Alteration of inflammation cytokines in lipopolysaccharide – activated lymphocyte by crude extract of taurine from sea slug *Paraonchidium* via down-regulation of NF-κB pathway

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Abstract. Free amino acid taurine is found in sea slugs. It has been used as a preventive agent for cardiovascular disease and inflammatory disorders. Inflammation takes place in the presence of lipopolysaccharide (LPS), as a specific inducer, which provokes activation of molecular signals controlling the development of pro-inflammatory and anti-inflammatory cytokines. This current work investigated the effects of taurine extract from shell-less sea slugs on the inhibition of IFN-γ as pro-inflammatory cytokine and IL-10 as anti-inflammatory cytokine in LPS-induced mice lymphocytes. The crude taurine was extracted using water solvent maceration of the sea slug's mucus. Culture of mice splenocytes in RPMI-1640 medium underwent for 5 days and they were then analysed by flow cytometry. As the result, crude taurine extracts substantially suppressed NF-κB expression in T cells, found in all concentrations. The best inhibitory effect was attributed to 500 µg/mL, which was significantly different over control groups (p<0.05). Additionally, the extract inhibited expression of CD4⁺ IFN-γ⁺ pro-inflammatory cytokines at 500 µg/mL, being stronger than inhibition by control (p<0.05), while it improved expression of CD4⁺IL-10⁻ anti-inflammatory cytokines at 500 µg/mL compared with control (p<0.05). Our experimental results indicated potential use of taurine crude extract isolated from sea slug *Paraonchidium* as anti-inflammatory agent from marine source.

Keywords: anti-inflammatory; lipopolysaccharide; sea slug *Paraonchidium*; taurine

1. Introduction
Sea slug *Paraonchidium* is characterized by a specific marine gastropod with a soft body without a shell [1]. Although the species has sluggish movement and imperfect protection against the environment and predators, it is endowed with a splendid ability to camouflage the skin and to secrete acid [2]. The sea slugs inhabit intertidal areas and around mangroves with mud, sand, and coral fragments of substratum forms [3]. They have been traditionally explored for food and medicine by coastal communities [4].
Currently, the discussion on their further use has been increased, concerning protein profile including presence of taurine [5].

In mammals, taurine is present as a consequence of cysteine metabolism in the form of free amino acids containing sulphur groups [6]. In meat and fish, but a little bit in plants, fungi and bacteria, taurine is also present [7]. Since first discovered, taurine has been extensively researched particularly regarding to its essential role in modulating lipid metabolism [8], preventing cardiac disease [9], acting as an antioxidant and its association in inflammation control [10], building immunity to prevent and cure diabetes [11].

Inflammation relates to immune system of the body as physiological response towards threatening pathologies, including infection and irritation induced by chemicals, pathogenic microbes, and wounds [12]. This immune response is significant for fighting antigens and detrimental to the surrounding tissue [13]. This inflammatory response is significant for maintaining tissue homeostasis, which begins with a specific molecular recognition pattern related to certain infections or damaged tissue [14]. Inflammatory reactions are recognized macroscopically by four signs, namely heat, redness, swelling, pain, and depletion of particular function [15].

Inflammatory stimuli can be first indicated by host cells through specific transmembrane receptors which are known as pattern recognition receptors (PRRs). They are expressed by two immune systems: innate and adaptive system. In this case, PRRs enable to detect presence of microorganism infection and any cell damage [16]. A close indicator of a microbial attack is lipopolysaccharide (LPS) which is a toxic metabolite in the cell walls of gram-negative bacteria, enabling to cause an inflammatory response and as indicator of bacterial infection [17]. Macrophages and dendritic cells can respond to LPS through PRRs on their surface.

To recognize the threat on cell surface, a protein called as toll-like receptors (TLRs) is responsible for detecting it. The attachment between ligands and TLR complex stimulates cytoplasmic signaling proteins, such as transcription factor NF-kB. NF-kB translocates into the nucleus and adheres to the promoter gene. Such actions regulate the transcription of genes encoding proinflammatory mediators, namely TNF-α, IL-1, IL-6, and iNOS [18]. IL-1 functions to activate various proinflammatory mechanisms, while IL-18 works together with IL-12 for prompting expression of IFN-γ via NK cells and T cells [19]. Furthermore, IL-10 retards activation of macrophages and dendritic cells which can suppress IL-12 and IFN-γ release and ultimately interferes with the IL-12/IFN-γ mediated proinflammatory response [20].

Research on how taurine may regulate inflammation is still inconclusive, particularly taurine derived from sea slug *Paraonchidium* in suppressing IFN-γ and IL-10. Current study was aiming at determining the effects of taurine crude extract of sea slug *Paraonchidium* on expression of inflammatory cytokines (IFN-γ and IL-10) in rats induced with lipopolysaccharide through inhibition of the NF-kB pathway.

