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Original Research

Comparison of the Effects of Dienogest and Leuprolide Acetat on Serum Interferon (IFN-ɣ) Levels in a Mouse Model of Endometriosis *(Mus musculus)*

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ABSTRACT

Introduction: Endometriosis is a medical condition in which there is abnormal growth of tissue resembling the endometrium outside the uterus. It can cause symptoms of pain and/or infertility. The current medical therapy focuses on reducing estradiol levels or stimulating progesterone response. One of the most effective options for medical treatment is the use of GnRH analogs. Dienogest have a direct anti-inflammatory effect on endometriotic stromal cells. However, a comparison of the anti-inflammatory effects of these drugs on IFN-ɣ has not been previously investigated. Therefore, this study aims to compare the effects of reducing IFN-ɣ by both Dienogest and Leuprolide Acetat in a mouse model.

Material and Methods: This study employed a post-test only control group design and involved 4 groups, namely the negative control group, positive control group, and two treatment groups: one group administered Dienogest at a dosage of 0.0052 milligrams per day for 14 days, and the other administered Leuprolide Acetat at a dosage of 0.00975 milligrams once every 5 days for a period of 14 days. Serum IFN-ɣ levels were measured using Enzyme-Linked Immunosorbent Assay (ELISA). The data were subsequently analyzed using IBM SPSS 25 with One-Way ANOVA test.

Results: This study demonstrated that Leuprolide Acetat significantly decreased serum levels of IFN-ɣ, whereas Dienogest actually increased the levels .

Conclusion: In a mouse model of endometriosis *(Mus musculus),* Leuprolide Acetat effectively reduced the levels of IFN-ɣ (pro-inflammatory). Conversely, Dienogest increased the levels of IFN-ɣ (anti-inflammatory) in the mouse model of endometriosis.

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INTRODUCTION

Endometriosis is a condition in which the growth and development of endometrial glands and stroma occur outside the uterus, affecting various pelvic organs such as the ovaries, Douglas pouch, uterine ligaments, and fallopian tubes [1]. It is a chronic estrogen-dependent disease that affects women during their reproductive years. It is characterized by the presence of tissue resembling the endometrium outside the uterine cavity, causing chronic inflammation, ovarian cyst formation, and fibrosis [2]. Chronic pelvic pain complaints can occur in up to 70% of cases [3]. Symptoms often worsen without effective treatment, leading to anxiety and frustration [4].

Dienogest, a synthetic progestin derived from progesterone, has emerged as a promising first-line long-term treatment option for endometriosis symptoms [5]. Administration of 2 mg of Dienogest per day has been proven effective in relieving endometriosis-related pain, reducing lesions, and improving the quality of life for patients [4]. Studies have also shown that 2 mg of Dienogest is more effective than placebo in alleviating pelvic pain in patients with varying degrees of endometriosis severity and improving symptoms of dysmenorrhea and irregular bleeding [6]. On the other hand, Leuprolide is an analog of gonadotropin-releasing hormone (GnRH), a ten amino acid peptide synthesized and released by neurons in the anterior hypothalamus [7]. The use of GnRH analogs post-surgery helps reduce pain and delay the recurrence of symptoms in patients who do not receive adequate treatment [8].

It is known that endometriosis triggers an abnormal inflammatory response [9]. Pain associated with endometriosis is caused by various factors, including inflammation [10]. Serum levels of IFN-ɣ have been identified as a potential non-invasive biomarker for endometriosis [11]. IFN-ɣ levels significantly increase in the peritoneal fluid of endometriosis patients compared to healthy individuals. However, other studies have reported a significant decrease in peritoneal IFN-ɣ levels in endometriosis patients. Therefore, the role of IFN-ɣ in the pathogenesis of endometriosis is not yet fully understood due to contradictory findings [12]. To date, there have been no specific studies explaining the effects of Dienogest and Leuprolide Acetate on IFN-ɣ levels in endometriosis. Hence, this study aims to compare the effects of Dienogest and Leuprolide Acetate on IFN-ɣ levels in a mouse model of endometriosis.

MATERIAL AND METHODS

Research Design

This study is a pure experimental study that used mice *(Mus musculus)* as experimental subjects (*in vivo* study). The research design employed was a posttest-only control group design. There were two control groups and two treatment groups. The control groups consisted of healthy mice (K-) and diseased mice (K+), both of which did not receive any treatment (no intervention). On the other hand, the treatment groups consisted of mice treated with Dienogest (P1) and mice treated with Leuprolide Acetat (P2). After undergoing the 14-day treatment period, the mice were sacrificed, and blood samples were collected to measure the serum IFN-ɣ levels. The independent variables in this study were Dienogest and Leuprolide Acetat, while the dependent variable was the serum IFN-ɣ levels.

