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Betok Fish (*Anabas testudineus*) Oil Decreases Inflammatory Cytokine through Increasing Vitamin D Level in Rats-induced Systemic Lupus Erythematosus

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Abstract

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Keywords: Betok fish oil; *Anabas testudineus*; Inflammatory cytokine; Vitamin D; Systemic lupus erythematosus

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BACKGROUND: Systemic lupus erythematosus (SLE) is an autoimmune disorder marked by the production of antibodies to the cell's core components. Vitamin D is a steroid hormone that plays a role in the regulation of cell growth, proliferation, apoptosis, and regulation of the immune system. Vitamin D deficiency is a clinical condition that worsens clinical SLE. Betok fish commonly found in South Sumatra, Indonesia, is rich in vitamin D content

AIM: The aim of the study was to explore the utilization of betok fish oil as a natural source of Vitamin D that could reduce inflammation in SLE.

METHODS: A total of 30 male Wistar rats (200 ± 20 g) were obtained from Eureka Research Laboratory (Palembang, Indonesia). About 30 kg of betok fish were obtained from the Palembang Fish Auction Center, South Sumatra, Indonesia. Betok fish were extracted to obtain their oil by heating and centrifuging processes. Thirty male Wistar rats were randomly divided into the six groups: (1) Betok fish oil Group 1 0.1 mL/kg BW group, (2) Betok fish oil Group 2 0.2 mL/kg BW group, (3) Betok fish oil Group 3 0.4 mL/kgBW group, (4) normal control group, (5) negative control group, and (6) positive control group. Enzyme-linked immunosorbent assay (ELISA) method was used to assess ds-DNA levels and Vitamin D levels. Western blot assay was used to confirm the ELISA examination of pro-inflammatory cytokine (tumor necrosis factor-alpha [TNF-α]). All data were presented as mean ± standard deviation and all statistical analyzes are performed with the SPSS 25 (IBM) program.

RESULTS: This study presented that the Vitamin D content in betok fish oil is quite high at 2021.43 IU/mL. That was higher than other Vitamin D regiments on the marketplace. Moreover, this research showed that giving fish oil at a dose of 0.4 mL/kg BW is more effective than the positive control in increasing serum Vitamin D levels. These results indicate the effectiveness of betok fish oil in increasing serum Vitamin D levels in SLE-induced rats. In addition, betok fish oil was able to reduce the expression of pro-inflammatory cytokine, TNF-α, in serum of SLE rats.

CONCLUSION: Betok fish oil has efficacy in reducing levels of ds-DNA autoantibodies in SLE rats by decreasing the inflammatory response through upregulation of Vitamin D in serum.

Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by the production of antibodies to the cell's core components. Pathological conditions found in SLE patients are associated with inflammatory processes, vasculitis, deposition of immune complexes, and vasculopathy [1]. The inflammatory process is a major pathological condition in SLE disorders. SLE begins with the formation of antibodies against the individual cell components themselves, and anti-double stranded DNA (dsDNA) is found in 95% of cases. The prevalence of SLE in the Asia Pacific region is 4.3–45.3 per 100,000 population. Meanwhile, the prevalence of SLE in Indonesia is 0.5%, and the prevalence is increasing from year to year. There are 16,000 new cases of SLE in the United States each year. This showed that the number of

new cases of SLE is very significant and continues to grow every year. Therefore, optimal comprehensive efforts are needed to explore pathophysiology and a comprehensive therapeutic approach [2].

The increase in ds-DNA in SLE is triggered by an increase in B cell population due to decreased T cell production, which is initiated by interleukin-2 (IL-2) production [3]. Increased IL-2 will encourage the activation of Th-17 cells to produce IL-17. Furthermore, this condition will activate the inflammatory cascade, which is mediated by IL-1, IL-6, and tumor necrosis factor-alpha (TNF-α), which is responsible for damage in various target organs and will eventually cause damage and target organ failure [4], [5]. Compounds 2, 6, 10, 14-tetramethylpentadecane or commonly known as pristane are compounds used for the induction of SLE in Wistar rats (Strain of mice: *Mus musculus*). This compound will induce chronic inflammation so that it initiates an increase in ds-DNA

autoantibodies which will trigger an inflammatory cascade and target organ damage in SLE [6], [7], [8].

Vitamin D is a steroid hormone that plays a role in the regulation of cell growth, proliferation, apoptosis, and regulation of the immune system. Vitamin D deficiency is a clinical condition that worsens clinical SLE. Decreased levels of Vitamin D are common in SLE patients. Giving immunosuppressant drugs (glucocorticoids) will trigger a decrease in vitamin D levels. However, this drug is a standard drug consumed by SLE patients. Vitamin D has the capacity to suppress the immune response so it will suppress the inflammatory cascade. Vitamin D supplementation in SLE patients becomes strategic management, because it can reduce clinical problems optimally in SLE patients [8], [7], [9].

