

# The Effects of Oral Plain Kefir Supplementation on Proinflammatory Cytokine Properties of the Hyperglycemia Wistar Rats Induced by Streptozotocin

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## ABSTRAK

**Tujuan:** untuk memvalidasi pengaruh Kefir Bening terhadap respons imun pada tikus Wistar hiperglikemia yang diinduksi dengan Streptozotocin. **Metode:** penelitian dengan desain acak untuk kelompok kontrol pra uji dan pasca uji (pretest - posttest control group) dilakukan pada tikus Wistar jantan hiperglikemia yang diinduksi streptozotocin (STZ). Tikus-tikus diacak menjadi empat kelompok: (1) Kelompok yang diinduksi STZ dan diberi perlakuan insulin 0,76 UI/200 g BB, (2) Kelompok yang diinduksi STZ dan diberi kefir bening 3,6 cc/200 g BB/hari selama 30 hari, (3) kelompok yang diinduksi STZ sebagai kelompok kontrol, (4) kelompok hewan normal sebagai kontrol negatif. Kadar gula darah diukur berdasarkan pemeriksaan darah lengkap yang diperoleh dari vena retroorbitalis sebanyak 0,1 mL dengan menggunakan mikrohematokrit pada hari ke-1 (pretest) dan hari ke-30 (post test) melalui metode enzimatik. Respons imun (sitokin IL1, IL6, IL10, TNF $\alpha$ ) diukur dengan teknik ELISA. Data dianalisis dengan One Way ANOVA, uji Mann Whitney dan Duncan dengan nilai  $p < 0.05$  dianggap bermakna. **Hasil:** suplementasi kefir bening 3,6 cc/hari memengaruhi kadar gula darah, sitokin proinflamasi (IL1, IL6, TNF $\alpha$ ) dan sitokin antiinflamasi (IL10). Analisis statistik menunjukkan penurunan glukosa  $-111,00 \pm 44,23$  ml ( $p < 0,001$ ) dan penurunan sitokin proinflamasi sitokin IL1 sekitar  $-18,62 \pm 23,59$  dan IL6  $-3,21 \pm 7,57$  mU/mL ( $p < 0,001$ ) dibandingkan dengan kelompok kontrol. Kadar TNF $\alpha$  menurun  $1,65 \pm 4,62$  mU/mL, tetapi tidak bermakna ( $p > 0,05$ ), kecuali pada kelompok kontrol. Selain itu, sitokin antiinflamasi (IL10) juga meningkat sekitar  $15,11 \pm 2,16$  ( $p < 0,05$ ), kecuali pada kelompok kontrol. **Kesimpulan:** suplementasi kefir bening secara bermakna menurunkan kadar glukosa darah, kadar sitokin (IL1, IL6) dan menurunkan kadar TNF $\alpha$ . Sedangkan kadar IL10 meningkat dibandingkan kelompok kontrol.

**Kata kunci:** probiotik, kefir bening, diabetes melitus, hiperglikemia, radikal bebas, sitokin proinflamasi.

## ABSTRACT

**Aim:** to validate the effect of plain kefir on immune responses of hyperglycemia wistar rats induced by Streptozotocin. **Methods:** the randomized pretest - posttest control group study design was conducted in male hyperglycemia Wistar rats induced by streptozotocin (STZ). Rats were randomized into four groups: (1) STZ-induced group were given insulin treatment 0.76 UI/200 g bw, (2) STZ-induced group and treated with plain kefir 3.6 cc/200 g bw/day for 30 days, (3) STZ-induced group as control, (4) normal animal group as a negative control. Blood glucose was measured from whole blood that was taken 0.1 ml from retroorbitalis vein by microhematocrit on day 1 (pretest) and day 30 (post test) by enzymatic methods. Immune responses (cytokines IL1, IL6, IL10, TNF $\alpha$ ) were measured by ELISA. Data were analyzed by one way Anova, Mann Whitney test and Duncan with significant level of  $p < 0.05$ . **Results:** plain kefir supplementation 3.6 cc/day affect blood glucose,

proinflammatory cytokines (IL1, IL6, TNF $\alpha$ ) and antiinflammatory cytokine (IL10). Statistical analysis showed decrease of glucose  $-111.00 \pm 44.23$  ml ( $p < 0.001$ ) and proinflammatory cytokines IL1 about  $-18.62 \pm 23.59$  and IL6  $-3.21 \pm 7.57$  mU/mL ( $p < 0.001$ ), respectively compared to the control groups. TNF $\alpha$  decreased  $1.65 \pm 4.62$  mU/mL, but not significant ( $p > 0.05$ ), except for controls group. In addition, antiinflammatory (IL10) showed also increase about  $15.11 \pm 2.16$  ( $p < 0.05$ ), except for the control. **Conclusion:** plain kefir supplementation significantly decreased blood glucose, level of cytokines (IL1, IL6) and lowered TNF $\alpha$  level. On the contrary, the level of IL10 is increased compare to control groups.

