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Effects of glutamine and arginine combination on pro- and anti-inflammatory cytokines

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Abstract

Background: Cytokines were beneficial for diagnosis and treatment, which in clinical situations introduced from the perspective of pro and anti-inflammatory effects. An inflammatory response is commonly associated with various severe traumatic insults that consequently recruit the immune cells into the target organs and causing systemic inflammatory response that can lead to sepsis. Immune-modulating nutrients, such as glutamine and arginine, are known as pathophysiological modulate in inflammation.

Aim: The aim of this study was to evaluate the effect of oral gavage supplementation with a combination of glutamine and arginine on inflammatory cytokines in intestinal mucosa, specifically jejunum.

Methods: Sixteen *Rattus norvegicus* rats (average weight 150–200 g) were randomly divided into two groups: groups A and B, both intraperitoneal injected by 2 ml NaCl 0.9%. Group A orally supplemented with 1 ml dextrose 5% daily, meanwhile, group B orally supplemented with 1 ml combination of glutamine and arginine (contains 250 mg/kg glutamine and 250 mg/kg arginine) daily. The experiment lasted for 3 days. We compared the pro and anti-inflammatory cytokines (IL-10, NF-κB, TNF-α, IL-8, and MMP-8) between the two groups by the Mann–Whitney test.

Results: More IL-10, TNF-α, and IL-8 cytokine-produced cells found in group A. Group B produced significantly lower TNF-α ($p = 0.009$) and IL-8 ($p = 0.003$). The number of NF-κB and MMP-8 were slightly higher in group B.

Conclusion: Giving a combination of glutamine and arginine as nutrition supplementation has beneficial effects in decreasing almost half of the cells that produce TNF-α and IL-8. Further studies must be carried out to support a standard guideline for this recommendation.

Keywords: Immunonutrient, Glutamine, Arginine, Inflammatory, Cytokine.

Introduction

Cytokines are defined as low-weight molecules of non-structural proteins that have critical roles in regulating inflammation and the immune system through complex interactions (Gulati *et al.*, 2016). Cytokines are known as biomarkers for various diseases and are beneficial for diagnosis and treatment, which in clinical is introduced from the perspective of pro and anti-inflammatory effects. Pro-inflammatory cytokines (such as TNF-α, IL-8, IL-1β, and others) facilitate the reaction of inflammation and stimulate immunocompetent cells. In contrary, anti-inflammatory (such as IL-10, IL-6, IL-4, and others) major role was inhibiting the inflammation (Liu *et al.*, 2021).

In the biomolecular model, the T regulator plays a vital role together with TGF-β in suppressing the immune response, which has both positive and negative impacts on the immune system (Wan and Flavell, 2007). T regulator involves making a balance condition between Th1 and Th2 (Lin *et al.*, 2019). Th1 affects the secretion of pro-inflammation cytokine such as TNF-α, IL-8, IL-6, and IL-1. Meanwhile, Th2 affecting to the IL-10 production (Dembic, 2015).

Th1 and Th2 were affected by macrophage type 1 (M1) and type 2 (M2). Macrophage has two side effects, which can attenuate nitric oxide (NO) or promote ornithine. During inflammation, macrophage improves ornithine which leads to the wounds healing. Macrophage also has suppressor activity that inhibits specific T-cell responses during several chronic infections. In addition, the suppressor activity was correlated with NO production. Excessive production of NO could inhibit beneficial immune response (Mills, 2015).

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Response of inflammation is commonly associated with various severe traumatic insults. Traumatic injury increases pro-inflammatory mediators that consequently recruit the immune cells into the target organs and cause systemic inflammatory response (Yu *et al.*, 2015). During inflammatory conditions, NF-κB is activated by local cytokines to promote macrophage activation at the site of infection (Baker *et al.*, 2011). Elevation of NF-κB enhances the MMP-8 production, which is suspected as the main collagen in the process of mucosal damage (O'Sullivan *et al.*, 2015; Lee, 2019). Recently, inflammation was correlated with the alteration of the gut microbiome, which is known as intestinal dysbiosis (Levy *et al.*, 2017). This intestinal dysbiosis condition can lead to increase gut permeability and induce mucosal immune dysfunction (Wang *et al.*, 2019).

