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Original Research Article

Evaluation of the Effect of Cow's Milk Kefir on Serum Tumor Necrosis Factor-alpha (TNF-α) and Expression of Inducible Nitric Oxide Synthase (iNOS) Gene in Liver Rats

Sunita Sunita[1]*, Mohammad Rizki Akbar[2], Mohammad Ghozali[3], Mas Rizky Anggun Adipurna Syamsunarno[3], Zamharira Muslim[4], Jon Farizal[1], Agung Riyadi[5], Ervan Ervan[5]

[1]*Department of Technology Laboratory Medic, Poltekkes Kemenkes Bengkulu, Bengkulu, Indonesia*
[2]*Department of Cardiology and Vascular Medicine, Faculty of Medicine, University Padjadjaran, Bandung,*
[3]*Department of Biomedical Sciences, Faculty of Medicine, University Padjadjaran, Bandung, Indonesia*
[4]*Department of Pharmacy, Poltekkes Kemenkes Bengkulu, Bengkulu, Indonesia*
[5]*Department of Nursing, Poltekkes Kemenkes Bengkulu, Bengkulu, Indonesia*

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ABSTRACT

TNF-α is a pro-inflammatory cytokine in insulin resistance. Cytokine-induced increase in TNF-α production by the iNOS pathway plays an important role in diabetes. This study was designed to determine the effect of cow's milk kefir as an antidiabetic and expression of the iNOS gene. Twenty-five Wistar rats were divided into 5 groups. Group cow's milk kefir dose variation (50%, 75%, and 100%). This study showed that TNF-α levels in the DM control rat group were higher compared to other DM rat groups that were given cow's milk kefir treatment. The cow's milk kefir treatment group (50%) had a higher average TNF-α content (146.3± 6.68) compared to the other two treatment groups given 75% cow's milk kefir (169.9± 6.39) and 100% cow's milk kefir (175.3± 4.66). There were differences in TNF-α levels between treatment groups (p=0.0013). The expression of the iNOS gene in the healthy control group was highest found in the DM rat group given 50% milk kefir and the lowest in the DM rat group given 100% milk kefir. There were significant differences in iNOS gene expression between groups (p = 0.0019). There was a significant difference between the DM rat group that was treated in the form of 50% group with DM Rats (positive controls) (p= 0.0048) and DM cow's milk Kefir rats 75% milk with DM Rats (Positive controls) (p=0.0128). This study shows that there is an effect of cow's milk kefir as an antidiabetic on TNF-α and iNOS gene expression.

Keywords: Tumor Necrosis Factor-alpha, Nitric Oxide Synthase gene, cow's milk kefir

Introduction

Chronic hyperglycemia in DM is associated with long-term damage, impaired function, and malfunction of various organs, in particular hepatic, kidneys, nerves, heart, and blood vessels.[1] There is an increase in TNF-α production in the pathogenesis of DM.[2] This increase in TNF-α levels occurs due to the dysregulation of the work of insulin so that hepar glucose production increases and causes chronic hyperglycemia. The increase in TNF-α levels in hepar also causes an increase in the activity of Inducible Nitric Oxide Synthase (iNOS) or known as NOS2 in the liver by 45-70% through up-regulation of the Nuclear Transcription Factor pathway.[3]

Kefir seeds are a mixture of lactic acid bacteria (*Lactobacilli, Lactococci, Lactobacillus kefir, Lactobacillus parakefir, Lactobacillus kefiranofaciens, and Lactobacillus kefirgramum*) with yeast and acetic acid bacteria.[4]

Supplementation of clear kefir at a dose of 3.6 mL / 200 grams of body weight for 4 weeks can lower blood glucose levels and levels of pro-inflammatory cytokines such as IL-1, IL-6, IL-8 in Wistar hyperglycemia rats induced with streptozotocin.[5]

*Corresponding author. E mail: sunita@poltekkesbengkulu.ac.id
Tel: +62-8137-373-6131

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The high nutritional value and benefits of dairy products have made more and more research developed for the use of these dairy products, especially kefir with a combination of various raw materials such as cow's milk as an effort to prevent several diseases including DM type 2 due to the antidiabetic, anti-inflammatory, anticancer, antioxidant, immunomodulatory, and probiotic effects of the product.