2. Materials and methods

2.1. Extraction of taurine

*Paraonchidium* sea slug was caught by hand from the seawaters of the island of Talango in Sumenep, Indonesia. After sea slug was washed and sacrificed, the mucus was collected for extraction. The process of extraction refers with minor modifications to the study of Lee *et al.* [21]. 100 grams of sample in total, then surgically separated by muscle, viscera, and mucus. The obtained mucus fluid was heated for 8-10 min at 90 °C, then centrifuged for 15 min at 5000 rpm. The precipitated fraction was collected, dried (using freeze-dryer) and stored for in vitro anti-inflammatory analysis.

2.2. Medium preparation

At Roswell Park Memorial Institute 1640 (RPMI), T cells were cultured, and complemented by 10% Fetal Bovine Serum (FBS). Next, we prepared glutamine medium (30 μg/mL), penicillin (100 U/mL), streptomycin (100 μg/mL), anti-CD3 (2% culture supernatant, LPS of 500 ng/mL). The media as
treatment included pure taurine (250 µg/mL) and crude extract of taurine from sea slug (prepared at 3 levels: 50 µg/mL, 250 µg/mL and 500 µg/mL).

2.3. Isolation of lymphocyte, intracellular staining, and flowcytometry analysis
Spleen lymphocytes were isolated from pathogen-free BALB/c mice (4 weeks old). The spleen was washed twice in a petri dish using PBS (phosphate-buffered saline) and then squeezed to release the tissue using a sterile syringe base. To collect a suspension containing lymphocytes, the crushed spleen was homogenized with 10 mL of PBS, and then centrifuged at 2500 rpm and 10 °C for 5 min. After centrifugation, the pellet was added with 1 mL of RPMI (Roswell Park Memorial Institute-1640) following the removal of the supernatant. The mixture (5 µL) was then substituted with 95 µL (20 dilution) of 10× evans blue, gradually homogenized by the pipette. The cell was counted under the microscope with hemocytometer and quantified using the following formula:

\[ \sum \text{cells} = \sum \text{cells count} \times 5 \times \text{times dilution} \times 10^4 \text{ cell mL}^{-1} \]

The cells obtained (1.5-2 x 10^6 cells/mL) were cultivated in 48 well plates divided into 6 treatment classes with taurine doses as follows: taurine of 0 µg/mL (K-), LPS of 500 ng/mL (K+), pure taurine 250 µg/mL (Tau), taurine crude extract of 50 µg/mL (T1), 250 µg/mL (T2), and 500 µg/mL (T3). With 3 million/mL cells, each medium was added, then softly mixed. The cell culture was performed for 5 days in the controlled incubator (temperature 37 °C, CO2 5%). After harvested, the cells were moved into a 15 mL-plastic tube and centrifuged for 5 min at 2500 rpm and 10 °C. Following pellet removal, 1 mL of PBS was transferred to the tube for intracellular antibody staining preparation.

The staining technique followed principles compatible to the combination of antibody, prepared as follows: A = FITC-conjugated rat anti-mouse CD4, PE/Cy5-conjugated rat anti-mouse NF-κB, and B = FITC-conjugated rat anti-mouse CD4, PE/Cy5-conjugated rat anti-mouse IL-10, and PE-conjugated rat anti-mouse IFN-γ. In short, cells were incubated in the ice box at 4 °C for 20 min with extracellular antibody CD4, then added with 50 µL of cytofix buffer and resuspended for 20 min under dark conditions. With 500 µL of wash-perm, the suspension was added and centrifuged at 2500 rpm and 10 °C for 5 min. While the pellet was added with 50 µL of intracellular antibody, the supernatant was discarded. The pellet was incubated under low light condition for 20 min at 4 °C. Each sample was moved into cuvettes for analysis in flowcytometry instrument (FACSCalibur; BD Biosciences, New Jersey, USA) and for quantification, BD CellQuest PRO software was employed.

2.4. Data analysis
BD CellQuest PRO software performed analysis of flowcytometry data, which was then tabulated and statistically analysed using analysis of variance (One-Way ANOVA), enabling to evaluate difference between control and treated groups. A large difference in significance of 95 percent was then confirmed by the Tukey test. Procedure in statistical analysis employed SPSS version 16.0 for Windows (SPSS Inc., Chicago, IL, USA).

3. Result and discussion

3.1. Inhibition of NF-κB by taurine crude extract
Protein signal was released from cytoplasm as response to pathogens. In this case, transcription factor NF-κB serves important role in immune system and regulates expression of inflammatory cytokines. The results showed that taurine crude extract from sea slug could inhibit expression of NF-κB in mice lymphocyte induced by LPS via in vitro protocols. NF-κB release in CD4+ T-cells could be retarded by the extracts (50, 250, and 500 µg/mL) in comparison with positive control LPS (figure 1). All treatments showed the reduction of NF-κB lower than pure taurine. The most desirable dose was achieved at 500 µg/mL (T3), resulting in expression of NF-κB in CD4+ T-cells of 0.45% ±0.26 (p<0.05).

