



# The Effect of Ascorbic Acid on Interleukin-10 and Tumor Necrosis Factor- $\alpha$ Cytokines in *Rattus norvegicus* with Endometritis

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## Abstract

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**BACKGROUND:** Endometritis is a gynecological disease characterized by inflammation of the endometrial glands and stroma. Inflammatory stimuli or tissue injury induce inflammatory pain through the release of cytokines. Ascorbic acid (AA) is a water-soluble Vitamin that plays a role in inhibiting the production of proinflammatory cytokines and increases the expression of anti-inflammatory cytokines.

**AIM:** The purpose of this study was to find out the association between administration of AA and inflammatory cytokines in experimental animals *Rattus norvegicus* with endometritis.

**METHODS:** The research was conducted using virgin female *R. norvegicus* laboratory mice weighing 250–300 g and aged 11–12 weeks with an estrus cycle of 5–6 days. Mice with regular oestrous cycles were randomly divided into three groups: group 1 was given 200 L of water orally without *Escherichia coli* inoculation and represented a negative control. Groups 2 and 3 were inoculated (50 L/rat) *E. coli* intravaginally, 10<sup>6</sup> colony-forming unit/mL, Group 2 was not given AA and the other side Group 3 was assigned AA. The interleukin (IL)-10 and tumor necrosis factor (TNF)- $\alpha$  cytokines examination was carried out by histopathological examination through a biopsy of the endometrial tissue. Hypothesis testing on the data was analyzed by the Kruskal Wallis test using Statistical Package for Social Sciences.

**RESULTS:** Data from the current study revealed that the highest mean value of IL-10 was found in the negative control group (2.5) and the lowest value in the positive control group (1.3). Regarding TNF- $\alpha$  the highest mean value (2.8) was found in the treatment group and the lowest mean value (2.1) was found in the treatment group. Using the Kruskal Wallis test, IL-10 and TNF- $\alpha$  showed insignificant results ( $p =$  value 0.304 and 0.145 respectively).

**CONCLUSIONS:** The administration of AA did not affect the decrease in TNF- $\alpha$  or the upregulation of IL-10 as anti-inflammatory cytokines.

## Introduction

Endometritis is a gynecological disease characterized by inflammation of the endometrial glands and stroma, furthermore, it is seen as one of the components of pelvic inflammatory disease that causes infertility in women and animals [1]. Microorganisms associated with endometritis include *Escherichia coli*, *Enterococcus faecalis*, *Streptococcus* group, *Staphylococcus* group, and *Mycoplasma* group (*Mycoplasma genitalium* and *Ureaplasma urealyticum*) [2], [3]. *E. coli* is the most common bacteria causing endometritis which causes implantation failure [4], [5], [6].

Changes in gene expression of endometrial genes encoding proteins involved in inflammatory, proliferative, and apoptotic responses have been found in women with chronic endometritis [7], [8]. Oral antibiotic therapy, such as doxycycline has been shown to help clear the cytoplasm from stromal cells in endometritis. Nevertheless, pregnancy rates from the

endometrium treated with oral antibiotics in patients with endometritis remain poor or inconclusive. In addition, the number of resistance to antibiotics is also increasing [9], [10], [11].

Ascorbic acid (AA) or commonly referred to as Vitamin C is a water-soluble vitamin that inhibits the production of proinflammatory cytokines, namely, interleukin (IL)-1 $\beta$ , IL-6, IL-12, and, tumor necrosis factor (TNF)- $\alpha$ . On the other hand, the expression of anti-inflammatory cytokines such as IL-4 and IL-10 is increased with the administration of AA [12], [13], [14], [15]. Splenocytes produce TNF- $\alpha$ , IL-6 and, IL-10 by stimulation or tissue injury. Several studies have shown that inflammation is reduced by taking AA. However, there is limited information on the effects of Vitamin C on cytokine production [13]. This encourages further research to address the need for alternative options for the management of endometritis. The purpose of this study was to find out the association between administration of AA and inflammatory cytokines in experimental animals *Rattus norvegicus* with endometritis.

## Methods

### Study design

This research is quasi-experimental research conducted from August to November 2020 at the Biology Laboratory of the Faculty of Mathematics and Natural Sciences and the Anatomical Pathology Laboratory, Faculty of Medicine, Universitas Sumatera Utara. The study was approved by the Ethics Committee of the Faculty of Mathematics and Natural Sciences Universitas Sumatera Utara with No. 00713/KEPH-FMIPA/2020.

### Subject selection

Virgin female *R. norvegicus* laboratory mice (with an estrus cycle of 5–6 days), weighing 250–300 g and aged 11–12 weeks. Mice with signs of infection were excluded from this study. Female mice were acclimatized for 7 days which were placed in plastic cages measuring about 30 cm  $\times$  40 cm  $\times$  15 cm with the bottom of the cage given a husk mat which was changed every day. The lighting conditions are approximately 12 h/12 h (lights are turned on at 7 am and turned off again at 7 pm) and controlled room temperature is around  $27 \pm 5^\circ\text{C}$ . Relative humidity ranges from 85% to 88%. Maintenance is carried out as well as possible following the correct animal treatment procedure in terms of the 3R principles (Reduction, Replacement, Refinement), as well as the 5F principle (Freedom of Hunger and Thirst, Freedom from Discomfort, Freedom from Pain, Injury or Disease, Freedom to Express Normal Behavior, Freedom from Fear and Distress).