Materials and Methods

Research Variables

The free variable in this study was cow's milk kefir and the bound variables were TNF-α levels and iNOS gene expression.

Materials

Filter paper, blender, 65 mesh sieve, watch glass, analytical scales, stirrer rod, stirrer, and rotary evaporator. Chemicals: Streptozotocin (STZ) dose 60 mg/kg BB dissolved in citrate buffer 1 M pH 4.5; Nicotinamide dose 120 mg/kg BB dissolved in saline solution (NaCl 0.9 %); Ether for inhalation of rat anaesthetics; ELISA kit TNF-α (BTLab); RNA isolation kit: Trizol reagent, cDNA synthesis kit (GENEzol), EvaGreen (GENEzol), primary analysis of iNOS gene and β-actin gene by RT-PCR.

Sample Size Calculation

This study used 5 test groups, so the number of experimental animals used was as many as 30. The number of experimental animals in each test group was 5. In anticipation of the death of experimental animals, each group had plus one experimental animal. The large calculation of the sample of animals was done using Federer's formula: (t-1) (n-1) > 15. Where: t (number of test groups); n (large sample per group).

Experimental Animal design
This study used Wistar rats[6] aged 3-4 months (280-300g) obtained from the Pharmacology Laboratory of Andalas University. Animals before giving treatment were acclimated for 7 days and fed with regular feeds. Prior to conducting the investigation using experimental animals, researchers had Ethical Clearance from the Health Research Ethics Commission (KEPK/128/04/2022). Wistar rats with blood glucose levels after five days post-induction with streptozotocin[7] 60 mg/kg body weight and nicotinamide 120 mg/kg body weight. The design of this study used 5 groups of test animals based on dose and the control group was Positive Control (Rats DM), Negative Control (Healthy Control Rats), DM + Cow's Milk Kefir Group 50% (P1), DM + Cow's Milk Kefir Group 75% (P2), DM + Cow's Milk Kefir Group 100% (P3). The dose was adjusted to changes in rat weight every week for 4 weeks of treatment, a one-day daily dose.

Kefir Setup
Cow's milk kefir was obtained from Bengkulu Province Indonesia and was made with a ratio of 1 liter of cow's milk (pH 6.5) and 50 gr kefir grains with a fermentation period of 17.5 hours incubated at room temperature (30-31ºC).

Glucose Level Check
Blood glucose levels were examined with capillary blood collection equipment (*Easy Touch* GCU No.GOS22211861) strip test method and the results were expressed in mg/dL units.[8]

TNF- α Level Check
TNF-*α* levels were examined by the Enzyme-Linked Immunosorbent Assay[9](ELISA, Rats Tumor Necrosis Factor *α*, TNF-*α* BT-LAB Kit Cat. No. E0764Ra) method using serum samples and then measured using an ELISA reader and the results were expressed in µg/dL units. TNF-*α* levels were determined using Standard Data Set from Current Experiment.

iNOS Gene Expression
The expression of the iNOS gene in the liver of rats was analyzed using the Real-Time Polymerase Chain Reaction (RT-PCR)[10] method with a comparison in the form of the β-actin gene and the results were expressed quantitatively. Expression of the iNOS gene in the liver were assayed through (1) Bioinformatic Study: The iNOS primers used were iNOS Rat-For Sequence (5' to 3'): CAGATCCCGAAACGCTACAC, iNOS Rat-Rev Sequence (5' to 3'): TGCGGCTGGACTTCTCACT; Primer β-Actin: Rat βActin-For Sequence (5' to 3'): AACCCTAAGGCCAACCGTGAAAAG, Rat βActin-Rev Sequence (5' to 3'): TCATGAGGTAGTCTGTCAGGT; (2) RNA isolation (GENEzol); (3) Calculate the RNA concentration of the isolated results, using the Nanodrop tool; (4) Sintesis cDNA (GENEzol); (5) Real Time PCR/Quantitative PCR (CFX96 Touch Real-Time PCR System Bio-Rad).

