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Bioactivity of *Moringa oleifera* and Albumin Formulation in Controlling TNF-α and lFN-γ Production by NK Cells in Mice Model Type 1 Diabetes

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Abstract

Type 1 diabetes (T1D) is a disease caused by pancreatic beta-cell injury due to high pro-inflammatory cytokines. TNF-α and IFN-γ are pro-inflammatory cytokines that play a role in T1D progression. *Moringa oleifera* (MO) and albumin (A) have anti-inflammatory and antidiabetic effects. The combination of both can works synergistically in suppressing the production of pro-inflammatory molecules. This study was conducted using five different groups (healthy mice, T1D, D1, D2, and D3). A dose of 145 mg/kg BW streptozotocin was used to induce T1D in mice. *Moringa oleifera* and albumin formulation **(**MOA) were orally administered for 14 days. Dose 1 (800 mg/kg MO:800 mg/kg A), dose 2 (615 mg/kg MO:615 mg/kg A), and dose 3 (800 mg/kg MO:615 mg/kg A). On day 15, hepatic cells from mice were isolated post-treatment, and the profile of $NK^+TNF\text{-}\alpha^+$ and $NK^+IFN\text{-}\gamma^+$ were analyzed by flow cytometry. This study reports that MOA administered in D3 more effectively suppresses TNF-α and IFN-γ produced by NK cells. MOA could be synergies work to decrease or suppress the level TNF-α and IFN-γ in T1D. So, administered MOA had the potential to be used as an alternative medicine for DM.

Keyword: *Moringa oleifera*, pro-inflammatory, T1D, TNF-α, IFN-γ

1. Introduction

Type 1 diabetes (T1D) is a disease caused by the destruction of pancreatic beta cells resulting in an absolute or relative deficiency in insulin production. The poverty of insulin leads to hyperglycemia (Simmons and Michels, 2015). The failure of pancreatic beta cells to produce insulin is closely related to NK and regulatory T cell function loss (Graham *et al.,* 2012). Infiltration of autoreactive T cells and Natural Killer (NK) cells in Langerhans islets lead to the progression of diabetes because of the increased secretion of pro-inflammatory cytokines (Nekoua *et al.,* 2020). *Tumor Necrosis Factor-Alpha* (TNF-α) dan *Interferon-Gamma* (IFN-γ) are pro-inflammatory cytokines most prominent produced by NK cells (Fauriat *et al.,* 2010). NK cells are one of the immunocompetent cells that increased inflammatory progression in diabetes mellitus (DM) by producing TNF-α and IFN-γ. Increased pro-inflammatory cytokines can cause chronic inflammation in the tissue (Abel *et al.,* 2018).

The high levels of TNF-α could induce dendritic cell activation that causes activation of T cells, which are mediators of damage to pancreatic β cells (Lee *et al.,* 2005). TNF-α can activate the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) which plays a role in the secretion of pro-inflammatory cytokines (Patel and Santani, 2009). IFN-γ and TNF-α play a role in autoimmunity in DM and apoptosis of pancreatic β cells. The increase in IFN-γ causes activation of macrophages and CD8 T cells, which causes damage to pancreatic β cells through TNF-α and interleukin (IL)-1β secretion (Tsiavou *et al.,* 2004). T1D is disease-related with numerous factors such as genetic, environmental, low insulin production due to pancreatic B cell damage, and uncontrolled secretion of pro-inflammatory cytokines (Marrack *et al.,* 2001; Cerf, 2013). Based on these factors, an effective treatment is needed in treating DM. Currently, treatment focuses on managing blood sugar levels with insulin and synthetic drug consumption to prevent complications. However, as we know, long-term use of synthetic drug lead to harmful side effects. Natural treatment is considered due to having many benefits without causing harmful side effects.

In this experiment, we have the purpose of investigating the effect of MOA on the T1D mice model

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** **Abbreviations:** ANOVA: analysis of variance;BW: body weight ;DM: diabetes mellitus ;H2O2: hydrogen peroxide ;IFN-γ: Interferon-Gamma;IL: interleukin;MO: Moringa oleifera ;NF-κB: transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells;NK: Natural Killer;PB: peripheral blood ;PBS: phosphate buffer saline; SH: sulfhydryl ; STAT4: signal transducer and activator of transcription4;STZ: streptozotocin ;T1D: Type 1 diabetes;TACE: tumor necrosis factor-alpha converting enzyme ;TNF-α: Tumor Necrosis Factor-Alpha

