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BIOMOLECULAR ASPECTS OF PLAIN KEFIR ANTIDIABETIC POTENTIALS

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Abstract: *Purpose and methodology:* This study investigated the effect of plain kefir on glycemic, antioxidants, immune response and pancreatic β cell regeneration of hyperglycemia Wistar Rats induced by Streptozotocin.

Findings: Kefir supplementation 3.6 cc / day affect significantly on blood glucose, antioxidants, lipid peroxidation, and pancreatic β-cells. Statistical analysis showed reduce of glucose ($p<0.001$), MDA ($p<0.001$), level of proinflamatory cytokines (IL_1, IL_6,) ($p<0.001$), except of controls. Antioxidant showed increase of catalase, GPx ($p<0.001$) and SOD ($p <0.05$). Similarly, there was increased of IL_{10} ($p<0.05$) and the normal cells pancreatic ($p <0.001$), except of control. TNFα reduced no significant ($p> 0.05$), except of control. Ancova test showed MDA and IL_{10} were the most contributed to the pancreatic β cells regeneration by 91.0% and 9% determined by TNF-α, antioxidants, blood glucose, body weight.

Value: Kefir is significantly reduced of glucose, lipid peroxide, level of cytokines (IL1, IL6) and enhanced IL10, antioxidants capacity and normal pancreatic β cell expression. Insulin and kefir descriptively reduced TNF α level.

Keywords: *Probiotic; Plain Kefir; Hyperglicemia;* β *Cells Regeneration; Proinflamatory Cytokines*

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INTRODUCTION

Diabetes mellitus type 2 (DMT2) is a metabolic disorder characterized by syndrome hyperglycemia. This disease is pandemic and WHO **predicts of people from 8.4 million in 2000 to 21.3 million in 2030** in Indonesia. The prevalence of type 2 diabetes increased yearly in this country. Consequently, it will be affecting on of diabetes complications, morbidity and mortality and a lower quality of human resources in the long term. *(Hadisaputro, S, et al. 2009)*

Excessive free-radicals in diabetes is due to hyperglycemia. It increases through various channels, such as: (1) non-enzymatic glycation (AGEs), (2) glucose auto-oxidation, and (3) polyol pathway and (4) protein kinase activation (PKC). **(Betteridge, 2000 Ceriello, 2000 Pfaffly, 2001 Djokomoeljanto, 2007 Soeyono, 2007).** Those mechanisms enhance the formation of free radicals and cause oxidative stress. Oxidative **stress damage** to proteins, enzymes, membrane lipids and DNA, reduce in antioxidants and immune response, increase lipid peroxidation and proinflammatory cytokines as well as pancreatic β cells damage. *(Moussa, 2008)* Brownlee (2000) **found that** hyperglycemia leads to damage the function and structure of pancreatic β cells. *(Brownlee, 2000)*

The impairment of immune response due to the chronic hyperglycemia triggers inflammation through activation of *Toll Like Recepor* (TLRs) 2 and 4, leading to an increase of proinflammatory cytokines secreation, namely; interleukin-1 (IL1), interleukin-6 (IL_6), tumor necrosis factor alpha (TNFα), interferon γ (IFNγ), whereas interleukin-10 (IL_{10}) is decreased. **(Mahardhika, et al. 2004 Rytter, et al. 2009)** TNFα reduces the glucose transport (GLUT) 1 and 4 in cell and effect decreases the glucose uptake. Proinflammatory cytokines inhibit insulin signal by activating receptor kinase inhibitor, NFkB to finnaly induces pancreatic β cell damage to pancreatic β cells apoptosis. *(Lee and Simin Liu, 2008)*

Diabetes therapy has been demontrated to unabale in achieving maximally blood glucose controlled by the availability of current therapy approaches, such as: (1) changes in behavior therapy, (2) diet, (3) exercise, (4) oral hypoglycemic medication and insulin. (*PERKENI,2007.a*) The use of insulin and medication are the most effective options, but it is difficult to implement on an ongoing basis, as they relate to socioeconomic, level of knowledge and understanding of residual effects after taking the medicine. Attempts on the induction of pancreatic β cell regeneration *(Meier, 2008)* by using probiotics are not 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. *(Hadisaputro, et al. 2009)*