Data Analysis
The results are presented as mean±standard deviation (S.D). Data were analysed using Office 365 Excel (Microsoft Corp., Redmond, Washington, USA), and Graph Pad Prism 9 was used to perform statistical analysis (Graph Pad Software Inc., San Diego, CA). The Shapiro–Wilk test was used to see if the data follows a normal distribution. The ANOVA parametric test was used to determine the difference in TNF-*α* levels between groups, and iNOS gene expression between groups and continued with the Tukey HSD post hoc test.

Results and Discussion

Tumor Necrosis Factor- Alpha (TNF-α) Serum Levels
The results of the examination of Tumor Necrosis Factor-alpha (TNF-*α*) levels after treatment are presented in Figure 1. TNF-*α* levels in the DM control rat group were higher than in other DM rat groups treated with Cow's milk kefir. The 50% had the lowest average of TNF-*α* levels compared to the 75 % and 100 %. The values were 50% (146.3± 6.68), 75% (169.9± 6.39) and cow's milk kefir of 100% (175.3± of 4.66) respectively.

The ANOVA parametric test was used to determine the difference in TNF-*α* levels between groups. The results of the ANOVA test showed that there were differences in TNF-*α* levels between treatment groups with a p-value = 0.0013 (Figure 1).

Ekspresi Gen İnducible Nitric Oxide Synthase (iNOS) Liver
Expression analysis of the hepar iNOS gene using the RT-PCR method. Gene amplification products are expressed by cycle threshold (CT) values and shown in the form of amplification curves in Figure 2. The specifications of the resulting amplification products are displayed in the form of melt curves and melt peak curves in Figures 3 and 4.
Amplification products of the same length are at the same melting temperature and form the same peak. The results of the calculation of the relative expression of the hepar iNOS gene are presented in Figure 5. The calculation uses the livak formula. iNOS expression was relative to the healthy control group and was highest found in the DM rat group given 50% milk kefir and the lowest in the DM rat group given 100% milk kefir. The Shapiro-Wilk test showed that the distribution of data was normal with a p-value ≥ 0.05. Data analysis continued with the ANOVA test and obtained a p-value = 0.0019 which means that there are significant differences between groups. The results of the Tukey HSD post hoc test showed that there was a significant difference between the DM rat group that was treated with the milk kefir 50% DM rats with DM rats (positive controls) (p= 0.0048) and DM kefir milk rats 75% milk (P2) with. DM rats (Positive control) (p=0.0128).
The highest average TNF-*α* levels in this study were found in the DM control rat group, with an average level of 178.3 pg/mL. The TNF-*α* DM control rat group in this study had levels of TNF-*α* that were significantly greater than those in the healthy control group. The DM group that was given kefir treatment for 4 weeks had lower TNF-*α* levels than DM control rats. This suggests that milk kefir has effective antidiabetic and immunomodulatory activity to lower serum TNF-*α* levels.
The superfamily Tumor Necrosis Factor (TNF) contains 19 ligands and 29 receptors that play a very diverse role in the body. All members of the TNF superfamily, without exception, showed pro-inflammatory activity, in part through the activation and involvement of transcription factors i.e. Nuclear factor kappa B (NF-κB). In the 1960s, a factor inducing tumor regression was discovered and named tumor necrosis factor-alpha (TNF-*α*), but the factor was actually identified when Aggarwal and colleagues isolated the cytotoxic factor TNF-*α* from macrophages. Complementary DNA (cDNA) TNF-*α* cloned resembles lymphotoxin both structurally and functionally. Later, it was also observed that TNF-*α* were prototypic members of the TNF ligand family[1].