through to control the TNF-α and IFN-γ production by NK cells. MOA consists of a combination of *Moringa oleifera* and Toman fish albumin (*Channa micropeltes*). Combining natural ingredients is expected to form a complex that works synergistically to maximize the natural compound's efficacy. *Moringa oleifera* has been known as a traditional medicine in various kinds of diseases. The effectiveness of *Moringa oleifera* was attributed to bioactive compounds such as phenol, flavonoid, alkaloid, glucosinolate, β-sitosterol-3-O-β-D-glucoside, isothiocyanate, tannin, terpenoid, and saponin (Lopez *et al*., 2018). In addition, MO has many beneficial properties such as anti-inflammatory, antimicrobial, antioxidant, anticancer, cardiovascular, antidiabetic, and diuretic effects (Osman *et al*., 2012; Bhattacharya *et al.,* 2018). Albumin from fish can serve as an alternative to fulfill albumin in the body. Moreover, albumin has a group sulfhydryl (-SH), which plays a role and functions as a free-radical scavenger (Quilan *et al*., 2005).

2. Materials and Methods

2.1. Experimental animals protocol

Animals used in this experiment were male BALB/c mice, obtained from Malang Murine Farm, Singosari, Malang, East Java, Indonesia. A total of 25 normal BALB/c male mice were 8-10 weeks with body weight (BW) around 25-30 g maintained in a pathogen-free chamber with controlled conditions. They had free access to standard pellet feed and water daily during the experiment period. Experimental mice were divided into five treatment groups: healthy mice, T1D mice model, and T1D mice model administered with three different doses (D1, D2, and D3). These experiments' protocols were carried out and internationally accepted and permitted by the Ethical Committee of Brawijaya University, Malang, Indonesia (Reg. No. 1180-KEP-UB).

2.2. Animal models type 1 diabetes

The induction of T1D was conducted by DiaComp Protocols (Brosius, 2015) with modification. T1D was induced by a single intraperitoneal (i.p.) injection of freshly prepared streptozotocin (STZ) (Bioworld, USA) at a dose of 145 mg/kg BW in 0.1 M citrate buffer (pH 4.5). The mice fasted for 4-6 hours before injection. The blood glucose levels were measured at six days post-injection of STZ with a glucometer. Mice were considered to suffer from diabetes if blood glucose level ≥200 mg dL^{-1}.

2.3. Preparation and oral treatment of Moringa oleifera-Albumin combination

MOA consists of a combination of *Moringa oleifera* and albumin from Toman fish (*Channa micropeltes*). *Moringa oleifera* (MO) leaves were collected from the Materia Medica Batu, Malang, Indonesia. A total of 50 g of MO powder were boiled in 500 mL of water for 5 minutes and filtered to obtain MO extract before stored in a freezer at -80ºC for 24 hours. The frozen extract was then evaporated with a freeze dryer. Albumin was obtained from IFALMIN® manufactured by Ismut Fitomedika, Makassar, Indonesia. IFALMIN® is a product derived from Toman fish extract. MOA combination was given orally to T1D mice for 14 days with three different doses. All doses we showed here are adjusted to mg/kg BW. Dose 1 (800 mg/kg BW MO:800 mg/kg BW A), dose 2 (615 mg/kg BW MO:615 mg/kg BW A), and dose 3 (800 mg/kg BW MO:615 mg/kg BW A). On day 15, all of the mice were sacrificed post-treatment for flow cytometry analysis.

2.4. Isolation of liver organ and Flow cytometry analysis

On day 15 of this experiment, the mice were sacrificed by dislocation and then dissected for liver isolation. In our study, we used the liver for analysis of the NK cell that produces TNF-α and IFN-γ. According to Sun et al (2013), the percentage of NK cells in the liver is higher than in the spleen and peripheral blood (PB). The liver was isolated and washed in a petri dish containing sterile phosphate buffer saline (PBS). Cells from mice's liver were isolated by crushing liver in PBS. The cell suspension was removed to the polypropylene tube, and added with PBS. Homogenates of the cell were centrifuged at 2500 rpm, 10 °C, for 5 min. Pellet was resuspended in 1 mL of PBS and divided into 1 mL microtube. Intracellular cytokine staining was performed with a Cytofix/Cytoperm kit (Biolegend BD Sciences) according to the protocol presented by the manufacturer. Cells were incubated with anti-TNF-α and anti-IFN-γ antibodies. Before intracellular staining, cells were subjected to surface molecules staining with anti-NK. The antibodies were applied at a concentration of 0.005 mg/100 μL. The cells stained with extracellular and intracellular antibodies were added with PBS of 300-500 μL and transferred to cuvettes to be analyzed with flow cytometry.

2.5. Data Analysis

The data from flow cytometry were analyzed by BD. cell quest PRO™ software, then tabulated using Microsoft Excel for statistical analysis. The statistical analysis was executed by SPSS version 16 for windows. Data were analyzed using a one-way analysis of variance (ANOVA) ($\rho \leq 0.05$) followed by the Tukey test to decide the significant difference among treatments.