Immune-modulating nutrients, such as glutamine, arginine, and others, could modulate pathophysiological processes in critical illness, such as inflammatory and oxidative stress responses (van Zanten *et al.*, 2014). In critically ill patients, glutamine supplementation was beneficial to reduce complication infection and shorter length of stay, which average, patients were to stay on 3 days only (Tao *et al.*, 2018). Glutamine also showed a significant reduction of neutrophil count in infection of burn injury during 3 days of administration (Sudarsa *et al.*, 2021).

Glutamine and arginine independently have a role in growth, tissue recovery, regeneration of cell, and reducing bacteria translocation in sepsis patients (Cohen and Chin, 2012). Lack of glutamine level was affected by the worsening in critically ill (Casaer and Van den Berghe, 2014). Arginine–proline metabolism change was found in bacteria induced-jejunum (Ilaiwy *et al.*, 2019).

The use of immunonutrition combination to improve ICU patient outcomes has been challenged (Arabi *et al.*, 2017). However, the recent studies showed that glutamine and arginine combination (GAC) supplementation significantly decreased pro-inflammatory cytokines (such as CRP, TNF-α, IL-1β, and IL-6), improve tissue repair, cell renewal, and collagen synthesis (Bakir *et al.*, 2019). Several studies found that supplementation of GAC had beneficial effects on the critically ill, but the specific benefit remained unclear (Bakir *et al.*, 2019; Zhou *et al.*, 2012). Here, we aim to present the effects of giving glutamine and arginine as a nutritional support for preventing immune system deterioration.

Materials and Methods

Animal and treatment

Sixteen adult male *Rattus norvegicus* strain Wistar (12–14 weeks) with average body weight between 150 and 200 g were chosen in this study. The animals were maintained in group cages of size 17.5 × 23.75 × 17.5 cm made from plastic with wire caps, each cage including eight rats, placed in a controlled environment at room temperature. They were given the same ratio of clean water and sawdust as food.

Trauma modeling in this study uses an injection of NaCl 0.9% intraperitoneally and an orogastric route of nutrition, which is commonly used and known, can cause high stress in animal models (Turner *et al.*, 2011; Sotocinal *et al.*, 2011). The injection was in the lower left side abdomen, which sign of inflammation in the injection area was observed.

All animals could adapt to the experimental conditions for 1 week. After the adaption period, the animals were weighed and randomly allocated into two groups: group A and group B. Each rat was injected with 2 ml of NaCl 0.9% intraperitoneally in the lower left side abdomen. One hour after injection, each rat started to have oral gavage supplemented, group A with 1 ml of dextrose 5% while group B with 1 ml of glutamine and arginine (containing 250 mg/kg glutamine and 250 mg/kg arginine). All animals were supplemented once a day for 3 days, at the same time every day. On the third day, 2 hours after supplementation, all animals were sacrificed. Injection and gavage were given by an expert veterinarian. Food intake, body weight, and body temperature were measured and noted by the team during the experiment.

Sample collection

After the rats were killed, the small intestine was removed. Jejunum was taken and washed in aquadest, then wrecked into small pieces and soaked in the plastic bottle containing 5 ml of phosphate buffer saline. The jejunum samples were processed by FACS Calibur flow cytometer.

Data analysis

This was a quasy experimental design. Inferential statistic test was used to analyze the number of cells producing anti-inflammatory (IL-10) and pro-inflammatory cytokines (NF-κB, TNF-α, IL-8, and MMP-8). Data were analyzed using Statistical Package for the Social Sciences with comparative non-parametric inferential method (Mann–Whitney test). Statistical significance was determined if p value < 0.05. Significant results then analyze continuance by using a histogram of flowcytometry (Bioscience, 2002).

Ethical approval

This study is ethically approved by the committee of ethics from the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya, Indonesia (2.KE.027.03.2021).

Results

We compared the number of cells producing pro and anti-inflammatory cytokine between group A and B. Results showed that there was no difference in the number of anti-inflammatory cells producing IL-10 between the two groups, nor there was any significant difference in the number of pro-inflammatory cells producing NF-κB and MMP-8. Meanwhile, there

was a significant difference in the number of pro-inflammatory cells producing TNF-α and IL-8 as shown in the Table 1.