Sample

The samples in this experimental study consisted of 20 adult female BALB/c mice. The procurement of these mice was conducted in collaboration with the Embryology Laboratory, Faculty of Veterinary Medicine, Airlangga University, Surabaya. According to Pehlivanovic *et al.,* (2019) cited in Mukherjee *et al.,* (2022), mice are commonly used experimental animals in *in vivo* models [13].

The inclusion criteria for the mice included being 6-8 weeks old, weighing 20-30 grams, [14,15], 2-3 months old [14,16], in a healthy condition [15], never mated [16], and without anatomical abnormalities (non-defective). Mice that died during the course of the study [15], showed signs of stress before the study, and/or had deteriorating health conditions [17] were excluded from the study.

In general, the mice (n=20) were acclimatized for 7 days before the start of the study. The mice were then randomly selected and placed in 4 cages. Fifteen mice were induced to develop an endometriosis model for 14 days. On the first day, three types of injections were administered: 1) intramuscular injection of cyclosporine A 0.2 ml/mouse; 2) intraperitoneal injection of adenomyosis tissue 0.1 ml/mouse; and 3) intramuscular injection of Ethynil Estradiol (Ovalumon) 0.1 ml/mouse. On the 5th day, an additional intramuscular injection of ethynyl estradiol (Ovalumon) was given at a dose of 0.1 ml/mouse. During the 14-day period, the mice were given ad libitum food and water. After 2 weeks, the endometriosis model was established (n=15).

Intervention

On the 16th day, the treatment was administered to the treatment group (DNG and LA). This study utilized four groups, namely the negative control group (n=5), positive control group (n=5), and two treatment groups (n=10). The negative control group consisted of healthy mice that did not receive Dienogest or Leuprolide Acetate therapy, while the positive control group (n=5) consisted of endometriosis model mice without receiving Dienogest or Leuprolide Acetate therapy. The two treatment groups included an endometriosis model group that received oral Dienogest at a dose of 0.0052 mg/mouse/day for 14 days (n=5), and a Leuprolide Acetate treatment group at a dose of 0.00975 mg/mouse via intramuscular injection once every 5 days for 14 days (n=5). On the 15th day, the mice were sacrificed, and intracardiac blood samples were collected, which were then centrifuged at 3,000 rpm for 10 minutes at a temperature of 4°C to obtain serum. The serum was stored in a freezer at -20°C for measuring the level of serum IFN-ɣ using the Enzyme Linked Immunosorbent Assay (ELISA) method.

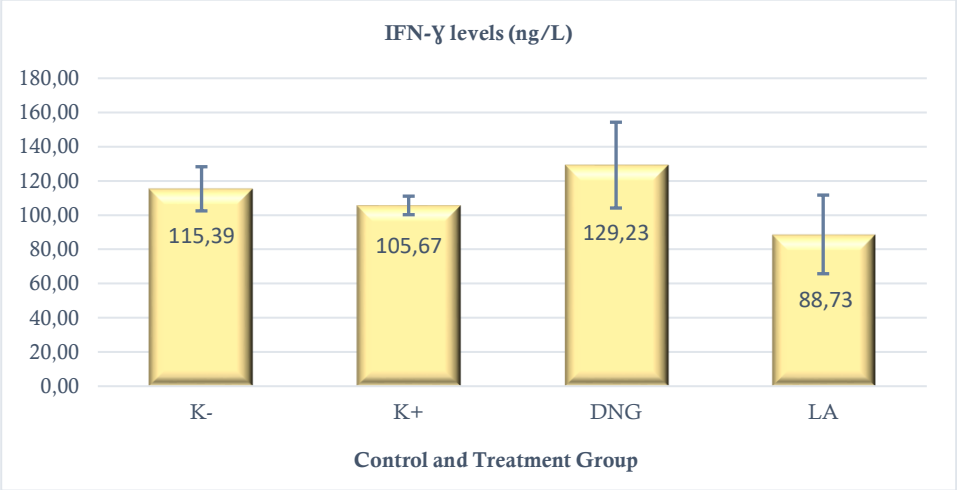


Fig.1. Comparison of Serum IFN-ɣ Levels between Dienogest and Leuprolide Acetat

Statistical Analysis

The data were analyzed using the One-Way ANOVA test due to fulfilling the assumptions of normality and homogeneity ($p>0.05$). The analysis was conducted using IBM SPSS Statistics 25.

Ethics

This study has obtained approval from the Ethics Committee of the Faculty of Medicine, Universitas Brawijaya, Malang, with the reference number 67/EC/KEPK/03/2023.