3. Results

T1D mouse groups models had the highest profile of cytokine pro-inflammatory than the normal groups ($p \leq 0.05$), including the relative number of TNF-α and IFN-γ production by NK cells ($NK^+TNF\text{-}\alpha^+$and $NK^+IFN\text{-}\gamma^+$). TID mouse groups were treated using three different doses of MOA. All of the doses giving various effects after post-treatment in suppressing TNF-α and IFN-γ production by NK cells. D1 and D3 have a significant impact on decreasing the relative number of $NK^+TNF\text{-}\alpha^+$ (figure 1). Meanwhile, D2 does not have a significant impact. Furthermore, D2 and D3 were significantly able to reduce the relative number of $NK^+IFN\text{-}\gamma^+$ compared to D1 (figure 2). Thus, in this study, D3 as a whole affected suppressing TNF-α and IFN-γ expressed by NK cells.

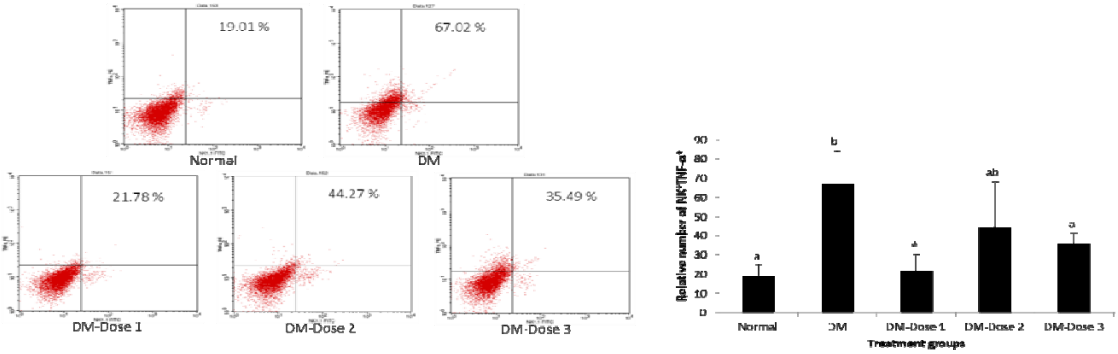

Figure 1. The administration of MOA in T1D mice model can suppress the relative number of $NK^+TNF\text{-}\alpha^+$. Normal = healthy mice; T1D = mice STZ induced; D1 = T1D + dose 1 (800 mg/kg BW (MOL) : 800 mg/kg BW (I)); D2 = T1D + dose 2 (615 mg/kg BW (MOL) : 615 mg/kg BW (I)); D3 = T1D + D3 (800 mg/kg BW (MOL) : 615 mg/kg BW (I)). Mice were sacrificed and analyzed using flow cytometry (left) on day 15 and tabulated into Microsoft Excel (right). The data are mean+SD in each group with p value ≤ 0.05.

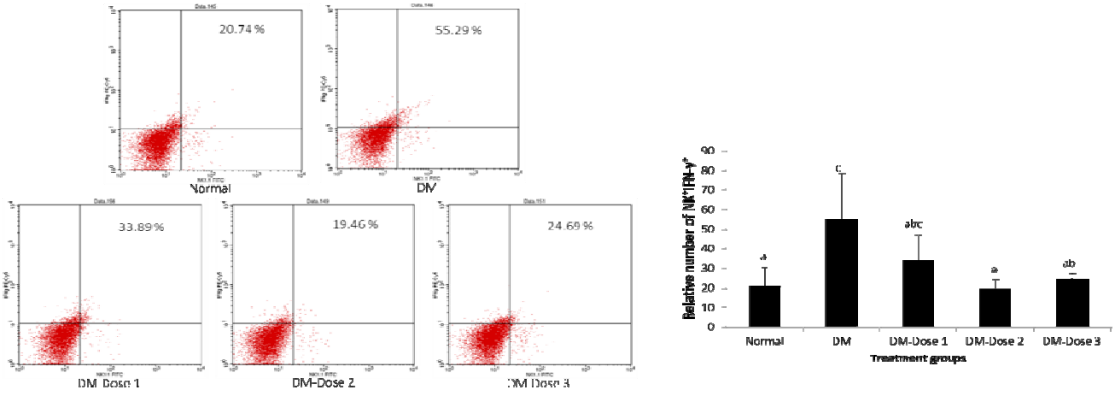

Figure 2. The administration of MOA in T1D mice model can suppress the relative number of $NK^+IFN\text{-}\gamma^+$. Normal = healthy mice; T1D = mice STZ induced; D1 = T1D + dose 1 (800 mg/kg BW (MOL) : 800 mg/kg BW (I)); D2 = T1D + dose 2 (615 mg/kg BW (MOL) : 615 mg/kg BW (I)); D3 = T1D + D3 (800 mg/kg BW (MOL) : 615 mg/kg BW (I)). Mice were sacrificed and analyzed using flow cytometry (left) on day 15 and tabulated into Microsoft Excel (right). The data are mean+SD in each group with p value ≤ 0.05.

