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Effect of Administration of Red Fruit (*Pandanus conoideus L.*) Extract in TNF-α, Microvessels Density Expression and Endometriosis Implant Area in Endometriosis Mice Model

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Endometriosis; TNF-α; Microvessel Density; Area of Endometriosis Implant; Red Fruit

ABSTRACT

Introduction: The prevalence of endometriosis in women of reproductive age was still high. A previous study showed that the red fruit (*Pandanus conoideus L.*) extract consisted of alpha-tocopherol and beta carotene as antioxidants that could potentially be an alternative treatment in endometriosis. This study aimed to determine the effect of Red Fruit Extract Administration on the expression of TNF-α, microvessels density (MVD), and the area of endometriosis implant in endometriosis mice model.

Methods: This was an experimental study with a randomized post-test control group design only applied to the endometriosis mice model and conducted in the Veterinary Faculty of Medicine Airlangga University, Surabaya. Twenty-eight endometriosis model mice were randomly divided into two groups. The control group (K) received CMC Na 5% for 14 days, and the treatment group (P) was given Red Fruit extract of 0,05 ml/day for 14 days. After day 28, mice were sacrificed, and immunohistochemical analysis was performed for TNF-α, microvessel density (MVD), and measured for endometriosis implant area. A T-test was used to analyze the comparison between groups.

Results: Mean TNF-α, microvessel density, area of endometriosis implant in treatment group were 1,56±0,87, 1,26±0,48, 13,24±4,08, respectively. There was a statistically significant difference in TNF-α expression, MVD development, and area of endometriosis implant between the treatment and control groups ($p<0.05$).

Conclusion: There was decreased expression of TNF-α, development of microvessel density, and area of endometriosis implant in endometriosis mice model given red fruit extract and proved to have potential effect as endometriosis therapy.

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INTRODUCTION

Endometriosis is the most common gynecological condition in reproductive-age women, and it is characterized by the presence and proliferation of endometrial tissue outside the uterus. Endometriosis is estimated to affect 5-20% of women experiencing pelvic pain, 20-40% of women experiencing infertility, and 10-15% of women of reproductive age in general [1,2]. This disease is also associated with infertility and sometimes delay in endometriosis diagnosis, altering the quality of life [3]. Endometriosis is still a complicated reproductive endocrinology disease with a high recurrence rate [4]. There are three different approaches to endometriosis, surgical, medical, and both interventions. However, there are still many limitations and side effects of treatment [5,6].

The development of endometriosis implants in the uterine cavity is associated with a chronic inflammatory process with an increase in pro-inflammatory mediators such as TNF-α [1,7]. Red fruit (*Pandanus Cononideus L.*) is an endemic plant in Papua rich in carotene and tocopherol that can increase the number of natural killer cells, increase phagocytosis, number of NK cells, and lymphocyte proliferation. In the long term, alpha-tocopherol and beta carotene inhibit NF-κb activation with various mechanisms on TNF- α and function as antioxidants to counteract free radicals in the maternal reproductive system, expected to increase the success of the development of preimplantation embryo development [8,9]. Therefore, this study aimed to investigate the effects of red fruit extract on the expression of TNF-α, microvessels density (MVD), and the area of endometriosis implant in the endometriosis mice model.

MATERIAL AND METHODS

Study Design

This was an experimental study, randomized post-test control group design only, applied to endometriosis mice model and conducted in Veterinary Faculty of Medicine Airlangga University, Surabaya, during March-May 2016. Twenty-eight endometriosis model mice (*Mus musculus*), 12 weeks old, weight 20-30 g, were randomly divided into two groups, namely the control group (K) was received a placebo (CMC Na 5%) for 14 days. The treatment group (P) was given Red Fruit extract 0,05 ml/day for 14 days by oral lavage. Mice were acclimatized one week before being treated, given food and drink ad libitum, and kept in a standard cage. After day 28, mice were sacrificed, and immunohistochemical analysis was performed for TNF-α, microvessel density (MVD), and measured for endometriosis implant area.

Endometriosis Model Mice

After acclimatization for one week, mice received 0,2 cc intramuscular dose of cyclosporine A. We randomly assigned mice to two groups, and each mouse was injected with the female endometrium intraperitoneally and injected with intramuscular estrogen on the 1st and 5th day. Endometrial scraping was taken from endometrial biopsy made into supernatant containing stroma, glands, and epithelial cells. Mice injected 0,1 ml of endometrial tissue intraperitoneally. Estrogen was derived from Ethinyl estradiol at a dose of 30 micrograms/Kg body weight and injected 0,1 cc intramuscular [10].

Red Fruit Extract

The red fruit (*Pandanus conoideus*) without skin was cut into pieces and washed with clean water. The red fruit was steamed for 1-2 hours. After tendered, it was removed and desired. The fruit's flesh was pounded by hand and then filtered to separate the seeds. The filter result was re-cooked over medium heat (40^{0}C) for 5-6 hours while stirring, then removed and allowed to stand for one day to form 3 layers of dreg (bottom layer), water (middle layer), and oil (top layer). The top layer was taken and separated. Red fruit extract was administered at 0,05 ml/day through oral lavage.