Plain **kefir supplementation identified** potentially to reduce hyperglycemia. The mechanism underlying is probably via its bioactive components such as; exopolyssacharide, peptide, antioxidant and immunomodulatory properties. **(Brown,2004 Sybesma,2004 Khazrai,2004 Virtanen,2004)** Exsopolyssacharide (EPS) activates the hormone glucagon like peptide 1 (GLP 1), gastric inhibitory peptide (GIP) *(Khan, 2001)* and the enzyme adenylate cyclase through the cyclic adenosine monoposfat (cAMP), sensitization of Ca^2 ions and activation of protein kinase A, thus it released insulin from the pancreatic β cells. Consequently, the blood glucose can be utilized by the body tissues and cells. Research EPS from non-kefir sources in vivo, showed hypoglycemic via several mechanisms such as; stimulating the immune system, regulation of glucose and insulin signaling. *(Maeda, H. 2004 Inggrid-Surono, 2007)* Moreover, antioxidants capacity will diminish of lipid peroxidation process by *malondealdehid* (MDA) reduction and suppresses the level of proinflammatory cytokines ($IL_{1,}$ IL_6 and TNFα), so it expects the enhancement in pancreatic β cells through cell regeneration and improving pancreatic β cell organ.

Kefir is also be able to activate *regulator T cells (Treg)* whose functions are maintain homeostasis of Th1-Th2, with mechanisms suppress to inflammation cytokines and increase production of interleukin-10 (IL_{10}) in pancreatic β cells. *(Susetia-Totoprajogo, 2010)* IL_{10} suppress proinflammatory response and apoptosis. *(Dronavalli, et all. 2008)*

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 recognized by specific receptor- pattern recognition receptors (PRRs). One of PAMPs on probiotics is lipoteichoic acid (LTA). LTA is biologically active molecules that are characteristic of gram-positive bacteria and the same with lipopolysacharide (LPS). *(Hughes, et al. 2004)* Previous research suggests the reduction of inflammatory therapy on β cells in pancreas contributed the synthesis of proinsulin to insulin by increased cell mass and insulin sensitivity. *(Donath, et al. 2009)* Synergy of kefir's bioactive such peptides and immunomodulatory stimulate to the cell regeneration and restoration of cell mass physiology of pancreatic β cell. Regeneration of cell effect of restoration of pancreatic β cell mass, leading to restoration of physiology and insulin secretion. The prevention of hyperglycemia in order to reduce the occurrence of lipotoxicity and glucotoxicity. Kefir bening is a low-fat milk fermented by kefir grains. Kefir grains are symbiotic bacteria and yeast colonies. This fermentor containing is more than 35 beneficial to health probiotic bacterias. It is also a simple production technology and easy to implement in the household.

Based on the description as mentioned above, the formulation of the problem defined as follows: "Is there a difference in improvement of glycemic status, antioxidant, immune response and cell regeneration of pancreatic β-induced hyperglycemia rats streptozotocin (STZ) between groups of plain kefir with insulin treatment and group control?."

MATERIALS AND METHODS

The randomized pretest - posttest control group study design was conducted in male hyperglycemia Wistar rats. (*Campbell, et al. 1963 Gross Portney, et al. 1993*) The study was conducted in two phase: (1) The first phase was measured the parameters of glycemic status, antioxidants, lipid peroxidation and histochemistry of pancreatic β cells, and (2) the second phase was measured the parameters of the immune response. The animals were intra peritoneally injected by 40 mg/kg bw streptozotocin (STZ) dissolved in 0,1 M buffer citrate pH 4,5. Rats were randomized into four groups, namely: (1) STZ-induced animals group and recieved insulin treatment 0.76 UI/200 mg bw, (2) STZ-induced animals group and were given orally plain kefir 3.6 cc/200 g bw/day for 30 days, (3) STZ-induced animals group as a positive control *(ad libitum)*, (4) normal animals group as a negative control *(ad libitum).*

Kefir was made from the 24 hours fermented skim milk by kefir grains commercial inoculum that obtained from the House of Kefir Bening Semarang. Animals were feed by AIN 93 standard diet. *(Reeves, Philip G. 1997)* Blood glucose were measured with a *Super Glucocard II* by enzymatic methods. Lipid peroxide was measured by substance Tiobartituric Acid (TBARs) method. Antioxidants status (SOD, GPX) were measured by ELISA. Catalase was measured by Spectrofotometry. Lipid peroxide was measured MDA-TBAR by Spectrofometry. Immune response were measured to cytokines IL_1, IL_6, TNFα, IL_{10}) by ELISA. Pancreatic histology was measured by immunohistochemistry. Characterization kefir probiotic microorganisms were measured by Total Plate Count (TPC). Statistical analysis of univariate data presented (mean, SD), bivariate (Wilcoxon, Pairs t-test), bivariate (Kruskal Wallis, ANOVA / Post Hoc Duncan's Multiple Range Test, multivariate Anacova with significance level 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, Semarang and Dr. Kariadi General Hospital.