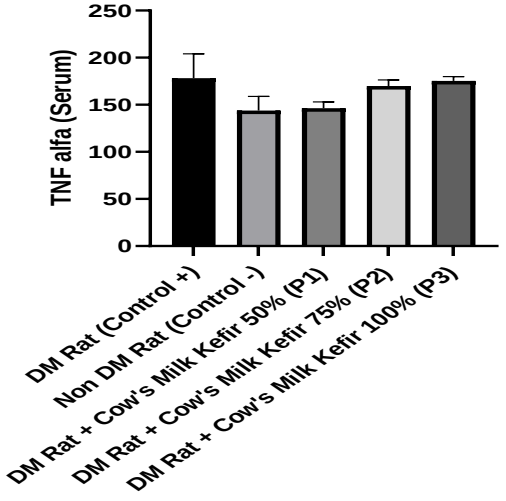

Figure 1: Plasma TNF-*α* levels after 4 weeks of treatment

Tumor Necrosis Factor Alpha is one of the proinflammatory cytokines that has been widely reported to be involved in the development of insulin resistance in type 2 DM. Among all the pro-inflammatory biomarkers, TNF-*α* was first known to be involved in the pathogenesis of insulin resistance, and glucose-related abnormalities associated with it. The inability of cells to respond to physiological levels of insulin is known as insulin resistance and is a characteristic condition of the development of DMT2. TNF-*α* levels in DM conditions will increase related to an increase in blood glucose levels. Hyperglycemia also causes the formation of reactive oxygen species (ROS) which also plays a role in increasing the formation of TNF-*α*. An increase in TNF-*α* can decrease insulin receptor autophosphorylation, change insulin receptor substrate1 (IRS-1) to an inhibitor of insulin receptor tyrosine kinase activity, as well as a decrease glucose transporter GLUT-4 thus worsening insulin resistance conditions.

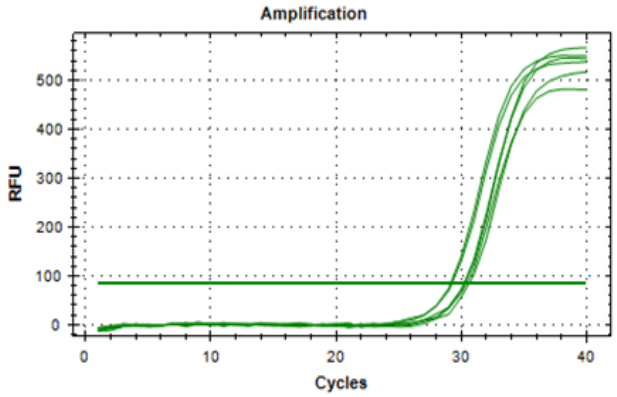

Figure 2: Amplification curve of the hepar iNOS gene

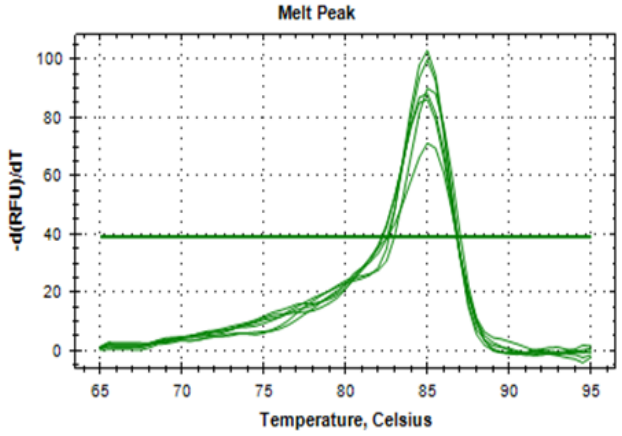

Figure 3: Melting curve of amplification products.