**Key words:** probiotic, plain kefir, diabetes mellitus, hyperglycemia, free radicals, proinflammatory cytokines.

## INTRODUCTION

Diabetes mellitus (DM) is a serious global health problem. World Health Organisation predicted an increased number of patients from 8.4 million in 2000 to 21.3 million in 2030 in Indonesia. The prevalence of the type 2 diabetes increases yearly in this country. Consequently, it will result in a lower quality of human resources in the long term.<sup>1</sup>

Excessive free-radicals in diabetes is due to hyperglycemia, increasing through various mechanisms, such as: (1) non-enzymatic glycation (AGEs), (2) glucose auto-oxidation, and (3) polyol pathway.<sup>2-6</sup> Those mechanisms enhance the formation of free radicals and cause oxidative stress. Oxidative stress will induce lipid peroxidation, proinflammatory cytokine production and decreases immune response, as well as pancreatic  $\beta$  cell damage. Hyperglycemia leads to damage the function and structure of pancreatic  $\beta$  cells.<sup>7</sup>

Chronic hyperglycemia modulates immune responses and triggers inflammation through activation of Toll Like Receptors (TLRs), leading to an increase of proinflammatory cytokine secretion; interleukin 1 (IL1), interleukin 6 (IL6), tumor necrosis factor alpha (TNF $\alpha$ ), interferon  $\gamma$  (IFN $\gamma$ ), whereas interleukin 10 (IL 10) is decreased.<sup>8</sup> Proinflammatory cytokines inhibit insulin signal by activating receptor kinase inhibitor, stimulate NF $\kappa$ B to finally induce pancreatic  $\beta$  cell damage and apoptosis.<sup>9-11</sup> TNF $\alpha$  reduces the glucose transporter (GLUT1 and GLUT4) in cells and decreases the glucose uptake.

Diabetes therapy has been demonstrated to be unable to achieve maximal glucose control by current therapy approaches.<sup>12</sup> The use of insulin and other medications are the most effective options, but it is difficult to implement on an ongoing basis for several reasons. Attempts on

the induction of pancreatic  $\beta$  cell regeneration by using probiotics are reported elsewhere, but the results have been conflicting in relation to the application of therapy. Further studies are therefore important to ensure the consistency of results.<sup>13</sup>

Plain kefir supplementation has been identified to potentially reduce hyperglycemia. The underlying mechanism is probably via its bioactive components such as: exopolysaccharide, peptide, antioxidants and through immunomodulatory properties.<sup>14-17</sup> The novelty in our findings is that kefir was able to activate regulatory T cells (Tregs) whose functions are to maintain homeostasis of Th1-Th2 responses. Through their cytokine products, these cells are able to suppress the inflammatory reactions, and increase production of interleukin 10 (IL10) in pancreatic  $\beta$  cells. IL10 suppress proinflammatory response and apoptosis.<sup>18</sup>

The aim of this investigation was to validate the effect of plain kefir on glycemic status and immune response of hyperglycemia Wistar rats induced by streptozotocin. This study used animal models due to the ease of handling, and intended to explore deeply in relation to the mechanisms of action of Kefir, before applying it in human being.

## METHODS

This study was a randomized control group pretest-post test design<sup>19</sup> on 84 male hyperglycemia Wistar rats aged 2.5 to 3 months, weighing 150-250 g. The animals intraperitoneally injected with 40 mg/kg bw streptozotocin (STZ) (Sigma, USA) dissolved in 0.1 M buffer citrate pH 4.5.<sup>20</sup> The rats were randomized into four groups, namely: (1) STZ-induced group which received insulin treatment 0.76 UI/200 mg bw/day for 30 days, (2) STZ-induced group and were given oral plain