RESULTS

Table 1 showed that the delta animal weight varried among the groups, except for the positive groups they gained with very small achievement bout 4,01 ± 16,82 g. Other groups were gained more than 13,80 ± 16,10 g. Statistical analysis were respectively found no difference among groups of animals (p> 0.05). The delta of blood glucose showed that there

Variabels	Delta Experimental Animal Groups				*P*
	Insulin χ ± SD	Plain Kefir χ ± SD	Positive Control χ ± SD	Negative Control χ ± SD	
Body Weight (gr)	13,80 ± 16,10	13,81 ± 21,29	4,01 ± 16,82	16,21 ± 6,13	0,492
Blood Glucose (mg/dL)	-162,29 ± 76,74	-111,00 ± 44,23	41,28 ± 53,19	41,285 ± 53,119	0,000a*
MDA (nmol/mL)	-2,86 ± 0,35	-1,83 ± 0,07	0,12 ± 0,20	0,012 ± 0,020	0,000b*
SOD (mU/mL)	8,86 ± 1,22	13,86 ± 1,28	-4,89 ± 6,87	4,08 ± 4,38	0,000a*
GPx (mU/mL)	-22,45 ± 7,89	-18,08 ± 7,26	-11,25 ± 5,96	1,43 ± 1,45	0,000a**
Catalase (mU/mL)	3,09 ± 0,33	3,32 ± 0,12	-0,42 ± 0,29	3,15 ± 0,34	0,000a*
IL1 (mU/mL)	-20,93 ± 34,59	-18,62 ± 23,59	25,00 ± 30,95	-9,74 ± 22,77	0,001a*
IL2 (mU/mL)	-2,69 ± 6,20	-3,21 ± 7,57	3,61 ± 3,37	2,39 ± 1,40	0,001b*
IL10 (mU/mL)	-0,15 ± 9,24	15,11 ± 2,16	-0,73 ± 5,69	-2,36 ± 2,84	0,038b**
TNF α (mU/mL)	0,06 ± 4,98	1,65 ± 4,62	3,49 ± 2,78	2,62 ± 6,34	0,323
Regenerasi sel β	14,94 ± 0,63	15,11 ± 4,17	-0,14 ± 0,64	-0,433 ± 0,76	0,000a*

a). Oneway Anova ** significance p<0,001* *** significance p<0,05*
b). Kruskal Wallis

Table 1: Summary Data of Changes in the Value of Various Variables Between Groups of Experimental Animals

were repectively reduced in the insulin group about -162,29 ± 76,75 mg/dL and plain kefir group about -111,00 ± 44.23 mg/dL, contrary the control group showed to increase. Insulin was the better reduction of blood glucose compare to kefir, but kefir has challenged to be blood glucose reduction.

Antioxidant status showed an increase of SOD the insulin treatment group about 8,86 ± 1,22 mU/mL, plain kefir group about 13,86 ± 1,28 mU/mL. GPx increased insulin treatment group about -16,94 ± 6,66, and plain kefir about -11,8952±8,1907 mU/mL. Catalase showed an increase the insulin treatment group about 3,10 ± 0,34 mU/mL, plain kefir group about 3,33 ± 0.13 mU/mL. Delta test () was significantly difference increase SOD ($p<0,05$), Catalase and GPx ($p <0.001$).

The delta immune response showed that there were decreased in the insulin group for IL1 level about -20.93 + 34,59 pg/mL and for IL6 level about -2.70 + 6.20 pg/mL, the plain kefir group for IL1 level about -18.62 + 23.59 pg/mL and for IL6 level also decreased about -3.21 + 7.57 pg/mL, while it was increased in the positive and negative control group. Delta test were significantly difference decrease in the insulin and kefir group ($p <0.001$). Moreover, IL10 level increased only in the plain kefir group about 15.11 + 2.16 pg/mL and the delta test was showed significantly only in the kefir group ($p<0.05$). TNFα level descriptively no significant decreased in the insulin group about 0.06 + 4.98 pg/mL and kefir with about 1,65 + 4,62 pg/mL, but control groups tend to increase among groups. Taking note kefir was respectively capable to control the TNF α level compare to control groups in descriptive manner. Finally, histology tests were detected to increase in pancreatic β cells among treatment groups, except the control positive group. Delta test obtained significantly difference to increase of the normal pancreatic β cells ($p <0.001$). This study has been able correctly to prove the hypothesis.

