		
P2	182,17 ± 5,981	181,5 (175 – 192)		

Information: * Significant (p < 0,05); ** Homogen (p > 0,05)

From table 1, it can be seen that the rat weight data are normally distributed and homogeneous. The highest rat weight was found in the group with topical administration of phenytoin, which was 182.17 ± 5.981 grams.

Distribution of data

Variable data on the number of fibroblasts obtained an average number of 69.50 ± 10.07 in the group without phenytoin (C) therapy, 155.50 ± 13.50 in the group of topical phenytoin (P1) and 182.16 ± 11.85 in the oral phenytoin therapy group (P2). From the results of the normality test obtained normal distribution results (p> 0.05

Statistical Test for the Number of Fibroblast

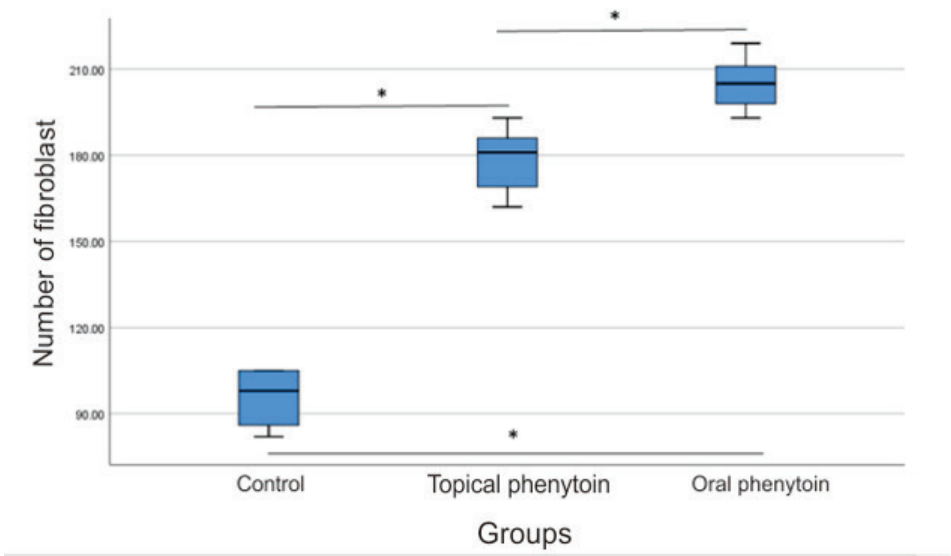

Figure 2. Boxplot graph of the number of fibroblasts from each group. C: fistula wound treatment with moist gauze, P1: fistula wound treatment with topical phenytoin, P2: fistula wound treatment with oral phenytoin (P2). There was a statistically significant difference (One Way ANOVA P <0.05). Also found significant differences when compared to each group (Post Hoc test); * significant P <0.05)

From the box above, it shows that there are differences in the number of fibroblasts. The lowest number of fibroblasts was seen in the control group without phenytoin therapy, 69.50 ± 10.07, while the highest number of fibroblasts was seen in the treatment group with oral phenytoin administration, which was 182.16 ± 11.85. Based on the One way ANOVA test, it can be seen that the p value <0.05, which means the difference is significant. In the Post-Hoc test there were differences in the number of fibroblasts from the groups given both topical and oral phenytoin therapy had a significant difference compared to the control group in each group (p <0.05), and there were significant differences in the groups given topical phenytoin compared the group was given oral phenytoin (p <0.05).