Exploration of Vitamin D sources is one of the important ways to improve the management of SLE disorders. Betok fish (*Anabas testudineus*) are a freshwater fish that are commonly found in the Musi River, South Sumatra, Indonesia. Like seluang fish (*Rasbora* spp.) which is a freshwater fish that are often found in the Musi River, Betok fish have similarities with seluang fish, both of which are first-rate consumers and both have similarities in terms of the amount of oil content found in scales and the bodies of the fish. Seluang fish oil has been shown to contain Vitamin D which is quite high, 2043.34 IU/mL [10]. As well as seluang fish oil, betok fish oil is rich in vitamin D content. High Vitamin D content is believed to be able to play a role in the regulation of the immune response and able to suppress the inflammatory cascade in SLE. Of course, this research was a significant and first attempt at utilizing betok fish oil to explore natural sources of Vitamin D that could reduce inflammation in SLE.

Methods

Animal

A total of 30 male Wistar rats (200 ± 20 g) were obtained from Eureka Research Laboratory (Palembang, Indonesia). Experimental animals were placed in cages under controlled conditions (12 h of light/dark cycles with temperatures of 22 ± 1°C and humidity of 40–60%), fed and drank *ad libitum*. All animal treatments and experimental procedures were approved by the Ethics and Humanities Commission of the Faculty of Medicine, Universitas Sriwijaya (No.176/kptfkunsri-rsmh/2019).

Fish oil processing and analysis of Vitamin D

About 30 kg of betok fish were obtained from the Palembang Fish Auction Center, South Sumatra, Indonesia, in May 2019. Fish were then kept in a

refrigerator –25°C to minimize biochemical changes during fish transportation from the Auction Center to the Biotechnology Laboratory, Faculty of Medicine, Universitas Sriwijaya (Palembang, Indonesia). Next, the whole body of betok fish were grinded and stored under –25°C until used.

Grinded betok fish were previously thawed at room temperature for 8 h, then as much as 1 L of water was added to every 200 g of flesh of the betok fish, then heating at 95°C for 30 min. Then, grinded fish were pressured, then, coextracted water together with oil. The third stage was centrifuging with a speed of 5000 rpm, temperature of 25°C, for 10 min. Furthermore, oil was separated from colloids and water. Around 15 µL of sample was injected to high-performance liquid chromatography (Shimadzu), spherical column C 18, mobile phase methanol: Aquadest (90:10), flow rate 0.8 ml/min, ultraviolet detector λ = 254 nm. The examination was carried out for analysis of Vitamin D content in betok fish oil [11], [12], [13], [14], [15].

Induction and treatment of experimental animals

Thirty male Wistar rats were randomly divided into the six groups: (1) Betok fish oil Group 1 0.1 mL/kg BW group: Five rats received a single intraperitoneal (ip) injection 0.5 ml pristane (Sigma-Aldrich, USA) on day 1 and given betok fish oil every day starting on day 2 for 3 months; (2) Betok fish oil Group 2 (BFO2) 0.2 mL/kg BW group: Five rats received 0.5 ml ip injection of pristane on day 1 and were given fish oil 0.2 mL/kg BW every day starting on day 2 for 3 months; (3) Betok fish oil Group 3 0.4 mL/kg BW group: Five rats received 0.5 ml ip injection of pristane on day 1 and were given fish oil 0.4 mL/kg BW every day starting on day 2 for 3 months; (4) normal control group: Five rats were not injected with pristane and were not given fish oil; (5) negative control group: Five rats received i.p 0.5 ml pristane injection on day 1 and were given Aquadest 0.4 mL/kg BW every day starting on day 2 for 3 months; and (6) positive control group: Five rats received i.p 0.5 ml pristane injection on day 1 and were given 100 IU/kg BW every day starting on day 2 for 3 months.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA method was used to assess ds-DNA levels and Vitamin D levels. Examination of ds-DNA levels and Vitamin D levels was performed first by taking blood from rats. Rats were first anesthetized with Ketamine 50 mg/kg BW and Xylazine 5 mg/kg BW. Blood was put into a non-EDTA tube, then centrifugation was carried out at a speed of 5000 rpm for 10 min, a temperature of 25°C. The supernatant from the centrifugation results was then taken and put into a

1.5 mL centrifuge tube to be stored at -20°C until used for ELISA examination.