Based on testing with modeling equation Ancova cell regeneration of pancreatic β = 0.733 - 5.545 (MDA) + 0.044 (IL10), indicating that lipid peroxidation (MDA) and IL10 contributed to changes in the regeneration of pancreatic β cells. Figure 1. Histologically pancreatic β cells expression.

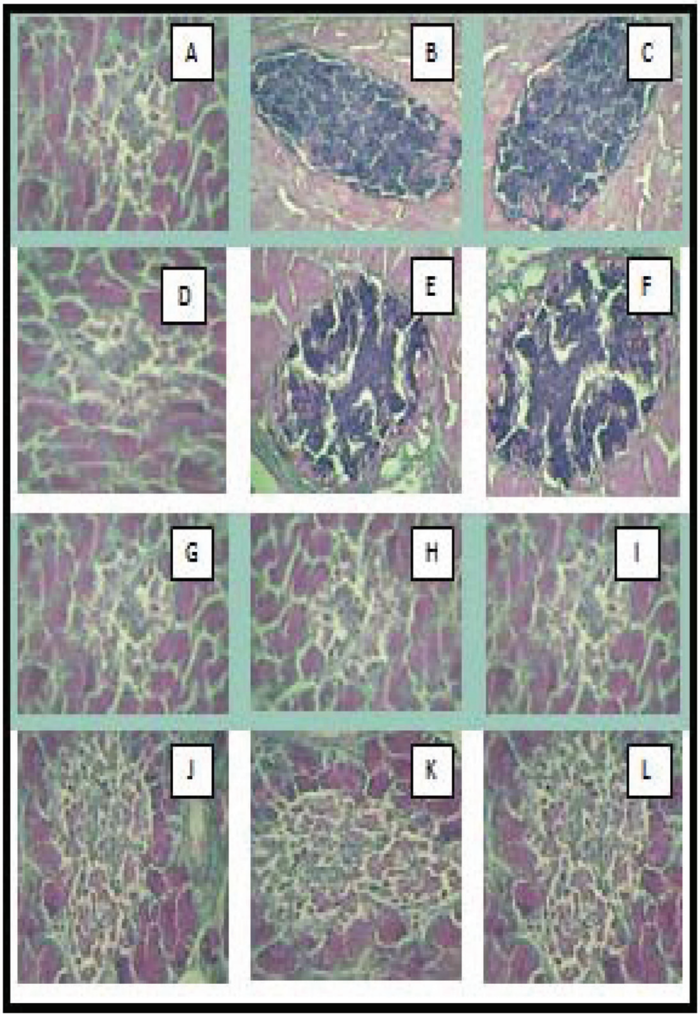

Figure 1: Histologically Normal Cells Increased Expression of Pancreatic β Cells. Description: Histology of Pancreatic β Cells Early Insulin Treatment Group (A), the Observation Day 15 (B) and Observation Day 30 (C). Kefir Early Treatment Group (D), Observation Day 15 (E) and Observation Day 30 (F). Initial Positive Control Group (G), the Observation Day 15 (H) and Observation Day 30 (I). Initial Negative Control gGroup (A), the Observation Day 15 (K) and Observation Day 30 (L).

DISCUSSION

This study has demonstrated the truth of hypotheses and build a new theory research, that the supplementation of plain kefir 3,6 cc/200 g BB / day for 30 days, significantly affect on blood glucose, antioxidants (SOD, Catalase, GPx), peroxidation lipids (MDA), immune response (cytokines IL_1, IL_6, IL_{10}) and pancreatic β-cell function. The research revealed that the mechanism of plain kefir played initially lowering blood glucose and pro inflamatory cytokine, also **reduced subsequent** effect of free radicals, lipid peroxidation. Reduction of peroxide molecules effects positively secretion pro-inflammation cytokines (IL_1, IL_6, IL_{10}), so the cell structure damage and function of pancreatic β inevitable. Kefir prevents against glucotoxiticity and lipotoxiticity and reduces the occurrence of hyperglycaemia. This ability is associated with bioactive found in kefir themselves.