Total angiogenesis

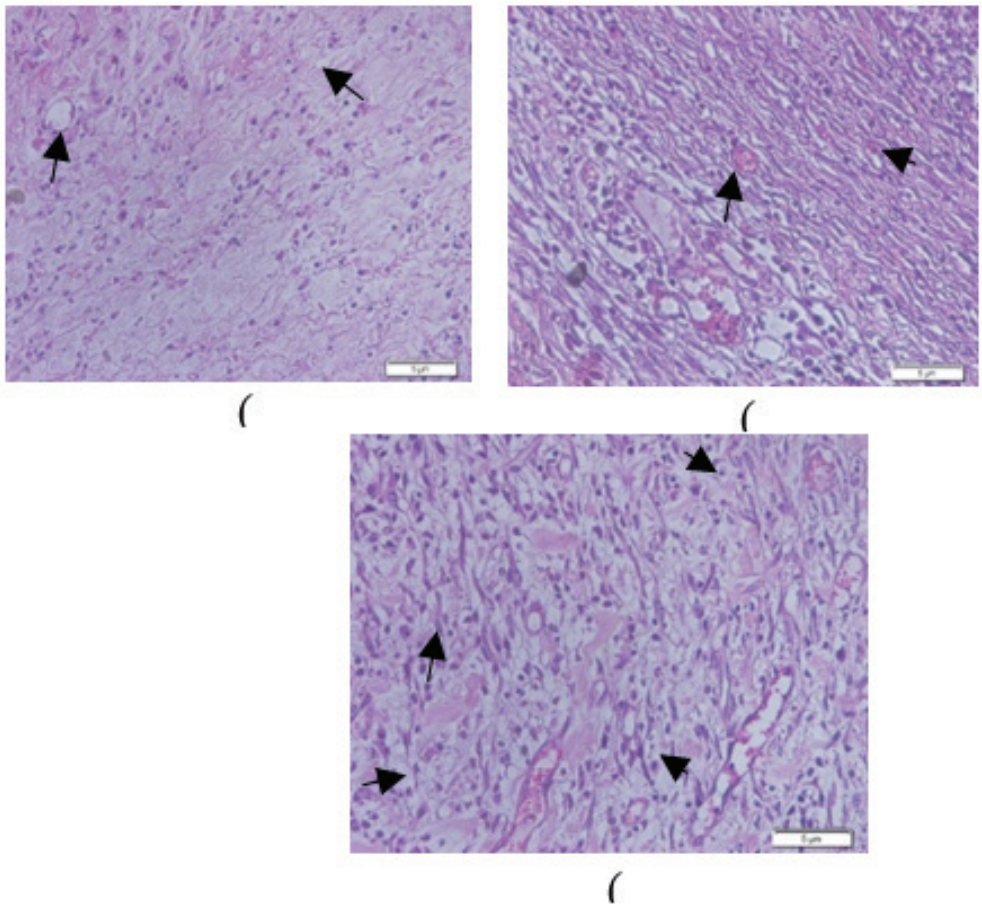

Figure 3. Histopathological picture of the amount of angiogenesis by painting hematoxylin-eosin with 400x magnification. a. Without treatment, b. Topical phenytoin, c. Oral phenytoin; arrows indicate angiogenesis.

Distribution of data

Variable number of angiogenesis data obtained an average number of 95.66 ± 9.72 in the group without phenytoin (C) treatment, 178.66 ± 11.75 in the topical phenytoin (P1) and 205.1 6 ± 9.74 groups in the oral phenytoin therapy group (P2). From the results of the normality test obtained normal distribution results (p> 0.05).

From the boxplot graph, figure 4 shows that there are differences in the number of angiogenesis. The lowest

number of angiogenesis in the control group without phenytoin therapy was 95.66 ± 9.72, while the highest number of angiogenesis was seen in the treatment group with oral phenytoin administration which was 205.16 ± 9.74. Based on the One way ANOVA test, it can be seen that the p value <0.05, which means the difference is significant. In the Post-Hoc test there were differences in the number of angiogenesis of the groups given both topical and oral phenytoin therapy had significant differences compared to the control group in each group (p <0.05), and there were significant differences in the groups given topical phenytoin compared the group was given oral phenytoin (p <0.05).

Statistical Test of Total Angiogenesis

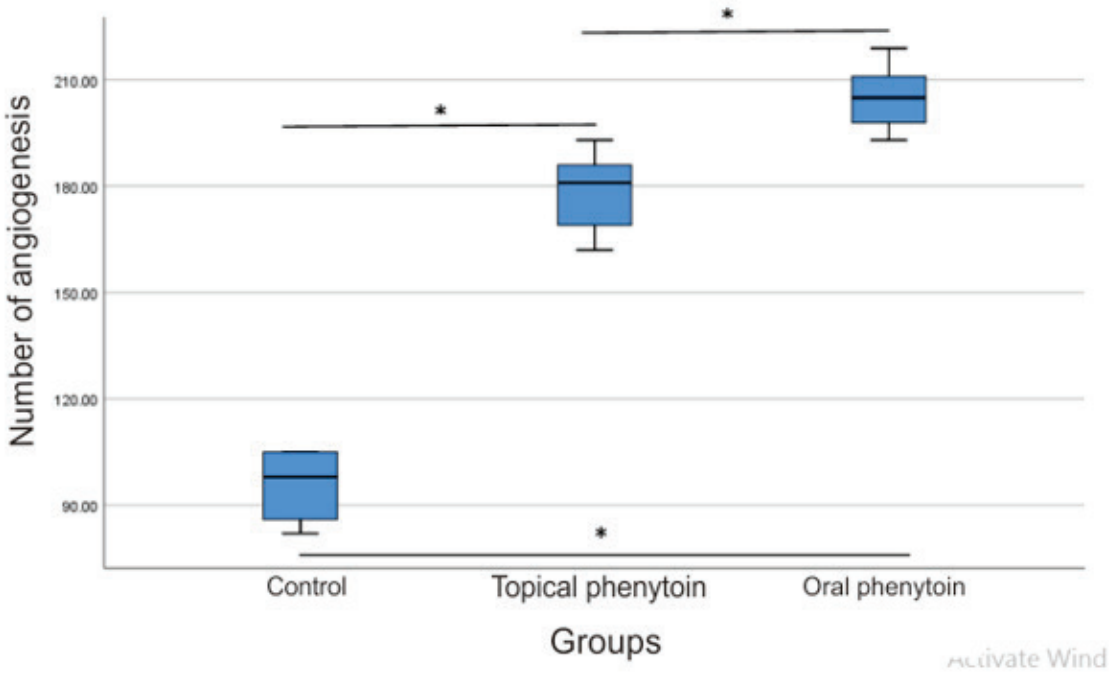

Figure 4. Boxplot graph of the number of angiogenesis of each group. C: fistula wound treatment with moist gauze, P1: fistula wound treatment with topical phenytoin, P2: fistula wound treatment with oral phenytoin (P2) There is a statistically significant difference (One Way ANOVA P <0.05), and also a significant difference when compared in each group (Post Hoc test); * significant (P <0.05)