RESULTS

Based on Fig.1, statistical analysis revealed that the levels of IFN-ɣ were actually higher in healthy mice compared to mice with endometriosis. The average level of IFN-ɣ in mice with endometriosis was 9.72 nanograms lower than in healthy mice, but the difference was not statistically significant (p = 0.159). Leuprolide Acetat effectively reduces serum levels of IFN-ɣ. The results of the One-Way ANOVA test (p = 0.021) indicate a significant difference between the effects of Dienogest and Leuprolide Acetat on serum levels of IFN-ɣ. Dienogest, on the other hand, further increases the serum levels of IFN-ɣ beyond the levels observed in healthy mice.

DISCUSSION

1. Healthy and Diseased Mouse Phenomenon

In this study, the serum levels of IFN-ɣ in the mouse model of endometriosis (EMM) were lower compared to healthy mice (Fig.1). IFN-ɣ is a cytokine that influences apoptosis and cell proliferation [18]. Administration of cyclosporine, which suppresses the immune response of IFN-ɣ in T cells, prevents apoptosis and promotes continuous cell proliferation, leading to the formation of granulomas in the peritoneal tissue.

In this study, mice that were used as an endometriosis model were also injected with a suspension of adenomyosis tissue. Despite cyclosporine weakening the immune response (immunocompromised), as indicated by lower levels of IFN-γ in the diseased mice group compared to healthy mice (Fig.1), the implantation of tissue still elicited an immune response through IFN-γ, which was able to differentiate between self (own cells and tissues) and non-self (foreign molecules and environmental microbes). Lymphocytes (T cells and B cells), the second most abundant cells in the immune response, are crucial in the normal immune response against infections and tumors, as well as in mediating transplant rejection and autoimmunity. The innate immune system recognizes infections and "alerts" the adaptive system through antigen presentation, facilitated by major histocompatibility complex (MHC) protein complexes. The innate immune cells also release other chemical signals such as cytokines and chemokines to fully activate the adaptive system. Despite its efficiency and high specificity, an imbalance in the immune response can lead to various disorders, such as autoimmune diseases and immunosuppression [19].

Endometriosis shares similarities with several autoimmune diseases. Genetic factors are associated with the pathogenesis of endometriosis. The major histocompatibility complex (MHC) gene, also known as a human leukocyte antigen (HLA) gene, is located on chromosome 6p. The HLA gene is polymorphic in its ability to bind and function in presenting antigen peptides to T cells. HLA molecules are key factors

involved in regulating the specificity of T cell-mediated immune responses in autoimmune diseases and infections. HLA-DR is one of the genes that encode class II MHC molecules [20]. A study reported that Dienogest induces the expression of HLA-DR and inhibits the production of TNF-α in the peritoneal fluid of women with endometriosis. IFN-ɣ is known to control the expression of HLA-DR and alter TGF-β [21].

Endometriosis is a chronic condition that occurs in women during their reproductive years and is dependent on estrogen. It is characterized by the presence of endometrium-like tissue outside the uterine cavity, leading to chronic inflammation, ovarian cyst formation, and fibrosis [2]. The endometriosis lesions in the peritoneum are initially supplied with blood by blood vessels in the peritoneum. Additionally, interactions take place between mesothelial cells (dominant cells) and the lesions [22]. Nearly 70% of patients with endometriosis experience pelvic pain, while endometriomas are found in 17-44% of patients [23].

It is known that endometriosis triggers pathological inflammatory responses [9]. Pain in endometriosis is caused by several factors, including inflammation [10]. Serum levels of IFN-ɣ have the potential to serve as a non-invasive biomarker for endometriosis [11]. Elevated levels of IFN-ɣ not only induce adhesion and invasion of ectopic lesions, promoting the development of endometriosis, but also inhibit apoptosis within the lesions. Dysregulation of the IFN-ɣ signaling pathway induced by the Estrogen/Erβ axis in the endometrium is involved in the development of endometriosis, particularly in relation to infertility, although this is not yet fully understood in humans [12].

2. Effects of Dienogest on Serum IFN-ɣ Levels

The analysis showed that the administration of Dienogest at a dose of 0.0052 mg/mouse/day for 14 days did not decrease the serum levels of IFN-ɣ (Fig.1). Dienogest appears to have variable effects on different mice, as it was observed that in other individuals within the group, the levels were successfully reduced below the control group (see appendix). When compared to the control group, the average IFN-ɣ level of 129.23 ng/L (SD = 25.08) indicates that Dienogest actually increased the serum levels of IFN-ɣ.

IFN-ɣ is primarily produced by activated T cells or NK cells, and its role is to regulate cellular immune responses, including macrophage activation and T cell development. The study by Hsu *et al.* (1997) demonstrated a decrease in IFN-ɣ levels in peripheral blood and peritoneal fluid. Another study conducted by Podgaec *et al.* (2007) reported higher expression of IFN-ɣ in the local environment of the lesions, specifically the peritoneal fluid, in patients with endometriosis compared to the control group, although there was no statistically significant difference in systemic IFN-ɣ levels in peripheral blood. Similar research findings indicate significantly higher mRNA expression of IFN-ɣ in ectopic tissue compared to normal endometrium. Interestingly, it was found that IFN-ɣ does not affect the proliferation and apoptosis of cells in ectopic endometrial implants originating from the ovaries. This may suggest that endometrial cells become resistant to apoptosis signals when they enter the peritoneal cavity through retrograde menstruation [24].