4. Discussion

T1D is also known as insulin-dependent diabetes mellitus (IDDM) (Arora *et al.,* 2009). People with T1D generally rely on insulin injections to regulate their sugar metabolism. Type I diabetes resulted from deficiency or failure of the pancreatic islet to secrete insulin. Insulin is a hormone with a pivotal role that served the body to make blood glucose available to cells (Rifa'i *et al.,* 2018). If the levels of insulin are low in the long term, it will cause hyperglycemia. Hyperglycemia is a condition of high *blood glucose levels exceeding normal limits (Balakrishnan et al., 2012; Mohamed et al., 2016), resulting* in oxidative stress that causes tissue damage in the liver. Tissue damage in the liver causes further disruption of glucose metabolism, resulting in increased ROS and pro-inflammatory cytokines production, such as TNF-α (Zwirner and Ziblat, 2017). NK cells can produce TNF-α. The high level of TNF-α in DM causes an inflammatory response that worsening health in DM. In addition to TNF-α, IFN-γ is also known to play a role in the inflammatory response. According to Zwirner and Ziblat (2017), the high levels of secretion of IFN-γ by NK cells were mediated by the activation of signal transducer and activator of transcription4 (STAT4) promoted by IL-12.

MOA consists of *Moringa oleifera* and Toman fish albumin. The administration of MOA in T1D mice can decrease inflammatory responses. The combination of bioactive compounds from MOA can work synergistically to reduce the level of TNF-α and IFN-γ produced by NK cells. The previous study showed that MO and albumin have a role as an antioxidant that suppresses ROS production. The high level of oxidative stress will trigger inflammatory factors such as the transcription factor Nf-κB and various cytokines such as IFN-γ and TNF-α. In this study, the MOA's high antioxidant content was able to work synergistically to prevent oxidative stress. Albumin has the amino acid cysteine (cyst 34), which acts as a ROS scavenger such as hydrogen peroxide (H_2O_2) and inhibits lipid peroxidation. Albumin also inhibits the activation of the transcription factor NF-κB and modulate intracellular GSH levels. Albumin has many sulfhydryl (-SH) groups that can bind free radicals that cause oxidative stress (Taverna *et al.,* 2013).

MOA administration of various doses has yielded varying results. The MOA administration at D3 was able to significantly suppress the level of TNF-α and IFN-γ produced by NK cells (Figures 1 and 2). Actually, administration of MOA at D1 and D2 was able to suppress, but not significantly. In figure 1, at D1 MOA could suppress the level of TNF-α, but the administration at D2 was increased and decreased in administration at D3, whereas, in figure 2, D2 and D3 significantly could

decrease the level of IFN-γ. Administration at D1 is still not able to significantly reduce IFN levels. Based on these results, it can be assumed that MOA is an immunomodulator. This is because MOA can control the production of cytokine by NK cells, wherein certain doses of MOA can suppress, but on the other hand MOA also increase the level of cytokine.

The decline production of TNF-α and IFN-γ by NK cells post-treatment indicates a reduction in inflammation and improved homeostasis in TID model mice. The presence of flavonoids in MOA plays a role in the mechanism of inhibiting the activation of the transcription factor NF-κB and the activity of tumor necrosis factor-alpha converting enzyme (TACE) in the synthesis of TNF-α. One of the receptors that regulate the activation of NK cells is the NKG2D receptor. Generally, the NKG2D ligand is expressed by stress cells. The binding between NKG2D and NKG2D ligands causes an increase in TACE activity and TNF-α secretion (Sharma *et al.,* 2017). Inhibition of TACE by flavonoids can reduce the production of TNF-α by NK cells (Gesso *et al.,* 2015). MOA is also known to contain β-sitosterol-3-O-β-D-glucoside, which suppresses IL-12 production by dendritic cell or macrophage, which activates STAT4 in NK cells, resulting in a decrease of IFN-γ production (Jimenez *et al.,* 2017; Ma *et al.,* 2018).

5. Conclusions

MOA has a potential role as an anti-inflammatory agent in mice models of TID by controlling TNF-α and IFN-γ production in NK cells. Administration of MOA decreases the number of NK^{+} IFN-γ^{+} and NK $^{+}$ TNF-α^{+} cell expression and is close to normal control.

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