Discussion

This study aimed to see the effect of phenytoin administration on the spontaneous closure of ECF. The effect was assessed on the number of fibroblasts and the number of angiogenesis in the healing process or spontaneous closure of ECF in Wistar rats. This study was conducted on Wistar rats that met the inclusion criteria and were treated for 7 days and subsequently assessed in fistula tissue by looking at the number of fibroblasts and angiogenesis through HE staining.

From this study, fistula tissue was taken on the 7th day after each group was treated, which time was the end of the inflamation phase of the wound healing process.[18] The number of fibroblasts and the number of angiogenesis should have increased at that time.[18] In the treatment group using topical and oral phenytoin therapy it was found that there was a significant difference in

the number of fibroblasts with the group giving oral phenytoin having the highest number. The higher number of fibroblasts in the administration of phenytoin therapy indicates the effect of phenytoin therapy on ECF, this is in accordance with the theory that the administration of phenytoin has a stimulating effect on fibroblasts proliferation.[14] Increased fibroblast proliferation in the ECF induction will have an effect positive in spontaneous closure of ECF. One of the factors expected to occur in fistula closure therapy is the presence of fibroblasts to support the wound healing process.[19]

In conventional ECF therapy currently popular using VAC which in its application has a mechanism of reducing proinflammatory cytokine and inducing microdeformation on the wound surface and complex wound healing.[20,21] Lu, Feng et alshow that microdeformation in wound healing is related by induction of fibroblasts.[21] The positive effect of the differences in the number of fibroblasts in this study could be a factor in the process of spontaneous closure of ECF. Intravenous administration of phenytoin to ECF in previous studies also showed good results with reduced output of the fistula.

This study also assessed the amount of angiogenesis in skin tissue, on the 7th day after each group was given treatment and found the highest value for the amount of angiogenesis found in the group giving oral phenytoin therapy. Whereas the treatment group that was not given phenytoin therapy had the lowest amount of angiogenesis. From these results it was found that there were significant differences in the number of angiogenesis between groups not given phenytoin therapy and groups who were given phenytoin therapy either topically or orally.

Angiogenesis is an important factor in the tissue's ability to repair itself and eliminate debris, providing nutrients and oxygen to the wound layer. The formation of granulation tissue which is a dense network of blood vessels, macrophages, and fibroblasts embedded in loose matrix fibronectin, hyaluronic acid, and collagen depends on the vascularization of the wound tissue and begins to appear in the wound about four days after injury.[22]

The higher number of angiogenesis in the treatment group of phenytoin proved that the effect of phenytoin therapy was most likely through M2 stimulation to increase the expression of growth factors, one of which was VEGF which played a role in increasing the amount of angiogenesis.[23,24] In the process of spontaneous closure of the fistula angiogenesis is also needed so that collagen deposits and granulation tissue can occur.[20]

The treatment using oral and topical phenytoin therapy also aims to see the difference from topical and systemic phenytoin therapy. Systemic administration of phenytoin is known to have side effects in long-term administration, namely osteomalacia, gingival hyperplasia, and hepatic and renal disorders in rare prevalence. The positive results of the phenytoin treatment group in this study also showed a significant difference between the groups given topical and oral therapy both in the number of fibroblasts and the number of angiogenesis observed, so that the study assumed the selection of phenytoin therapy in enterocutaneous fistulas was superior to oral administration. or systemic. Jaber reported in 2013 a case study conducted with intravenous phenytoin giving a positive effect on the healing of gastrointestinal fistulas, in this study, Jaber chose systemic therapy because healing that was considered more important in fibrosis in the fistulacan be achieved through systemic therapy compared to topical given on the surface of the fistula wound.[9]

Conclusion

This conclusion was followed by the number of fibroblasts and angiogenesis in the administration of topical phenytoin in the ECF of Wistar rats, higher than those not receiving phenytoin therapy. In addition, the number of fibroblasts and angiogenesis in oral phenytoin administration in the Wistar rat ECF were higher than those not receiving phenytoin therapy. It can also be concluded that the number of fibroblasts and angiogenesis in oral phenytoin administration in Wistar rat ECF, is higher than those without topical phenytoin.

The author recommends further research with a longer observation time to see the process of closing the ECF on the administration of phenytoin therapy and research on patients who have criteria for spontaneous closure with VAC and phenytoin therapy both oral and topical as additional therapy.

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Source of Fund : Self

Conflict of Interest : Nil

Ethical Clearance : The study was submitted and approved by the Diponegoro University Faculty of Medicine Research Ethics Commission No.78/EC/H / KPEK/FK-UNDIP/V/2019. Eighteen animals were used in the experiments

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