A study reported that Dienogest can restore the decreased/pathologically produced cytokines, thus restoring HLA-DR expression on macrophages to normal levels. In the case of endometriosis, decreased HLA-DR expression can indicate a weak response to endometriotic tissue during retrograde menstruation, and increased pro-inflammatory cytokines in the peritoneal fluid can worsen the development of endometriosis. The immunological effect of Dienogest is to increase HLA-DR expression on macrophages in the peritoneal fluid and reduce TNF-α production in the peritoneal fluid, thereby enhancing the peritoneal immune response. By enhancing the peritoneal immune response through the use of Dienogest, it may reduce endometriotic tissue through its direct effects on the tissue [21].

Dienogest also exhibits similar activity to Leuprolide Acetate, inhibiting gonadotropin secretion to a considerable extent, leading to a moderate reduction in endogenous estradiol production. When administered continuously, Dienogest creates a local endocrine environment with low estrogen and high progestin levels, causing decidualization of the endometrial tissue and subsequent atrophy of endometriotic lesions [4]. However, the ability of Dienogest and Leuprolide Acetate to reduce serum IFN-ɣ levels differs significantly (p = 0.021). It is understandable that the hypoestrogenic effect provided by Dienogest occurs gradually, making it suitable for long-term endometriosis treatment.

Long-term treatment (60 months) with Dienogest 2 mg/day in women with endometriosis effectively reduces pelvic pain and prevents the recurrence of pain after surgery. Dienogest is well tolerated, and its side effects are clinically managed [25]. Oral administration of Dienogest (2 mg/day) also efficiently reduces the volume of endometriotic cysts and prevents cyst recurrence [26]. Dienogest primarily targets the inhibition of NF-ϰβ, TNF-α, and IL-8 [27].

3. The Effects of Leuprolide Acetate on Serum IFN-ɣ Levels

This study demonstrates a significant decrease in serum IFN-ɣ levels caused by Leuprolide Acetate. The levels were even much lower than those of healthy mice (Fig.1). Leuprolide Acetate at a dose of 0.00975

mg/mouse was administered once every 5 days for a duration of 14 days. A study reported that in Sprague-Dawley rat models with endometriosis, a single subcutaneous dose of 1 mg/kg Leuprolide Acetate resulted in a significant reduction in IL-6, IL-8, and TNF-α levels in both plasma and peritoneal fluid [28]. Treatment with GnRH agonists restores NK cell activity in patients with peritoneal endometriosis, which may be due to direct effects on immune cells or indirect effects through the reduction of microperitoneal bleeding [29]. This advantage stems from its primary mechanism of action, which acts directly on the hypothalamus by disrupting pulsatile GnRH secretion, thus reducing FSH and LH regulation [7].

Furthermore, GnRH analogs can directly affect endometrial cells by inhibiting their growth and proliferation through the regulation of apoptosis and angiogenesis mechanisms. Therefore, Leuprolide Acetate has been proven effective in reducing the growth of endometrial cells, not only through its classical endocrine effects on the pituitary gland but also through direct effects on the endometrial cells themselves [30]. Leuprolide Acetate has been shown to significantly decrease serum VEGF levels in Wistar-Albino rats [31].

In this in vivo study, with 3 administrations of Leuprolide Acetat, it was effective in reducing IFN-ɣ levels in endometriosis mice (*Mus musculus*). In patients with endometriosis, the use of Leuprolide Acetat is limited to a maximum of 6 months. Besides its excellent anti-inflammatory capabilities, Leuprolide Acetat is known to have side effects, inducing a hypoestrogenic state that causes menopausal symptoms [32].

In other disease, administration of Leuprolide Acetate in experimental autoimmune encephalomyelitis (EAE) mice also reduces the significant activation of NF-ϰβ and pro-inflammatory cytokines IL-1ß, IL-17A, and TNF-α during the EAE recovery phase, this reducing clinical severity [33].

CONCLUSION

Dienogest does not decrease serum IFN-ɣ levels, likely due to its pharmacological effect on IFN-ɣ by increasing HLA-DR expression in peritoneal fluid macrophages. In other words, Dienogest induces the anti-inflammatory function of IFN-ɣ. On the contrary, Leuprolide Acetate reduces serum IFN-ɣ levels as a pro-inflammatory cytokine.

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CONFLICT OF INTEREST

There are no conflicts of interest in this study.

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