Randomly, mice were divided into three groups, with each group consisting of six samples: Group 1 (negative control) without *E. coli* inoculation was given 200  $\mu\text{L}$  of water orally. Groups 2 and 3 were inoculated with 106 colony forming unit/mL of *E. coli* each intravaginally (50  $\mu\text{L}$ /mouse) with Group 2 was not giving AA and Group 3 was given.

### Administration of AA

AA (Marin Liza Pharmacy, Indonesia) was given 1000 mg/kg body weight/day for 5 days 36 h after *E. coli* inoculation (based on previous research for diffuse acute endometritis) [5], [16], [17]. AA tablets were crushed, dissolved in aquades, and given orally, with a ten cc syringe. AA was given at 9–10 am every day.

### Sample collection

On the 5<sup>th</sup> day after the conception process (14<sup>th</sup> day of the study), mice were euthanized using

ketamine (Bernofarm, Indonesia) for histopathological examination. Histopathological examination, using a biopsy of endometrial tissue, was performed to determine the expression of IL-10 and TNF- $\alpha$ .

### Statistical analysis

The data were analyzed using Statistical Package for Social Sciences version 25.0. Hypothesis testing on the data was carried out with the Kruskal Wallis test because the data were not normally distributed.

## Results

In statistical analysis, the highest mean value of IL-10 was found in the negative control group (2.5) (There are no units for this measurement because histopathological examination works by looking at the expression of the antibodies examined in endometrial tissue) and the lowest value in the positive control group (1.3). For TNF- $\alpha$ , the highest mean value (2.8) was found in the treatment group (inoculated with *E. coli* and given AA), and the lowest mean value (2.1) was found in the positive control group. By using the Kruskal Wallis test, IL-10 and TNF- $\alpha$  showed insignificant results ( $p = 0.304$  and  $0.145$ , respectively). Hence, it can be concluded that there is no relationship between levels of IL-10 and TNF- $\alpha$  interleukins with AA administration.

## Discussion

AA is one of the important antioxidants for defense mechanisms and immune homeostasis of the body [13]. The antioxidant properties of AA are important because of its ability to donate electrons and protect the integrity of many cells, including lymphocytes, against damage from radicals generated in response to infection or toxin [18]. However, under stressful conditions such as vaccination, thermal stress, or infection, the need for AA increases. Therefore, supplementation with AA may reduce the side effects associated with stressful conditions [18].

As it is well-known, cytokines and chemokines are expressed in the uterus of many species in association with the inflammatory process [19]. TNF- $\alpha$  is a cytokine that plays a broad role in inflammatory and non-inflammatory processes such as cachexia, septic shock, disorders caused by inflammatory processes, and autoimmune diseases [19].

In a previous study [13], it was found that TNF- $\alpha$  expression was significantly inhibited at 72 h in mice treated with AA, and co-administration of Concanavalin A (Con A) and AA therapy in mice resulted in upregulation of IL-4 and IL-10 at 72 h compared to treatment with Con A alone [13]. However, in this study the results were not significant regarding the association between TNF- $\alpha$  and AA administration, meaning that AA administration did not decrease the regulation of TNF- $\alpha$  (Table 1).

**Table 1: Differences in the mean values of IL-10, IL-1 $\beta$ , and TNF- $\alpha$  in the three study groups**

Parameter	Group 1	Group 2	Group 3	p-value
IL-10	2.5	1.3	1.6	0.304
IL-1 $\beta$	2.6	1.2	2.5	0.036*
TNF- $\alpha$	2.3	2.1	2.8	0.145

\*Significant with the Kruskal-Wallis test. IL: Interleukin, TNF: Tumor necrosis factor.

IL-10 is an anti-inflammatory cytokine that plays a vital role in controlling inflammation. In addition, it acts as an active maternal immune suppressor during pregnancy, thereby enabling acceptance of the fetal allograft [5]. Xu *et al.* 2014 showed a decreasing trend of IL-10 after bacterial inoculation, but in Kim *et al.* 2014, IL-10 levels in uterine flush were increased in cows with endometritis, while no difference was observed in serum. In another study [5], data showed that bovine endometritis was not significantly associated with IL-10 gene expression. Administration of AA for the upregulation of IL-10 was found in a study conducted by Kong *et al.* 2015, but no significant difference was found in this study. However, previous studies [13] did not prove a dose-dependent relationship between AA administration and level of anti-inflammatory cytokines. The insignificant results obtained in this study may be due to the limitations of the study because several other types of proinflammatory cytokines such as Interferon gamma and IL-6 as well as other anti-inflammatory cytokines such as IL-4 and transforming growth factor beta were not examined due to laboratory limitations.

## Conclusions

Administration of AA did not affect the decrease in TNF- $\alpha$ , as a proinflammatory cytokines, or the upregulation of IL-10, as an anti-inflammatory cytokines. The difference between cytokine levels may be related to different sample size, animal species, and also sampling time points.

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