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Kefir Fermented Milk Ameliorates Serum Indicators of Hyperglycemia, Dyslipidemia, Pro-Inflammatory Cytokines and Hepatic Histology in Streptozotocin-Diabetic Rats

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Abstract **Objectives:** Diabetes mellitus has increased worldwide, including in Saudi Arabia. Probiotic foods can lower the consequence of diabetes complications. Kefir fermented milk (KM) is a valuable source of probiotics. It has attracted increasing attention in the field of preventive medicine. This is due to its antioxidant, immunomodulatory and anti-inflammatory characteristics. This study aimed to examine the impact of KM on markers of hyperglycemia and lipid profile, pro-inflammatory cytokines and hepatic damage in streptozotocin (STZ)-diabetic rats. **Methods:** Thirty-two male rats were distributed into four groups: I- Control; II- Diabetic (rats injected with 60 mg/kg STZ to induce diabetes); III- Diabetic + 10 ml/kg KM: diabetic rats treated with 10 ml/kg KM; IV- Diabetic + 20 ml/kg KM group: diabetic rats treated with 20 ml/kg KM. The KM treatment period was 35 days. Serum hyperglycemia markers (glucose, glycosylated hemoglobin (HbA1c) and insulin), lipid profile (total cholesterol (TC), triglyceride (TG), high-density lipoprotein (HDL-C), very low-density lipoprotein (VLDL-C), low-density lipoprotein (LDL-C)) and pro-inflammatory markers (tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)) were estimated. Furthermore, hepatic tissue histopathologic changes were assessed. **Results:** KM effectively relative to diabetic group reduced blood glucose, HbA1c, TG, TC, VLDL-C and LDL-C. Besides, KM significantly increased serum insulin and HDL-C. The levels of two inflammatory mediators, TNF-α and IL-6, were reduced in the KM-treated group. KM treatment improved hepatic inflammation and necrosis induced by STZ. These effects were most pronounced in the group receiving the high KM dose. **Conclusion:** KM administration effectively reduced elevated blood sugar levels caused by STZ by enhancing insulin secretion. KM administration reduced inflammation and markers of dyslipidemia. Furthermore, KM consumption appears to improve STZ-induced hepatic damage.

Key Words Hyperglycaemia, inflammation, dyslipidemia, hepatic, histopathology

INTRODUCTION

Diabetes is a systemic, long-term endocrine, metabolic condition distinguished by persistent high blood sugar levels via a deficit in insulin action and/or secretion. It is a multifactorial illness. Diabetic prevalence, considered a world epidemic, is increasing significantly and affecting approximately 5-10% of adults globally. According to WHO reports, diabetes become the 7th leading cause of death [1]. In Saudi Arabia, the incidence of diabetes is increasing due to socioeconomic changes affecting the lifestyle, including altering physical activity and diet [2]. Around 13 % of Saudis are diagnosed with diabetes relative to 2.8-4.4% global prevalence [3]. One in 10 are prediabetes (at risk of developing diabetes) of the remaining Saudi population [4]. Elevation diabetes prevalence is proportionally correlated with the high prevalence of cardiovascular diseases and premature death, accounting for 42% in Saudi Arabia [4].

Rationale and Knowledge Gap

Studies investigating the human microbiome's role in health and diseases have attracted more attention in the past decade. The human microbiome is essential for digestion, nutrition metabolism and immune system regulation [5]. It is vital in illness pathogenesis, including liver, diabetes, obesity and cancer [6]. The restoration of homeostasis of gut microbiota via consumption of probiotics and prebiotics resulted in

preventing as well as attenuating numerous metabolic complications and cardiovascular diseases [7].

Kefir grains are symbiotic associated with various microorganisms. It is made primarily of yeasts as well as lactic and acetic acid bacteria in a natural polysaccharide and protein matrix. Kefir-fermented dairy products are made through the fermentation via yeasts and bacteria naturally present in grains of kefir. Kefir milk (KM) has beneficial properties [8]. It contains many bioactive peptides with antibacterial, antihypertensive, immunomodulatory and antioxidative properties [9].

