The Study of Combination Ethanol Extract of *Averrhoa bilimbi* L. and *Momordica charantia* L. on CD4⁺CD25⁺TGF-β⁺ Spleenocytes of Hyperglycemia Mice

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Abstract

Diabetes mellitus is one of the four priority non-infectious diseases in the world. Plant-based medicine is an alternative treatment with few side effects. Star fruit (*Averrhoa bilimbi* L.) and bitter melon (*Momordica charantia* L.) are plants that have anti-hyperglycemic activity. Hyperglycemia produces Reactive Oxygen Species (ROS) that make the β-cells of the pancreas necrosis that decreasing insulin synthesis. The anti-inflammatory activity appears based on the relative levels of CD4⁺ and CD25⁺, which are TGF-β-producing regulatory T cells where TGF-β is a mediator that acts as an immunosuppressant. TGF-β would induce CD4⁺ + T lymphocytes into T reg. The purpose of this study is to determine the profile of TGF-β on CD4⁺ and CD25⁺ spleenocytes on hyperglycemia mice after ABMC (*Averrhoa bilimbi* *Momordica charantia* mix) treatment. Mice were divided into 5 groups, non-diabetic (N), hyperglycemia (H), hyperglycemia with extract doses of 10 mg.kg⁻¹ BW (P1), 40 mg.kg⁻¹ BW (P2), and 160 mg.kg⁻¹ BW (P3). Diabetic mice were obtained after a single injection dose of 145 mg.kg⁻¹ BW *streptozotocin* (STZ). The result showed that ABMC can reduce blood sugar levels faster and able reduce the number of CD4⁺TGF-β⁺ cells in hyperglycemia mice.

Keywords: *Averrhoa bilimbi* L., CD4⁺TGF-β⁺, CD25⁺TGF-β⁺, hyperglycemia, *Momordica charantia* L.

INTRODUCTION

Hyperglycemia is characteristic of diabetes mellitus (DM) due to decreased insulin secretion by pancreatic β cells. Hyperglycemia describes an increase in blood glucose levels in circulating blood and produces Reactive Oxygen Species (ROS) through various pathways, including redox balance dysregulation, augmentation of glycation products, activation of protein kinase C which ultimately leads to oxidative stress in various tissues [1]. A survey from the World Health Organization (WHO) shows that Indonesia has a high number of DM patients, ranking fourth in the world after India, China, and America [2]. The number of people with diabetes has quadrupled from 108 million in 1980 to 422 million in 2014 [3].

The main cause of complications in diabetes mellitus is the presence of oxidative stress conditions due to increased reactive oxygen species (ROS) [4-6]. Thus the islets contain very low levels of antioxidants, so the accumulation of ROS can produce *oxidative stress*, a well-known trigger for β cell apoptosis [7]. Here, the altered β-cell redox state, coupled with other factors such as nutrient-induced augmentation of insulin

synthesis, can lead to stress-induced apoptosis of the endoplasmic reticulum. The result is a reduced functional β cell mass, resulting in a further decrease in insulin secretion [8,9].

The hyperglycemia will also increase the expression of transforming growth factor-β (TGF-β). TGF-β signaling is one of the signaling pathways that affect β-cell differentiation and function. Impaired TGF-β signaling has the potential to be the center of β-cell dedifferentiation. TGF-β signaling is involved in almost all tissue types in the body and has been shown to play a role in the regeneration of β-cells in the islets of the pancreas. Failure of β cells in type II diabetes is a multifaceted process that can include inflammation of the islets of Langerhans, increased β cell apoptosis, decreased β cell proliferation, and dedifferentiation of β cells to a progenitor-like state [10].

Both TGF-β and CD4⁺-CD25⁺ regulatory T cells (Treg) play important roles in controlling immune responses and maintenance of immune homeostasis. TGF-β is a pleiotropic cytokine with a number of context-dependent effects on immune cells, including inhibition of T cell proliferation and differentiation, macrophage activation, and DC maturation [11]. The role of TGF-β1 in the regulation of CD4⁺CD25⁺ Treg cells in vivo was confirmed by a study using a type I diabetes mouse model. In that study, the transient TGF-β1 pulses in islets during the early phase of diabetes were sufficient to inhibit

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disease onset by stimulating expansion and expression of Foxp3 from CD4⁺CD25⁺ Treg intralyslet [12].