The levels of ds-DNA and Vitamin D in serum were carried out using the Rat ELISA kit ds-DNA and Vitamin D (Cloud Clone), based on the protocol found in the manufacturer's protocols. Briefly, 50 μl standard diluents or serum samples were added to wells that had been coated with anti-ds-DNA; the anti-Vitamin D was incubated at 37°C for 30 min. After the plates were washed, 100 μl of biotinylated antibody solution was added and incubated for 30 min at 37°C . After washing 3 times, 50 μl avidin-peroxidase complex solution was added and incubated for 15 min at 37°C . After washing, 50 μl of the tetramethylbenzidine color solution was added and incubated in the dark for 15 min at 37°C . Finally, 50 μl stop solutions were added to stop the reaction and optical density values were measured using an ELISA reader (Bio-Rad), wavelength 450 nm.

Western blot assay

This method was used to confirm the ELISA examination of pro-inflammatory cytokine (TNF- α). This process began with the stage of protein extraction. The sample supernatant was put into a RIPA buffer (Sigma-Aldrich) equipped with phenylmethanesulfonyl fluoride on ice for 5 min. After centrifugation at 12,000 rpm for 10 min at 4°C , the supernatant was collected as total protein lysate. Cytoplasmic protein and nucleus were extracted from synovial tissue using a protein extraction kit (Sigma-Aldrich), based on manufacturer's protocol. Concisely, synovial tissue was cut into small pieces and homogenized with a protein extraction agent. After incubation on ice for 15 min and centrifugation at 5000 rpm for 5 min at 4°C , the supernatant was collected as a partial cytoplasmic protein, while the pellet was re-extracted again in the extraction buffer. After incubation on ice for 15 min and centrifugation at 12,000 rpm for 5 min at 4°C , the supernatant was combined with the cytoplasmic protein above. The pellet was then extracted again in an extraction buffer and shaken violently for 30 min at 4°C . After centrifugation at 12,000 rpm for 10 min at 4°C , protein was obtained. Total protein concentration was quantified using the bicinchoninic acid assay protein assay (Sigma-Aldrich) kit, according to the manufacturer's protocol.

A total of 40 μg of protein extract was separated at sodium dodecyl sulfate polyacrylamide gel electrophoresis 10%. Furthermore, the protein that has been separated was transferred to the PVDF membrane (Millipore) and blocked with 5% nonfat milk in Tris-buffered saline with Tween 20 for 1 h at room temperature. The membrane was incubated overnight at 4°C with the rabbit polyclonal TNF- α 1:700 primary antibodies (Cloud Clone). Next, the membrane was incubated with horseradish peroxidase-conjugated antibodies at 1:5000 for 45 min within temperature of 37°C . Next, visualization of the results of blotting with chemiluminescence (Bio-Rad) was performed.

Standardization of blotting results was done by blotting on β -actin.

Statistical analysis

All data were presented as mean \pm standard deviation and all statistical analyses are performed with the SPSS 25 (IBM) program. One-way ANOVA followed by *post hoc* analysis was carried out to assess differences in the mean expression levels of each protein. $p < 0.05$ was determined as an indication that there were significant differences in the mean levels.

Results

Betok fish oil is fish oil derived from freshwater fish, where this fish are classified as the first level consumer. Table 1 shows that the Vitamin D content in betok fish oil is quite high at 2021.43 IU/mL. Vitamin D

Table 1: Vitamin D level in betok fish oil

Active compound	Quantity
Vitamin D	2021.43 IU/mL

levels contained in the fish oil are quite high compared to Vitamin D supplements that are sold on the market.

Pristane induction showed the potential for inducing autoimmune conditions through increased ds-DNA autoantibodies, as seen in negative controls. Table 2 shows the efficacy of betok fish oil in reducing the level of ds-DNA autoantibodies with increasing doses of fish oil.

Table 2: Level of ds-DNA in serum

S. No.	Group	ds-DNA (pg/mL) \pm SD	p value*
1.	Control normal	34.26 \pm 2.54	0.00
2.	Control positive	176.36 \pm 11.12	0.00
3.	Control negative	676.76 \pm 9.54	-
4.	Betok fish oil Group 1	476.34 \pm 23.76	0.00
5.	Betok fish oil Group 2	328.11 \pm 17.43	0.00
6.	Betok fish oil Group 3	154.77 \pm 11.87	0.00

*Versus negative control, ANOVA, *post hoc* Bonferroni; $p < 0.05$.