Previous animal studies have shown that administration of KM improved specific oxidative stress markers by restoring the ROS/NO imbalance, as well as showed hypoglycemic, hypolipidemic and pro-inflammatory cytokine expression [10]. Moreover, it has anticarcinogenic and anti-inflammatory activities [11,12].

Objective

As a result of the increasing prevalence of diabetes globally, especially in Saudi Arabia and given that fermented milk is one of the most popular consumed beverages among Saudis. Therefore, the objective of this research is to assess the effect of KM as anti-hyperlipidemic and hypoglycemic on streptozotocin-diabetic rats.

METHODS

Preparation of KM

Organic kefir grains were kindly provided from Honest Com., Jeddah, Saudi Arabian (SA). Fresh whole cow's milk (Almarai) with 3% fat, homogenized and UHT treated was obtained from a local market in Jeddah, SA.

The kefir fermented milk (KM) was prepared following a previously established protocol [13]. Kefir grains (10% w/v) were added to glass containers with cow's milk (3% fat) and kept at 25°C in a dark environment for 24 hours. After fermentation, the grains were separated from the fermented milk using a 1-mm² mesh sieve. The resulting liquid was referred to as kefir beverage. A portion of this liquid, termed cell-free supernatant (CFS), was centrifuged and filtered through membranes with a pore size of 0.22 µm, then stored at -80°C for later use. The kefir grains were reused for subsequent fermentations, stored at 4°C between cycles and inoculated in fresh milk for up to seven fermentation rounds. As stated in reference [13], the microbial composition in KM primarily consists of lactic acid bacteria (LAB), acetic acid bacteria (AAB) and yeasts. The dominant LAB species identified was Lactococcus lactis, while a single AAB species, Acetobacter orientalis, was also present in KM. Additionally, various yeast species, including Pichia kudriavzevii, Geotrichum candidum, Geotrichum bryndzae, Saccharomyces cerevisiae and Kazachstania unispora.

Standardization of KM preparation involves maintaining consistent conditions to ensure uniformity across batches. Key factors include using the same microbial composition of kefir grains, controlling fermentation conditions such as temperature (25°C) and duration (24 hours) and using the same type and fat content of milk (3% fat). Additionally, standardized processes like filtering kefir grains, maintaining consistent storage conditions (4°C) and monitoring parameters such as pH and acidity ensure that each batch has the same characteristics, which is crucial for reproducibility in both research and product quality.

The use of UHT milk in kefir fermentation supports better growth of L. acidophilus colonies by providing a sterile environment free from competing microbes. UHT-fermented kefir meets the minimum colony-forming units (CFUs) required for L. acidophilus to remain effective, ensuring consistent probiotic benefits. This makes UHT milk a reliable option for producing kefir with adequate probiotic content [14].

Animals

Thirty-two adult male Albino rats (190-210 g) were obtained from the Pharmacy College, KAU, SA. The sample size was calculated according to a crude method "resource equation" based on the law of diminishing return [15]. Animals were housed in cages and adapted to lab setting at a temperature of 24±2°C and a humidity of 60±10% during a 12:12 h dark/light cycle. All rats were fed a standard diet containing protein, soybean oil, sucrose, fiber, mineral mixture, vitamin mixture, choline chloride (14, 5, 10, 5, 3.5, 1 and 0.25, respectively) and the remainder was corn starch up to 100% [16].