Plant-based medicine is one of the alternatives used as diabetes therapy because of its low side effects and affordable cost. The active principles present in medicinal plants have been reported to have the ability to regenerate pancreatic β cells, release insulin and fight insulin resistance problems [13,14]. Bitter melon (*Momordica charantia* L.) has high antioxidant activity. It also has active compounds including saponins, flavonoids, polyphenols, and vitamin C, as well as insulin-mimetic compounds consisting of charantin, vicine, and polypeptide-p, which are considered the main hypoglycemic compounds [13,15,16]. Ethanol extract (95%) from *M. charantia* L. has significantly lowered blood glucose in streptozotocin-diabetic male albino rats at a dose of 35 mg.kg⁻¹ BW [17].

Star fruit (*Averrhoa bilimbi* L.) contains anti-diabetic substances, including flavonoids, saponins, and vitamin C [18]. Saponins function as anti-hyperglycemic by preventing glucose uptake at the brush border in the small intestine, while flavonoids are alpha-glucosidase inhibitors, which function to delay carbohydrate absorption. The ethanol extract of the fruit and leaves of starfruit can reduce blood glucose in diabetic rats [19]. The ethanol extract of leaves and fruit from *A. bilimbi* L. showed a very significant anticoagulant effect in normal and diabetic male Wistar rats by giving it for 14 consecutive days [20].

M. charantia L. has high antioxidant activity but takes longer to lower blood glucose levels, while *A. bilimbi* L. can lower blood glucose levels quickly but has low antioxidant activity. Based on this description, scientific research is needed to be related to the use of a combination of *M. charantia* L. and *A. bilimbi* L. extracts on blood sugar levels, transforming growth factor- β (TGF- β) of the spleen in mice (*Mus musculus*) hyperglycemia model.

MATERIAL AND METHOD

Preparing Combination Extract ABMC (*Averrhoa bilimbi*-*Momordica charantia*)

A. bilimbi L. and *M. charantia* L. fruit were processed to become *simplicia* at Balai Materia Medica, Batu, East Java. The *simplicia* extraction of *M. charantia* L. and *A. bilimbi* L. fruit used 96% ethanol [20]. Soaking was carried out with 1 liter of ethanol and stirred, followed by filtering after maceration for 1 x 24 hours, and replaced with

new ethanol solvent. This step was carried out 3 times. The macerated solution obtained is evaporated in a rotary evaporator for 1-2 hours until all the solvent evaporates. The extract was then further dried using freeze-dry to evaporate the remaining solvent. The dried extract is stored in a refrigerator at 4°C.

Animals

The research got approval from the ethics commission No. 1109-KEP-UB 2020. This study used 8-10 weeks old female Balb/c mice (*Mus musculus*) with an average weight of 25 grams obtained from the Animal Physiology Laboratory of the State Islamic University of Maulana Malik Ibrahim Malang.

Hyperglycemia Mice

Streptozotocin (STZ) was injected intraperitoneally at a single dose of 145 mg.kg⁻¹ BW [22]. The induction of STZ-induced diabetes was confirmed by measuring the blood glucose levels. Mice with glucose levels above 200 mg.dL⁻¹ were subjected to further treatment using ABMC.

Abmc Treatment

The diabetic mice were randomly divided into five groups consisting of five animals each. Group N is the non-diabetic (control), Group H is the diabetic mice. Group P1, P2, and P3 are diabetic mice who got ABMC 10, 40, and 160 mg.kg⁻¹ BW respectively for 14 days orally. Blood glucose levels were measured on days 1 and 15 after [16,20].