In this study, plain kefir have been proven to reduce hyperglycemia and lipid peroxidation, and increased IL10. Regeneration will be decreased by 5.545 when cells are exposed to lipid peroxidation (MDA) after the controlled by variable IL10. The R^2 (R square) of 0.910 means that the MDA and IL10 variables can explain the occurrence of regeneration of pancreatic β cells by 91.0%, while the rest is determined by other factors, such as: TNF-α exposure, antioxidant status, blood glucose, body weight of animals.

Exopolysaccharide (EPS) is a biopolymer lowering blood glucose, the mechanism is through intestinal microvilli coating process, so that inhibit glucose uptake and glucose does not increase in the body.*(Maeda, 2004)* Studies *in vitro* supported this rsearch that kefir lowers of blood glucose. Another mechanism EPS activates glucagon like peptide 1 (GLP 1), gastric inhibitory peptide (GIP) *(Khan, 2001)* and the enzyme adenylate cyclase through the cyclic adenosine monoposfat (cAMP) through sensitization of Ca2 ions and activation of protein kinase A, thus increasing insulin release from cells pancreatic β, the occurrence of blood glucose homeostasis and suppression glucotoxicity. *(Pickup and Gareth, 1998, Djokomoeljanto, 1999)*

Kefir's peptide improves biological value and digestibility of protein, so it affects the restoration of the pancreatic β cell mass, restoration of its physiology and insulin secretion; besides it strengthen the immune system through normallized the pro-inflammation cytokine. It also supported by Meier and Almatsier that the best efforts β cell regeneration is through a systematic effort from inside his own body, the provision of high quality nutrition intake, especially high biological value protein is very importand, because protein will maintain and regenerated the body cells. *(Meier,2008, Almatsier, 2001)*

Kefir's antioxidants inhibit oxidation, reducing hydroxyl radical, superoxide and lipid peroxidation. The antioxidant activity occurs in a way delivers on its hydrogen atoms NADP, which will further reduce the existing free radicals. Antioxidant effects to lower through the reduction process malondealdehid peroxidation (MDA) and suppress pro-inflammatory activity of IL_1 and IL_6, resulting in improvement of pancreatic β cells through cell regeneration and improvement in organ pancreatic β cells. *(Susetia-Totoprajogo, 2010)*

Intestinal immune system that works well modulate the innate and adaptive immune in the body. At the molecular level, the innate immune system is centered on the activation of NF-kB, induces transcription of several proinflammatory cytokines, response to stimulation by microbial or agent of AGEs. In its role to help bridge the innate immunity system to TLR adaptive system, able to induce a good immune response towards Th1 or Treg. Macrophage cells exposed to probiotics maintain immune cells in a state of homeostasis, through immunosuppression and immunomodulating with decreased production of cytokines (IL1, IL6) and increased production of IL10. The role of IL10 inhibit Th1 cells. Increased cytokine IL10, which is proven to maintain homeostatic proliferation of Th1-Th2, and proinflammatory cytokine production can be controlled and inflammation in pancreatic β cells unavoidable. *(Susetia-Totoprajogo, 2010)*

Probiotic bacteria and gut mucosal acts synergistic in form of immunomodulation. At the level of intestinal epithelium, probiotic bacteria provide beneficial effects through colonization and the release of bioactive mixture. Then it reinforce 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. (*Listiani,2005, Inggrid Surono, 2007, Corthesy,2007*).

Increased IL10 related to the period and pancreatic β cell phyisiological, this is supported IL10 suppress proinflammatory response and apoptosis in pancreatic β cells. *(Dronavalli, et all. 2008)* found an association between IL10 and insulin sensitivity. *(Bukhari, 2009)*, Decreased of inflammation in pancreatic β cells is closely related to the improvement of the synthesis of proinsulin to insulin and increase insulin sensitivity and pancreatic β cell mass. (Donath, et al. 2009)

CONCLUSIONS AND RECOMMENDATIONS

Supplementation of the plain kefir with dose about 3,6 cc/200 g bw / day for 30 days *in vivo* study of Wistar rats STZ induced hyperglycemia, was significantly decreased blood glucose, proinflammatory cytokines IL_1, IL_6 and lipid peroxidation (MDA) and increased antioxidants (SOD, catalase, GPX), anti-proliferation cytokine IL_{10} and improving of the normal of pancreatic β cells expression. Insulin and kefir descriptively reduced TNF β level and not significant. It is very challenging to study on characterization of probiotic properties of viable bacteria in kefir to find out the biomolecular mechanisms and apply it in clinically diabetes mellitus therapy.

BIOGRAPHY

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