Immunohistochemistry

The tissue was first made of paraffin blocks form. Then, the deparaffinization was done by soaking in Xylol and rehydrating with alcohol. Then, we rinsed in H2O for 5 minutes. The slides were stained with Haematoxylin Eosin. The expression of TNF- α was measured in tissue preparation using a light microscope at 400x magnification using the modified Remmele method. The Remmele index scale results from multiplying the percentage score of immunoprotective cells with the color intensity score produced on cells. MVD expression was measured by averaging the microvasculatures recorded in the five areas with the highest vascularity. Neovascularization was measured through antibody against von Willebrand antibody with Light Microscope at 400x magnification. The size of the endometrial implant area was assessed macroscopically in hyperemia and hypervascularization of the peritoneum wall. The total area of the reddish lesion was assumed to be the area of the endometriosis implant. The calculation was done by computer analysis using Motic Software, Kowloon, Hong Kong.

Ethical Statement

All procedures were performed under guidelines and regulations of animal welfare and approved by the Research Ethics Committee, Faculty of Medicine, Airlangga University, Surabaya, East Java.

Statistical analysis

Statistical analysis was analyzed using SPSS Version 20.0 for windows. Expression was represented as mean± standard deviation, area in mm^{2,} and independent t-test was used to analyze differences between groups.

RESULTS

A total of 28 mice were used in this study. The content of bioactive components of red fruit extract is shown in Table 1. There was a significant difference in TNF- α expression, MVD expression, and implant area

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Table 1. Bioactive Components of Red Fruit Extract (*Pandanus conoideus*)

Component	Number
Total carotenoid	12.000 ppm
Total tochoperol	10.000 ppm
Betacarotene	3.581 ppm
B-Cryptoxanthin	1.4660 ppm
Oleic Acid	74,6%
Linoleic Acid	8%
Linolenic Acid	8,36%
Decanoate	2,1%
Natrium	21.0 mg

Table 2. TNF- α Expression, MVD Expression and Implant Area in Control and Treatment Group

Variable	Control Group (n=14)	Treatment Group (n=14)	*p*
TNF- α (IRS)	4,73±2,16	1,56±0,87	0,000
MVD Expression (IRS)	2,84±0,86	1,26±0,48	0,000
Implant area (mm^2)	41,32±15,90	13,24±4,08	0,000

between the control and treatment groups (p<0,05), as shown in Table 2.

DISCUSSIONS

This study showed a significant decrease in TNF-α, MVD expression, and implant area in the endometriosis mice model given red fruit extract. This decrease was caused by the high tocopherol content of red fruit [11]. In an in-vivo study, Tocopherol acts as an antioxidant by protecting unsaturated fatty acids on cell membranes from peroxidative degradation. Alpha-tocopherol and beta carotene can inhibit NF-kB activation with various mechanisms that form phorbol ester to TNF-α hydrogen peroxide. Other studies have shown that vitamin E's alpha and beta tocopherol derivatives can inhibit TNF-α induced NF-KB activation [12–14]. In endometriosis, the inflammatory process often involves pro-inflammatory cytokines, particularly TNF- α. TNF-α levels in peritoneal fluid increase with the severity of endometriosis and play a role in facilitating the attachment of ectopic endometrial tissue to the peritoneum [15,16]. Microvascular density observation is often used in some studies to assess angiogenesis, so it is known that red fruit extract can suppress angiogenesis. Wang *et al.,* 2014, perform an MVD measurement to determine the anti-angiogenesis endostatin effect in endometriosis. This study found that the MVD was decreased in endometriosis treated with anti-angiogenesis. Angiogenesis in endometriosis was related to hypoxia in the endometrium [17,18]. As a proangiogenesis regulator factor, part of VEGF plays a vital role in Endometriosis angiogenesis. Based on the previous study, Anti-angiogenesis therapy significantly decreased VEGF expression, number of lesions, and MVD compared to the control group. Vascular supply is required for ectopic endometrial lesion persistence, and as a result, an angiogenesis inhibitor is effective for the initial prevention of Endometriosis [19]. Recent studies in Indonesia stated that the red fruit extract contained natural substances such as alpha-tocopherol, oleic acid, linoleate acid, decanoate, calcium, fiber, protein, vitamin B1, and niacin. According to Sauqi et al., *P. conoideus* fruit extract was investigated to suppress NF-κB [20] activation and reduce VEGF. Both of these are accompanied by inhibition of TNF- α formation can cause a decrease in the area of endometrial implants [21].

CONCLUSION

The administration of red fruit (*P. conoideus*) extract decreased expression of TNF-α and development of microvessel density, that reduce the area of endometriosis implant in the endometriosis mice model. Thus, this proved that *P. conoideus* has potential effects as endometriosis therapy.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest.

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