Taurine enables to react with hypochlorite acid produced by neutrophil via myeloperoxidase pathway. The reaction produced taurine chloramine (Tau-Cl) that shows anti-inflammatory activity [10].
Previous research explained that taurine could regulate antiinflammation. Tau-Cl is able to reduce activation of NF-kB, which is a strong transducer signal for inflammatory cytokines through oxidation of IkB-α [22]. In addition, Tau-Cl could diminish activity of NF-kB activated by immune cells by oxidizing inhibitor IkB-α [7]. IkB-α is a member of protein IkB able to inhibit NF-kB binding with DNA [23]. Furthermore, taurine was reported able to down-regulate the expression of NF-kB, which is linked to immunomodulation via TLR-2 and TLR-4 in mice induced with *Streptococcus uberis* [24].

![Figure 1. Taurine crude extract inhibits expression of NF-kB in CD4 T-cells in vitro.](image)

(A) calculation of CD4 T-cells enabling to express NF-kB in spleen cells of mice induced with LPS; (B) flowcytometry of NF-kB expression in spleen cells of mice induced with LPS. K(-): negative control, health lymphocyte cells without LPS; K(+): positive control, lymphocyte cells with LPS; Tau: pure taurine 250 µg/mL; T1: taurine crude extract 50 µg/mL; T2: taurine crude extract 250 µg/mL; T3: taurine crude extract 500 µg/mL.
3.2. Inhibition of IFN-γ by taurine crude extract
IFN-γ constitutes a proinflammatory cytokine in which the activation mechanism relates to interleukin 1 (IL-1). IL-1 primarily expressed as IL-12 altogether with IL-18 stimulates release of IFN-γ. The result showed that taurine crude extract from sea slug could attenuate expression of IFN-γ (figure 2). The extracts (50, 250, and 500 µg/mL) showed inhibitory properties on IFN-γ in CD4+ T-cells in mice lymphocyte via in vitro in comparison with control groups. In this regard, T1 (50 µg/mL) is the best treatment for reducing the expression of IFN-γ, i.e. 0.54% ±0.13, compared to positive control, and being better compared with pure taurine, i.e. 2.21% ±0.69 (p<0.05). in addition, no significant difference occurred between pure taurine and negative control, but the taurine could inhibit expression of IFN-γ more strongly compared with positive control (p<0.05).

![Figure 2](image-url)

Figure 2. Taurine crude extract from sea slug mucus inhibits expression of IFN-γ in CD4 T-cells in vitro. (A) calculation of CD4 T-cells able to express IFN-γ in spleen cells of mice induced with LPS; (B) flowcytometry of IFN-γ expression in spleen cells of mice induced with LPS. K(-): negative control, health lymphocyte cells without LPS; K(+): positive control, lymphocyte cells with LPS; Tau: pure taurine 250 µg/mL; T1: taurine crude extract 50 µg/mL; T2: taurine crude extract 250 µg/mL; T3: taurine crude extract 500 µg/mL.
Taurine crude extract prevents the release of NF-kB, which in turn reducing the binding between NF-kB and DNA stimulating expression of proinflammatory cytokines, primarily those from interleukin (IL) [19]. Expression of IFN-γ in CD4+ T-cells of mice lymphocyte cells could be hampered due to absence of transduction signals from NF-kB which inhibit release of IL-12 and IL-18. Previously, taurine could reduce level of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ) and interleukin (IL) in traumatic brain injury (TBI) in mice [25].

3.3. Enhancement of IL-10 by taurine crude extract

Interleukin-10 (IL-10) is known as essential anti-inflammatory cytokines and can be classified into Th2. It is important for maintaining homeostasis of all immune responses. Our present work revealed that IL-10 in mice lymphocyte cells induced by LPS could be enhanced with addition of taurine crude extract from sea slug (figure 3). Administration of taurine crude extracts could enhance concentration of IL-10, being significantly different compared with positive control (p<0.05). As depicted in figure 3, higher level of taurine could proportionally increase the quantity of IL-10 in mice lymphocyte cells. Thus, the best dose was achieved at 500 µg/mL (T3), with IL-10 reaching up to 1.24% ±0.25 compared to positive control, i.e. 0.06% ±0.03 (p<0.05). Additionally, T3 is also better than pure taurine (P<0.05), though not differed in comparison with negative control.

![Figure 3](image)

Anti-inflammatory modulator is essential for reducing immune response at cellular level. Regarding the properties of immunosuppressive activity, release of IL-10 is linked to monocyte and T helper cell,
including activation of T cell and B cell, which also able to suppress the inflammation response at a wide spectrum [26]. Former studies mentioned that the use of taurine in mice BALB/c treated with cardiac myosin for inducing experimental autoimmune myocarditis (EAM) could reduce level of TNF-α, IFN-γ, and IL-2, but increase expression of Th2 (IL-4 and IL-10) [27].

4. Conclusion
Our present work found that taurine crude extract from sea slug could be a potential source as anti-inflammatory agent. The extract at 500 µg/mL demonstrated the strongest effect on suppressing expression of NF-kB in mice lymphocyte cells induced by LPS. Besides, the extract inhibited release IFN-γ most properly acquired at dose of 50 µg/mL. Meanwhile, the extract could enhance production of anti-inflammatory cytokines IL-10.

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