Figure 1 shows that giving fish oil at a dose of 0.4 mL/kg BW is more effective than positive control

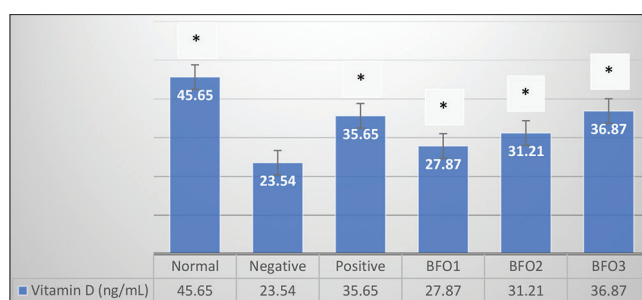


Figure 1: Effects of betok fish oil in Vitamin D in systemic lupus erythematosus rats. * $p < 0.05$ versus negative group; ANOVA, *post hoc* Bonferroni

in increasing serum Vitamin D levels. These results indicate the effectiveness of betok fish oil in increasing serum Vitamin D levels in SLE-induced rats.

Figure 2 shows that the supplementation of betok fish oil was able to reduce the expression of

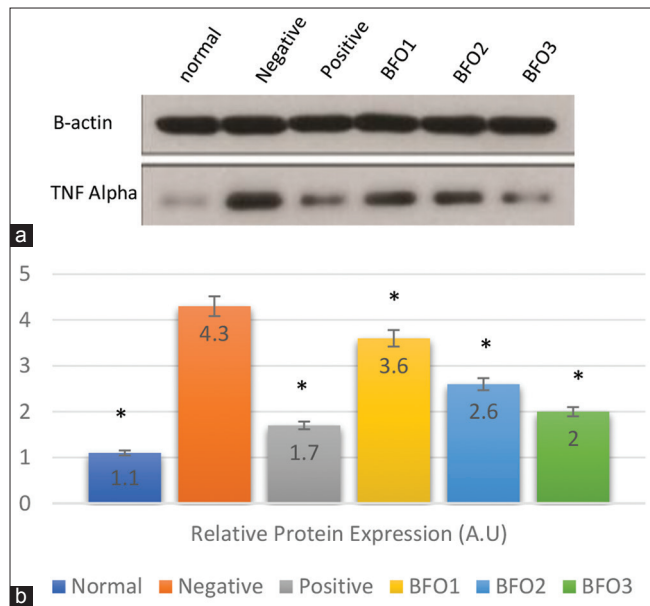


Figure 2: (a and b) Effects of betok fish oil in tumor necrosis factor-alpha expression in systemic lupus erythematosus Rats. * $p < 0.05$ versus negative group; ANOVA, post hoc Bonferroni

pro-inflammatory cytokines in TNF-alpha in serum of SLE rats. Increasing the dose of betok fish oil showed more effectiveness in decreasing the expression of pro-inflammatory cytokines, TNF-alpha. This showed the potential of betok fish oil to reduce inflammation in SLE cases.

Discussion

SLE is an autoimmune disorder characterized by misinterpretation and recognition of cell parts by individual body's immune cells. In normal conditions, parts of the cell nucleus (ds-DNA or anti-nuclear antibody [ANA]) produced by cell damage are recognized as foreign antigens (immunogen antigenic) [16], [17]. This causes the ds-DNA or ANA will cause activation of the immune system, which will cause the binding of antigens to the body's antibodies. These conditions will cause the activation of various immune systems and cause greater damage to individual body cells. Pristane is a compound that is highly immunogenic so that it will spur increased ds-DNA and ANA. At the same time, this compound will activate from immune cells so that it will further stimulate immunogenic activation which results in more severe cellular damage. The induction process caused by

pristane is believed to be able to be a model of SLE induction in experimental animals [18], [19], [20].

Damage caused by antigen binding (ds-DNA and ANA) with antibodies will cause activation and recruitment of various immune responses by releasing various pro-inflammatory mediators, such as TNF α and IL-1 β . Subsequent pro-inflammatory mediators will cause more massive damage due to immunogenic inhibitory activity [21], [22], [20]. Vitamin D is a regulator that plays a role in maintaining the body's defense system homeostasis. Vitamin D acts as a regulator of various gene expressions that play a role in the activation and inhibition of immune cells. In an autoimmune condition where immune system reactivation occurs, Vitamin D will act as a regulator of the expression of anti-inflammatory cytokine genes, which will provide negative feedback from immune cell activation, which in turn leads to inhibition of immune cells. Betok fish oil, which is rich in Vitamin D, is a potential for exploration and development of new therapeutic modalities for SLE treatment [23], [24], [25].

This study showed that the provision of fish oil can reduce the expression of pro-inflammatory cytokines, TNF- α , along with the ability of fish oil to reduce the expression of autoantibodies, ds-DNA, and fish oil also can increase vitamin D levels in serum induced white rats with pristane.

Conclusion

Betok fish oil has efficacy in reducing levels of ds-DNA autoantibodies in SLE rats by decreasing the inflammatory response through upregulation of Vitamin D in serum.

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