Induction of Diabetes

Streptozotocin (STZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA), it is used to induce diabetes in the animal. STZ solution prepared freshly in 0.01 citrate buffer (pH = 4.5). After fasting overnight, rats were injected intraperitoneally with STZ (60 mg/kg), while control rats were injected with the citrate buffer only. As the number of early deaths was less than 20%, rats were allowed to drink glucose solution (5%) within 6 hours after STZ injection to overcome death from rapid and massive β-cell necrosis that results in the release of large quantities of insulin, causing fatal hypoglycemia [17]. Diabetes was confirmed after 72 h. of STZ injection. Glycemia was measured by glucometer; blood was drawn from the tail vein. Rats with fasting blood glucose (FBG) levels exceeding 250 mg/dL were classified as diabetes [18]. The success rate of diabetes induction was 80 (24 rats out of 30).

Study Protocol

This study is an experimental study. Thirty-two rats were randomly assigned into two main groups: a control group consisting of 8 rats and a diabetes group with 24 rats. The diabetes group was then further randomly subdivided into three groups, each with 8 rats: a diabetic group, a diabetic group treated with KM (10 ml/kg) by gavage and diabetic group treated with KM (20 ml/kg) by gavage. The control and

diabetic untreated groups received saline via oral gavage in the same manner as in diabetic treated groups to ensure consistency across all groups. The dose 10 ml/kg/day was chosen based on prior study according to [19]. All groups were fed a standard diet for a long experiment period (35 days).

Analytical Measurements

Serum samples obtained from animals after an overnight fast of 12 h were used to determine biochemical parameters. The glycemia and lipid profile were measured using colorimetric enzymatic kits (BioMerieux, France) followed the standard procedures as described in kits' protocol. The insulin, glycosylated hemoglobin (HbA1c) and proinflammatory cytokines (IL-6 and TNF-α) were measured using a fully automated enzyme-linked immunosorbent assay ELISA kits (Abcam, UK). A monoclonal antibody specifically targeting rat insulin, HbA1c, IL-6 and TNF-α were employed. All parameter concentrations were quantified by comparing them to a standard curve.

Histopathology Examination

Hepatic sections of all groups underwent standard histopathology procedures including trimming of the samples, fixation within neural buffer formalin, clearance by xylene and embedding in paraffin wax. Samples were section by microtome at 5 μm thickness before being stained by the hematoxylin and eosin (H and E) stain. For identification of the histological alterations in each group, the stained hepatic slides were examined under a microscope.

Quantitative scoring of the hepatic lesions was done through blind examination of the slides of the different groups based on the hepatic degeneration, necrosis and inflammation As stated in reference [20].

Statistical Analysis

The normality of the distributions was evaluated using the Kolmogorov-Smirnov normality test for all measured parameters. The one-way analysis of variance (ANOVA) test followed by Tukey's multiple comparisons test was used to compare the results in normally distributed data. The Kruskal-Wallis test followed by Dunn's multiple comparisons test was used to compare the hepatic lesions score (no normal distribution) of all groups. The research utilized statistical analysis through the Statistical Package (SPSS) (Version 27) to analyze the results. The main data analyses consisted of descriptive statistics in the form of means, SD (standard deviations) and confidence interval (CI, 95%) . Data with the p-value $\leq$0.05 were regarded as significant. The data was blinded collected and statistically analyzed to reduce the bias.

RESULTS

Impact of Kefir Milk (KM) on Glucose, Insulin and HbA1c in Diabetic Rats

Table 1 represents the impact of KM on glucose, insulin and HbA1c measured in diabetic rats. Serum glucose and HbA1c concentrations of STZ-induced hyperglycemic rats were significantly elevated, whereas serum insulin was significantly reduced relative to the control rats. Diabetic animals received KM significantly improved glucose, insulin and HbA1c concentrations toward normal levels. The KM treatment was significantly effective in lowering the blood glucose and HbA1c, concurrent with significant elevate insulin concentration relative to the diabetic untreated group. The effectiveness of KM was dependent on the dose administered. This was evident by the significant differences in blood glucose levels, HbA1c and insulin concentrations between the 10 and 20 ml/kg KM groups.