kefir 3.6 cc/200 g bw/day for 30 days, (3) STZ-induced animal group as a positive control, (4) normal animal group (Non STZ-induced) as a negative control. Kefir was made from 24 hour fermented skim milk by kefir grains commercial inoculum obtained from the House of Kefir Bening Semarang. Animals were fed by AIN 93 standard diet.<sup>21</sup> Blood glucose was measured with a Super Glucocard II from whole blood that was taken 0,1 ml from retroorbitalis vein by microhematocrit on day 1 (pretest) and day 30 (post test) by enzymatic methods. Immune response (cytokines IL1, IL6, IL10, TNF $\alpha$ ) were measured from blood serum on day 1 (pretest) and day 30 (post test) by ELISA.<sup>22</sup> Statistical analysis were performed with univariate (mean, SD), bivariate (Kruskal Wallis, ANOVA/Post Hoc Duncan's Multiple Range Test) with significance level of 0.05. Rats were obtained from the Integrated Research and Development Institute of Unit IV (LPPT ) University of Gajah Mada Yogyakarta. This study was approved by The Research Ethics Committee for Health Research, Faculty of Medicine, Diponegoro University/Dr Kariadi General Hospital, Semarang.

## RESULTS

**Table 1** showed that the delta of animal weight gain varied among the groups, except for the positive groups, with very small gains of  $4.01 \pm 16.82$  g. Other groups gained more than  $13.80 \pm 16.10$  g. Statistical analysis found no difference among groups of animals ( $p > 0.05$ ).

The delta of blood glucose showed, respectively reduction in the insulin group of  $-162.29 \pm 76.75$  mg/dL and in plain kefir group of  $-111.00 \pm 44.23$  mg/dL. On the contrary the control group showed an increase. Insulin administration

was associated with better reduction of blood glucose. In addition, plain kefir is also potential to reduce blood glucose closely with insulin treatment.

The immune responses showed that there were respectively decreases of IL1 level in the insulin group about  $-20.93 \pm 34.59$  pg/mL, and in the plain kefir group about  $-18.62 \pm 23.59$  pg/mL, while it tends to increase in the positive and negative control group ( $p < 0.001$ ). IL6 level respectively decreased in the insulin group about  $-2.69 \pm 6.20$  pg/mL, and in the plain kefir group about  $-3.21 \pm 7.57$  pg/mL ( $p < 0.001$ ), while in control group, the IL6 was increased. Moreover, IL10 level increased only in the plain kefir group about  $15.11 \pm 2.16$  pg/mL and the delta test showed significantly only in the kefir group ( $p < 0.05$ ). TNF $\alpha$  level was not significantly decrease in the insulin group ( $0.06 \pm 4.98$  pg/mL) and in kefir group ( $1.65 \pm 4.62$  pg/mL), but control group tends to increase among groups. Taking note kefir was capable to control the TNF $\alpha$  level compare to control group in a descriptive manner.

## DISCUSSION

This study has proven the hypotheses and built a new theory for research, that the supplementation of plain kefir of 3.6 cc/200 g. bw/ day for 30 days, significantly affected blood glucose, immune response (cytokines IL1, IL6, TNF $\alpha$ ) and antiinflammatory cytokine (IL10). The study suggest that the mechanism of action of plain kefir in initially lowering blood glucose and pro inflammatory cytokine, also decrease subsequent effect of free radicals, and lipid peroxidation. Reduction of peroxide molecules affects positively the secretion of

**Table 1.** Changes in the value of various variables between groups of experimental animals

Variables	Delta Experimental Animal Groups				P
	Insulin $\bar{x} \pm SD$	Plain Kefir $\bar{x} \pm SD$	Positive Control $\bar{x} \pm SD$	Negative Control $\bar{x} \pm SD$	
Body Weight (gr)	$13.80 \pm 16.10$	$13.81 \pm 21,29$	$4.01 \pm 16.82$	$16.21 \pm 6.13$	0.492
Blood Glucose (mg/dL)	$-162.29 \pm 76.74$	$-111.00 \pm 44.23$	$41.28 \pm 53.19$	$41,285 \pm 53.119$	0.000a*
IL1 (mU/mL)	$-20.93 \pm 34.59$	$-18.62 \pm 23.59$	$25.00 \pm 30.95$	$-9.74 \pm 22.77$	0.001a*
IL6 (mU/mL)	$-2.69 \pm 6.20$	$-3.21 \pm 7.57$	$3.61 \pm 3.37$	$2.39 \pm 1.40$	0.001b*
IL10 (mU/mL)	$-0.15 \pm 9.24$	$15.11 \pm 2.16$	$-0.73 \pm 5.69$	$-2.36 \pm 2.84$	0.038b**
TNF $\alpha$ (mU/mL)	$0.06 \pm 4.98$	$1.65 \pm 4.62$	$3.49 \pm 2.78$	$2.62 \pm 6.34$	0.323

a) Oneway Anova; b) Kruskal Wallis; \* significance  $p < 0.05$ ; \*\* significance  $p < 0.001$

pro-inflammation cytokines (IL1, IL6, IL10), so the cell structure damage and function of pancreatic  $\beta$ -inevitable. Kefir prevents against glucotoxicity and lipotoxicity and reduces the occurrence of hyperglycaemia, associated with synergism bioactives compound found in kefir themselves.