Table 1 showed that GAC effect on the cell that produces IL-10, NF-κB, and MMP-8 are not significant with $p = 0.208$, $p = 0.916$, and $p = 1.000$, consecutively. The combination of glutamine and arginine significantly decreases the cell that produces TNF-α ($p = 0.009$) and IL-8 ($p = 0.003$). This significancy also showed in the flowcytometry histogram of macrophage proliferation into M1 type on TNF-α examination for both groups (Fig. 1). Group A, which received 5% dextrose, showed a higher peak of M1 proliferation, with 8.54% cells producing TNF-α in macrophage. In group B, only 5.69% of cells have been found to produce TNF-α in macrophages that proliferate into M1.

Figure 2 showed macrophage proliferation of M1 type on IL-8 examination of both groups. Group A showed a higher peak of M1 proliferation with 19.80% of cells producing IL-8, meanwhile in group B only 2.86% of cells have been found to produce IL-8.

Discussion

Injection of 0.9% NaCl intraperitoneally can lead to inflammation and causing pain in rats (Coria-Avila *et al.*, 2007). Our study used the injection of 2 ml 0.9% NaCl intraperitoneally in the lower left abdomen. The location for peritoneal injection refers to the study of Coria-Avila *et al.* (2007) that found the lower left injection showed more lesions in the cecum than the lower right injection. Hopefully, this treatment can stimulate the inflammation process in this study.

We found that TNF-α significantly decreased in the group supplemented by a combination of glutamine and arginine. This result is in line with a study by Bakir *et al.* (2019) that showed GAC led to lower serum levels of TNF-α in the liver of rats with sepsis, and also a study by Zhou *et al.* (2012) that found decrease TNF-α mRNA in the intestinal mucosa of endotoxemic rats.

We chose jejunum as the sample and found that TNF-α was significantly low for the combination of glutamine and arginine treatment. Another study found significantly low TNF-α in the combination of glutamine and arginine treatment in the ileum segment of endotoxemic rats, but in the jejunum segment even though decreasing TNF-α was found but not significantly. Another study also found decreased TNF-α in the enterally supplementation of GAC, and also for glutamine or arginine only (Zhou *et al.*, 2012).

Our study found a significant decrease in IL-8. We could not find the same studies that used a combination of glutamine and arginine to decrease IL-8 expression. However, Huang *et al.* (2021) used glutamine gavage supplemented only for mice with acute lung injury induced by lipopolysaccharide (LPS) and showed that glutamine adequately depressed IL8 production. Several studies also found low levels of IL-8 in arginine treatment. Arginine with a dose of 8 mM effectively decreased IL-8 in the IPEC-J2 cells induced by LPS (Lan *et al.*, 2020). Another study also found that the administration of arginine topical showed low levels of IL-8 in laparotomy mice (Jeronimo *et al.*, 2016).

Low levels of TNF-α and IL-8 might be caused by the inhibition of M1 macrophage proliferation pathways. Our study found short peaks of M1 macrophage proliferation in cells that produce TNF-α and IL-8 in a group with a combination of glutamine and arginine. M1 macrophage was activated by IFN-$_{\gamma}$ which was secreted by CD4+ helper T cells, NK cells, and NKT cells (Wu *et al.*, 2014; Kanno *et al.*, 2019). Unfortunately, we did not measure those parameters in this study.

M1 macrophage plays a role in the secretion of pro-inflammatory cytokines such as TNF-α and IL-8 (Cruzat *et al.*, 2018; Gomarasca *et al.*, 2020). Glutamine availability was regulating the activation of M1 macrophages (Cruzat *et al.*, 2018). In addition, glutamine, known as essential amino acid in several conditions because of the widely used glutamine in the intestinal cell, renal, and immune cells, exceeds endogen production (Kim and Kim, 2017). During inflammation, glutamine was beneficial in modulating the imbalance production of cytokine (Cruzat *et al.*, 2018).

Meanwhile, arginine was involved in any of the various processes one of which is related to macrophage polarization into M1 and M2 type (Ley, 2017). M1 macrophages release NO synthesize that metabolized arginine into NO and cytruline, which is NO will metabolize into reactive nitrogen species which is commonly followed by tissue damage, hence M2 macrophage were needed for tissue purify and renewal. M2 macrophage expressed arginase that hydrolyze arginine into ornithine and urea, which ornithine

Table 1. Comparison of the number of cells that produce cytokines.