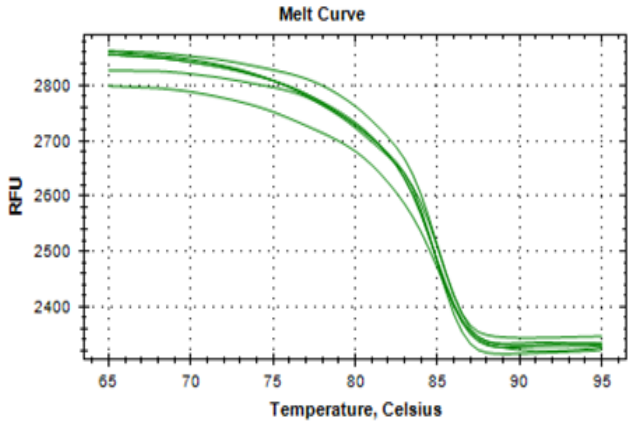

Figure 4: Melt peak curve of amplification products

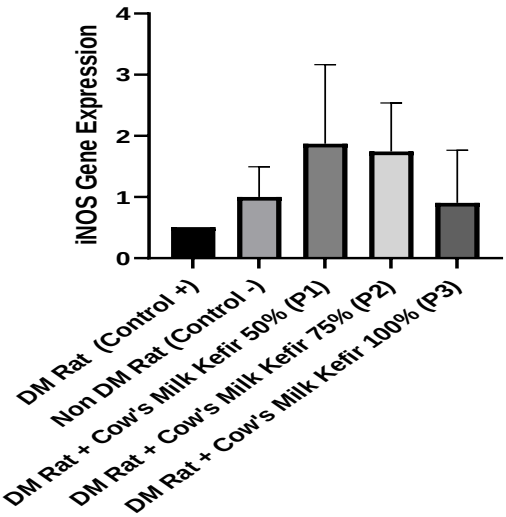

Figure 5: Expression of the hepar iNOS gene relative to the control of healthy rats after 4 weeks of treatment.

TNF-*α* plays an important role in the development of insulin resistance in such a way that it reduces the expression of glucose transporter type 4 (GLUT4) which is an insulin-regulated glucose transporter and is mainly located in the adipocytes, skeletal muscles and heart. Frequent phosphorylation of insulin receptor substrate-1 (IRS-1) induced by activation of TNF-*α*, also works as an inhibitor of insulin receptors and lowers the activation signal of phosphatidylinositol-3 kinase. TNF-*α* plays an important role in the overall pathophysiology of insulin resistance especially in men with a greater body mass index compared to women. An overview of the overall scheme of action mechanism of TNF-*α* in the development of insulin resistance has been reported that the administration of TNF-*α* into experimental animal models and cell cultures may also interfere with the work of insulin compared to experimental animal models deficient in TNF-*α* and its receptors.[3]

In diabetic individuals, the mRNA level of TNF-*α* and its protein increases in adipose tissue. TNF-*α* alters lipid and protein metabolism in adipose tissue (insulin-target tissues that lack a hormonal response will result in the development of insulin resistance). In isolated adipocytes, TNF-*α* suppresses the action of genes responsible for regulating the rate of absorption of fatty acids in tissues. TNF-*α* is also responsible for inhibiting lipoprotein lipase and initiating the breakdown of lipid molecules (lipolysis) in adipose cells. As a result of this lipolysis, levels of non-esterifying fatty acids increase which results in the development of insulin resistance.[1]

NO is derived from L-arginine in a reaction catalyzed by several isoenzyme nitric oxide synthase (NOS). The NOS protein family is classified into constitutive type, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible type (iNOS). iNOS can be expressed by various types of cells, including macrophages, vascular smooth muscle cells, and glomerular mesangial cells, which leads to the formation of large amounts of NO and can be induced by endotoxins and cytokines. It has been proposed that the stimulation of iNOS due to hyperglycemia can cause an increase in the generation of NO, which in turn contributes to the hyperfiltration of diabetes and glomerular abnormalities in diabetes.[4] Recent large-scale clinical studies have shown that poor glycemic control, activation of the renin axis of angiotensin, and hypertension play an important role in the development of diabetic nephropathy. NO has the potential to play a major role in determining the effects of hyperglycemia, hypertension, and activation of angiotensin. In the kidneys, NO controls afferent and efferent vascular tone, ultrafiltration coefficient, and medullary blood flow. Some of the findings support the role of excessive NO production in mediating increased perfusion and intraglomerular pressure during the early stages of diabetes as our study findings. On the contrary, there is evidence that a decrease in production or inactivation of NO in long-term diabetes and

changes in vascular reactivity and tone contribute to the development of nephropathy.[4]