Spleenocytes Isolation

The mice were killed by cervical dislocation then placed on the operating table with a dorsal position and all four limbs fixed. Then an incision was made on the left side of the mice's abdomen. The spleen organs were taken and rinsed using PBS twice, homogenized by crushing, put in a different petri dish. Homogenate was centrifuged at 2500 rpm for 5 minutes 4°C. The pellets were resuspended in 1 mL of PBS.

Antibody Staining

A total of 100 μ L of cell suspension was added with 500 μ L of PBS, centrifuged at 2500 rpm for 5 minutes at 4°C. The pellets were added 50 μ L of anti-CD4 and anti-CD25, incubated for 30 minutes at 4°C. Then, the suspension was added Cytoperm/Cytofix kit according to the manufacturer's protocol (BDBiosciences Pharmingen) and modified by Rifa'i and Widodo [23]. After centrifugation, the pellet was

incubated with FITC-conjugated rat-antimouse CD4, PE-conjugated rat-antimouse CD25, and PE/Cy5 conjugated rat-antimouse TGF- β (from BDBiosciences Pharmingen), respectively at 4°C for 20 minutes. The cell was resuspended with 400 μ L PBS and running to *flow cytometry* (BDCellquest ProTM Software).

Data Analysis

Statistical Data was analyzed using one-way ANOVA (Analysis of Variance) with α 5% followed by Tukey Test Honestly Significant Difference (HSD) test to evaluate the significant difference among treatments.

RESULT

ABMC Decrease The Blood Glucose Level on Diabetic Mice

The non-diabetic mice had blood sugar levels of 143 ± 7.24 mg.dL⁻¹ ($p < 0.05$) on the first day and 145 ± 4.20 mg.dL⁻¹ on day 15, whereas the hyperglycemia mice had blood sugar levels of 462 ± 15.17 mg.dL⁻¹ on the first day and 412 ± 9.78 mg.dL⁻¹ on day 15. There was a decrease blood glucose level in group P1 from 439 ± 11.64 mg.dL⁻¹ on the first day and became 346 ± 6.18 mg.dL⁻¹ on day 15 (21.1 %), P2 was 436 ± 9.35 mg.dL⁻¹ on the first day and 295 ± 6.16 mg.dL⁻¹ on day 15 (32.3 %) and P3 430 ± 11.05 mg.dL⁻¹ on the first day and 345 ± 15.54 mg.dL⁻¹ on day 15 (19.7 %) (Fig. 1). On day 1, hyperglycemia control was not significant with the treatment group because the ABMC treatment needed time to lower blood glucose. On day 15, the treatment groups were significantly decreased blood glucose level faster-compared hyperglycemia control, as shown by the stars above the graph in Figure 1.

Figure 1. The decreasing fasting blood sugar levels.

Description:

N = non-diabetic

H = hyperglycemia control

P1 = hyperglycemia mice + treatment 10 mg.kg⁻¹ BW

P2 = hyperglycemia mice + treatment 40 mg.kg⁻¹ BW

P3 = hyperglycemia mice + treatment 160 mg.kg⁻¹ BW

ABMC Cannot Decrease The Number CD25⁺TGF- β ⁺ Cells

The hyperglycemia mice (H) had an average number of CD25⁺TGF- β ⁺ 0.47% $\pm$ 0.09 ($p < 0.05$), not significantly different from non-diabetics (N) and all treatment groups as shown with no stars above the graph (Fig. 2). Meanwhile, group P1 had an average relative number of CD25⁺TGF- β ⁺ 0.55% $\pm$ 0.08, which was significantly different from group P3 with an average relative number of CD25⁺TGF- β ⁺ cells 0.38% $\pm$ 0.05. So statistically ABMC cannot decrease the number of CD25⁺TGF- β ⁺ cells.

Figure 2. The number of CD4⁺TGF- β ⁺ and CD25⁺TGF- β ⁺ cells in hyperglycemia mice.