Variable	Mean ± SD	*p*
IL-10		
Group A	4.18 ± 1.30	0.208
Group B	3.02 ± 1.33	
NF-κB		
Group A	14.89 ± 5.93	0.916
Group B	15.05 ± 4.90	
TNF-α		
Group A	11.37 ± 5.94	0.009*
Group B	6.06 ± 2.37	
IL-8		
Group A	16.62 ± 5.31	0.003*
Group B	7.40 ± 3.87	
MMP-8		
Group A	4.22 ± 4.52	1.000
Group B	4.27 ± 4.56	

*Mann–Whitney test significance ($p < 0.05$). Group A. Supplemented with dextrose 5%. Group B. Supplemented with a combination of glutamine and arginine.

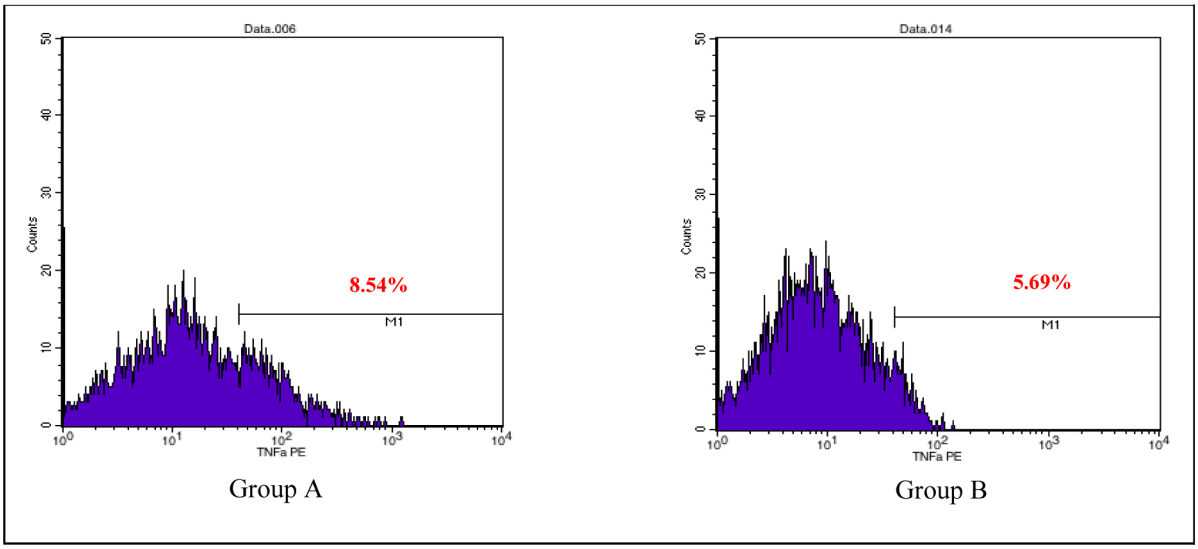

Fig. 1. Flowcytometry of M1 proliferation in TNF-α. Group A: Supplemented with dextrose 5%. Group B: Supplemented with a combination of glutamine and arginine.