Thus, pagan feeding of milk for 4 weeks in rats with diabetes improved proteinuria and reduced the level of glomerulosclerosis. Some difficulties preclude the investigation of the role of NO in diabetic nephropathy. First, NO provides, many biological functions in one organ system, including the kidneys, making it difficult to make a definitive statement about the beneficial or detrimental effects of NO under certain conditions.[5] Acute inhibition of NOS with low doses of nonselective NOS inhibitors (L-NAME) lowered GFR and RPF significantly in diabetic animals back to normal control values, whereas this dose did not affect renal hemodynamics in control rats. These findings suggest that glomerular hyperfiltration is highly dependent on the increased formation and action of NO in the early stages of this experimental diabetic nephropathy. These results are consistent with previous reports, which postulate the significant role of NO in diabetic hyperfiltration, although the involvement of specific NOS isoforms in this process is still unclear[1]. Since there is an increased production of various cytokines including TNF-α and IFN-γ in diabetes and macrophages infiltrated into the glomerulus of rats in the early stages of diabetes, it can be postulated that cytokine-induced iNOS pathways may play an important role. role in glomerular injury seen in diabetic kidneys. The exact role of iNOS in mediating DM nephropathy has not been fully explained, and the results of previous studies have been inconsistent. Some authors have determined inhibition of induced NO production in rat MC cultured by certain pro-inflammatory stimuli. Others were able to show an increase in LPS-induced production of NO or cytokines in MC.[11]

The decrease in serum TNF-α levels in all three kefir treatment groups was caused by immunomodulatory activity in BAL-derived kefir. Lactic acid bacteria will modulate the immune system by producing anti-inflammatory cytokines such as IL-10 and TGF-β. These anti-inflammatory cytokines inhibit the activity of phagocyte cells. Lactic acid bacteria can also induce innate and adaptive immune responses through specific molecules on their cell walls called pathogen-associated molecular patterns (PAMPs) which will then be recognized by specific receptors called pattern recognition receptors (PRRs). One of the PAMPs found in BAL is lipoteichoic acid (LTA) which has similarities with lipopolysaccharides (LPS).[12] The role of LTA in BAL is to be involved in Toll-Like Receptor (TLR) signals which are a link between the innate and adaptive immune systems. Another mechanism is through the activation of T-regulator (T-reg) cells which play a role in maintaining the response balance of Th1-Th2 so that it can suppress inflammatory reactions and increase IL-10 production in pancreatic beta cells. IL-10 cytokines play a role in suppressing the pro-inflammatory response and apoptosis.[12]

Other immunomodulatory activities also come from the content of biogenic peptides from milk such as lactoferrin. Lactoferrin inhibits the production of local pro-inflammatory cytokine macrophage products including IL-1, TNF-α, IL-6, and iNOS.[13] Lactoferrin also acts as an extracellular iron binding. Extracellular iron will be bound by transferrin or lactoferrin proteins so that it will inhibit the formation of reactive oxygen species (ROS). The formation of ROS is also inhibited by the antioxidant activity obtained by the combination of soy milk. The mechanism of isoflavone bioactive compounds contained in soy milk as antioxidants and preventing damage due to free radicals is by donating hydrogen ions and acting as free radicals directly.[14] The meta-5,7-dihydroxyl structure in ring A of the isoflavones shows the ability to donate hydrogen ions so as to form more stable compounds and less reactive phenolic compounds[15], while the 4-hydroxyl group in ring B of the isoflavone compound has the ability to be a scavenger of ROS compounds.[14] Antioxidants from kefir also play a role in suppressing the activity of pro-inflammatory cytokines so that they can improve the regeneration of pancreatic beta cells, with a decrease in inflammatory conditions in pancreatic beta cells related to the synthesis of proinsulin into insulin and an increase in insulin sensitivity and pancreatic beta cell mass.[12]

Conclusion

This study showed that there was an effect of cow's milk kefir as an antidiabetic against serum TNF-α and expression of the iNOS gene of the Wistar rat liver organ.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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