In this study, plain kefir has been proven to reduce hyperglycemia and proinflammatory cytokines IL1 and TNF $\alpha$ . The mechanism relying on bioactive exopolysaccharide (EPS) from kefir activating the hormone glucagon-like peptide 1 (GLP 1), gastric inhibitory peptide (GIP) and the enzyme adenylate cyclase through the cyclic adenosine monophosphat (cAMP), sensitization of Ca<sub>2</sub> ions and activation of protein kinase A. Thus, affects to increase insulin release from the pancreatic  $\beta$  cells, so the glucose can be utilized by the body tissues and cells. Exopolysaccharide (EPS) is a biopolymer lowering blood glucose, whose mechanism is through intestinal microvilli coating process, that will inhibits glucose uptake and glucose will not increase in the body. Maeda's (2004) *in vitro* studies supported this study that kefir lowers blood glucose.<sup>23</sup> Another mechanism is that EPS modulates insulin signaling through c-AMP. Increased c-AMP in pancreatic cells, contributes to increase insulin secretion from pancreatic  $\beta$  cells.

Probiotic bacteria and gut mucosa act synergistically in of immunomodulation. At the level of intestinal epithelium, probiotic bacteria provide beneficial effects through colonization and the release of bioactive mixture. Then it reinforces barrier function through modulation of intestinal epithelial cells including the release of cytokines and chemokines. State of good intestinal immune system will affect the whole body immune system.

Plain kefir probiotics are able to induce both the innate and adaptive immune responses, due to their specific molecules on the cell wall, known as pathogen-associated molecular patterns (PAMPs), through immunomodulatory mechanisms. PAMPs are recognized by specific receptor-specific pattern recognition receptors (PRRs). One of PAMPs on probiotics is lipoteichoic acid (LTA). LTA is biologically active molecule that are characteristic of gram-positive bacteria and are the same as lipopolysaccharide (LPS).

In addition, LTA from kefir has the role of TLR signals. Toll-like receptors (TLRs) play a

role in bridging the innate immunity system to the adaptive system. TLRs are PRRs (pattern recognition receptors) that function as signal transducers, which help the cells to recognize pathogens and initiate the signal cascade. TLRs also helps to bridge the innate immunity system to the adaptive system by inducing various effector molecules and co-stimulators. Effectors downstream of TLR2 and TLR4 is MyD88 adapter protein that interacts with transmembrane receptors through the C-terminal TIR domain. MyD88 recruits Ser/Thr kinase IRAK (IL-1R associated kinase) to form a receptor complex. IRAK associated with the adapter molecule TNF receptor associated factor (TRAF6). TRAF6, MAP3K family member then activates NIK (NF- $\kappa$ B-inducing kinase), which would activate NF- $\kappa$ B inhibitor of kinases (IKKs). Degradation of NF- $\kappa$ B inhibitor I- $\kappa$ B NF- $\kappa$ B release an immediate translocation to the nucleus to induce gene expression.<sup>24</sup>

Kefir's peptide improves biological value and digestibility of protein, so it strengthens the immune system through normalization of the pro-inflammation cytokine. It is also supported by Meier and Almatsier, i.e. that the best efforts for  $\beta$  cell regeneration is through a systematic effort from inside the body, the provision of high quality nutrition intake, especially high biological value protein is very important, because protein will maintain and regenerate body cells.<sup>25,26</sup>

## CONCLUSION

Supplementation of the plain kefir 3.6 cc/200 g BW/day for 30 days significantly reduced blood glucose, proinflammatory cytokines (IL1, IL6) and slightly lowered TNF $\alpha$ . In contrast IL10 showed also increase compare to control groups.

Futher studies are necessary to explore the role of plain kefir on free radicals (glutathione peroxidase, enzym superoxide dismutase, catalase) and lipid peroxidation before applying clinically to diabetic patients.

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