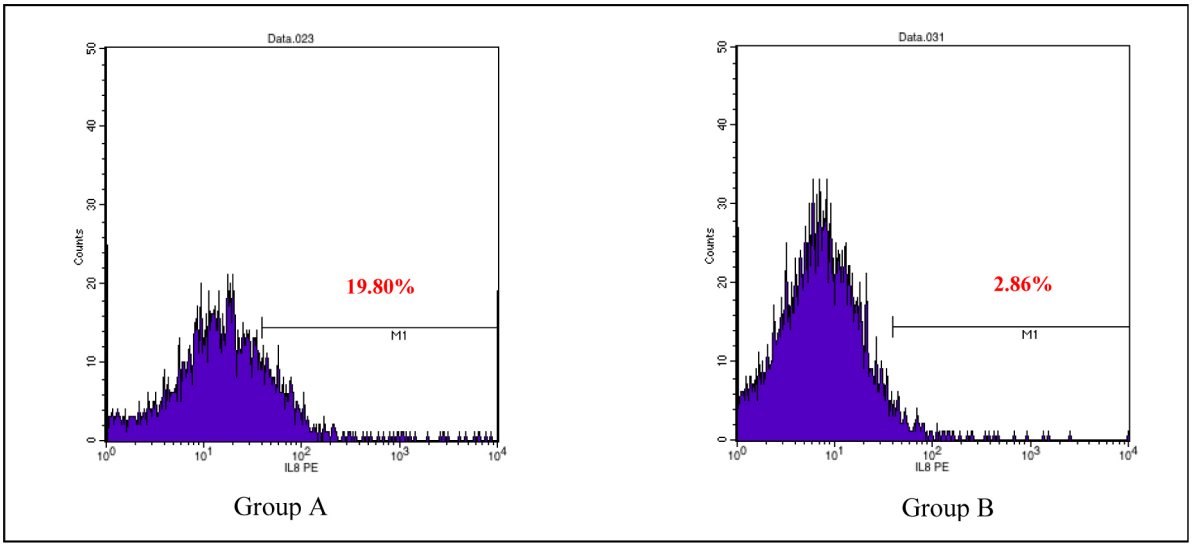

Fig. 2. Flowcytometry of M1 proliferation in IL-8. Group A: Supplemented with dextrose 5%. Group B: Supplemented with a combination of glutamine and arginine.

changes into polyamine, glutamate, and proline for cell proliferation and tissue repair (Rath *et al.*, 2014).

The immune system showed that TNF-α and IL-8 were correlated. TNF-α could activate the secretion of IL-8 (Baratawidjaja, 2012). Both TNF-α and IL-8 were affected by NF-κB activation (Sudiana, 2017; Kany *et al.*, 2019). Glutamine could suppress NF-κB production by stimulating Ikβ (Kim and Kim, 2017). Meanwhile, arginine attenuates NF-κB by increasing IL-10 production (Rath *et al.*, 2014; Lee, 2019; Bergmann *et al.*, 2021). Our study found that GAC had higher NF-κB production and lower IL-10.

This result is in line with a study by Zhou *et al.* (2012) in endotoxemic rats, which showed a decrease in IL-10. Arginine supplementation only showed that topical administration did not affect IL-10 production in mice with laparotomy (Jeronimo *et al.*, 2016). In Contrary, several studies showed that glutamine only affects the high production of IL-10 in lobectomy patients (Wang *et al.*, 2018) and in bone marrow-derived macrophages induced by LPS (de Oliveira *et al.*, 2018).

Our study did not find a significant difference in cells that produce NF-κB. Until now, we could not find another study that uses a combination of glutamine and arginine and its effects on NF-κB expression. We found several studies used glutamine only or arginine only with a different result for the expression of NF-κB. Glutamine administration could inhibit

the expression of NF-κB and showed advantages effect to protecting the intestinal sepsis rats (Wu and Zhang, 2019). As well as glutamine, 500 µM arginine significantly inhibits NF-κB in IPEC-J2 cells injected with LPS (Qiu *et al.*, 2019).

MMP-8 is suspected as the main collagen in the process of mucosal damage (Lee, 2019). MMP-8 production was affected by NF-κB presence (O'Sullivan *et al.*, 2015). In our study, there is no difference in cells producing MMP-8 between the two groups. It might be caused by no differences in NF-κB cell production for both groups.

No differences in cells that produce IL-10, NF-κB, and MMP-8 in our study may be caused by the dose and the time of administration. A study by de Oliveira *et al*. (2018) found that glutamine modulation in cytokine expression depended on the time of administration, in which high dosages could increase IL-10 affecting the inflammation response. A review by Sepandi *et al*. (2019) mentioned that a previous study reported contradictory evidence of arginine effects on indices of lipid profiles and inflammatory markers. Several studies showed the beneficials of arginine for lipid disorder and on inflammatory markers, but some studies have been failed to prove it.

Conclusion

Studies about giving a combination of glutamine and arginine are limited. In our study, nutritional supplementation with GAC has beneficial effects in decreasing almost half of the cells that produce TNF-α and IL-8. Further and larger studies are required to make a more established and standard guideline.

Conflict of interest

The authors declare that there is no conflict of interest in this study.

Author contributions

M, NMR, and SMS have made substantial contributions to the conception; design of the work; the acquisition, analysis, and interpretation of data; the creation of new software used in the work; have drafted the work substantively revised it.

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