Impact of Kefir Milk (KM) on Lipid Profile Indices in Diabetic Rats

In diabetic untreated rats, the indices of hyperlipidemia (TC, TG, VLDL-C and LDL-C) concentrations were significantly elevated. In contrast, serum HDL-C was significantly reduced relative to the control rats. Diabetic rats treated with KM exhibited significant improvement in lipid profile indicators toward normal levels. The KM treatment was significantly effective in lowering the TC, TG, LDL-C and VLDL-C, concurrent with significantly elevating HDL-C levels relative to the diabetic untreated group. The effectiveness of KM was dose-dependent, as evidenced by significant differences between KM at a low dose (10 ml/kg) and high dose (20 ml/kg) in TC, TG, VLDL-C and LDL-C, as well as HDL-C concentrations (Figure 1).

Table 1: Impact of KM on glucose, insulin and HbA1c in diabetic rats

Experimental groups	Glucose (mg/dl)	Insulin (mIU/L)	HbA1c (%)
Control			
95% CI	80.75±6.50 (75.32-86.18)	19.59±1.66 (18.19-20.98)	4.79±0.55 (4.33-5.24)
Diabetic			
95% CI	253.62±22.21$^{a\#}$ (235.05-272.19)	4.43±0.86$^{a\#}$ (3.71-5.14)	10.53±0.76$^{a\#}$ (9.89-11.16)
Diabetic + KM (10 ml/ kg)			
95% CI	106.13±17.02$^{b\#}$ (91.89-120.35)	16.11±3.15$^{b\#}$ (13.48-18.74)	5.59±0.68$^{b\#}$ (5.02-6.15)
Diabetic + KM (20 ml/ kg)			
95% CI	84.38±13.18$^{b\#,c*}$ (73.36-95.39)	20.54±2.63$^{b\#,c\wedge}$ (18.34-22.74)	4.93±0.57$^{b\#,c*}$ (4.45-5.40)

All values are offered as Mean±SD (n = 8). CI: Confidence interval, [a]: Significant versus the control, [b]: Significant versus the diabetic, [c]: Significant versus the diabetic +KM (10 ml/kg) group, (*p$\leq$0.05, ^p$\leq$0.01, #p$\leq$0.001).