A. Dot Plot of CD4⁺TGF- β ⁺ cells number

B. Dot Plot of CD25⁺TGF- β ⁺ cells number

C. Relative Number of CD4⁺TGF- β ⁺ and CD25⁺TGF- β ⁺

Description:

N = non-diabetic

H = hyperglycemia control

P1 = hyperglycemia mice + treatment 10 mg.kg⁻¹ BW

P2 = hyperglycemia mice + treatment 40 mg.kg⁻¹ BW

P3 = hyperglycemia mice + treatment 160 mg.kg⁻¹ BW

ABMC Decrease The Number CD4⁺TGF-β⁺ Cells

The hyperglycemia control (H) had an average relative number of CD4⁺TGF-β⁺ cells 0.71%±0.06 (p <0.05) but not significantly different from the non-diabetic (N) and treatment group 2 (P2), which has an average relative number of CD4⁺TGF-β⁺ 0.58%±0.08. Treatment group 1 (P1) had the highest average number of CD4⁺TGF-β⁺ cells 0.92%±0.05. Treatment group 3 (P3) had the lowest average number of CD4⁺TGF-β⁺ 0.49%±0.07 and significantly different from the hyperglycemia control (H), as shown by the stars above the graph (Fig. 2).

DISCUSSION

Streptozotocin (STZ) causes toxicity to pancreatic-β cells by damaging DNA through the mechanism of Poly-ADP ribosylation polymerase (PARP) activation, decreasing cellular NAD⁺ and ATP. Diabetogenic effects of streptozotocin are also initiated by reactive oxygen species (ROS) via a direct toxic effect on GLUT 2 [20]. High levels of ROS will inhibit T cell proliferation, which leads to apoptosis and interferes with the process of T cell differentiation and regulation of its function. Disruption in the process of differentiation and proliferation of T cells will cause a decrease in the number of regulatory T cells, which are a subset of cluster differentiated T cells (CD) 4⁺ that maintain peripheral tolerance and suppress adaptive immune responses by secreting anti-inflammatory cytokines, such as Transforming growth factor-β (TGF-β). CD4⁺CD25⁺ Treg cells can inhibit the inflammatory response through various pathways, such as increasing the secretion of anti-inflammatory cytokines, modulating the microenvironment, and altering cell receptor expression [20,21,24].

TGF-β signaling is one of the signaling pathways that affect β-cell differentiation and function. The disruption of TGF-β signaling has the potential center of β-cell dedifferentiation. Intact TGF-β signaling can modulate β-cell response to increase glucose levels. TGF-β can also maintain Foxp3 expression in CD4⁺CD25⁺ Treg to enhance immunosuppressive function [25].

In Figure 2, Treatment ABMC extract can decrease the number of CD4⁺TGF-β⁺ cells on hyperglycemia mice. TGF-β is a multifunctional cytokine that plays various roles in cellular differentiation and immune regulation [25]. TGF-β regulates the inflammatory response through activation control or chemotaxis, and the survival of various immune cells such as lymphocytes,

natural killer cells, dendritic cells, macrophages, mast cells, and granulocytes [26,27].

The *momordicine* 1 found in *M. charantia* L. fruit can significantly improve glucose-induced ROS by activating the Nrf2/HO-1 pathway. Momordicine 1 can inhibit fibrogenesis through modulation of Nrf2-mediated TGF-β1-Smad2/3 signal transduction [28]. While the *charantin* compound from *M. charantia* L. fruit can stimulate the pancreas-β cells to produce insulin, increase glycogen sugar reserve deposits in the liver. In addition, there are polypeptide-P insulin compounds that directly reduce blood glucose levels [29].

Starfruit contains flavonoids, triterpenoids, and quercetin. Flavonoids are active antihyperglycemic compounds by stimulating insulin secretion [28,29]. Also, the flavonoid is alpha-glucosidase inhibitors which function to delay carbohydrate absorption [30]. Meanwhile, quercetin can improve kidney function in diabetic nephropathy rats by inhibiting the overexpression of TGF-β1 and CTGF [31].

CONCLUSION

ABMC combination able reduce 19.7%-32.3% blood glucose levels on hyperglycemic mice. ABMC dose of 40 and 160 mg.kg⁻¹ BW can decrease (18.3-30.9%) the number of CD4⁺TGF-β⁺ cells.

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