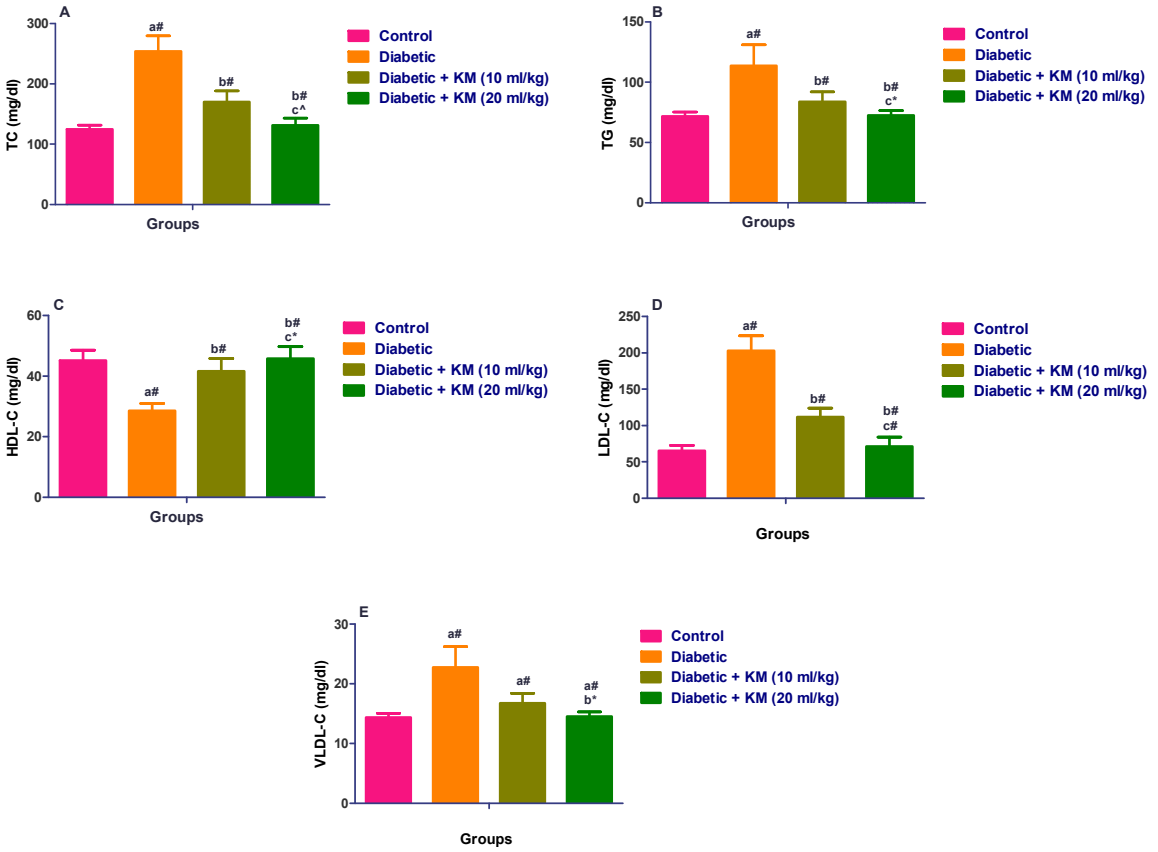

Figure 1: Impact of KM on markers of dyslipidemia (TC, TG, HDL-C, LDL-C and VLDL-C) measured in STZ-induced hyperglycemic rats. All values are offered as Mean±SD (n = 8). [a]: Significant versus the control, [b]: Significant versus the diabetic, [c]: Significant versus the diabetic +KM (10 ml/kg) group, (*p≤0.05, ^p≤ 0.01, [#]p≤ 0.001). 95% CI (confidence interval) for TC [118.52-130.48; 232.88 -275.37; 154.71- 185.54; and 121.20- 141.30]; TG [68.79- 74.71; 99.09- 128.15; 76.86-90.64; and 69.19-75.81]; HDL-C [42.25-48.00; 26.45-30.55; 38.11-45.14; and 42.41-49.09]; LDL-C[72.93-85.82- 205.71-245.79; 103.08-151.92; and 78.16-92.84]; and VLDL-C [13.76-14.94; 19.82-25.63; 15.37-18.13; and 13.84-15.16] for control; diabetic; diabetic+ KM (10 ml/kg); and diabetic+ KM (20 ml/kg), respectively]

Impact of Kefir Milk (KM) on Pro-Inflammatory Biomarkers in Diabetic Rats

Diabetic rats exhibit significant inflammation, as evidenced by significantly elevated IL-6 and TNF-α relative to the control rats. Diabetic rats treated with KM exhibited significant improvement in pro-inflammatory cytokines (IL-6 and TNF-α) toward normal concentrations. The KM treatment was significant and effective in lowering the IL-6 and TNF-α levels relative to the untreated diabetic group. The effectiveness of KM was dependent on the dose administered. This was evident by the significant differences in IL-6 and TNF-α concentrations between the 10 and 20 ml/kg KM groups (Table 2).

Table 2: Impact of KM on pro-inflammatory indices (cytokines IL-6 and TNF-α) in diabetic rats

Experimental groups	IL-6 (pg/ml)	TNF-α (pg/ml)
Control		
95% CI	5.75±0.79	13.27±1.46
	(5.09 - 6.41)	(12.04-14.48)
Diabetic		
95% CI	18.04±1.40[a#]	40.38±3.19[a#]
	(16.86-19.21)	(37.71-43.04)
Diabetic + KM (10 ml/ kg)		
95% CI	6.86±0.86[b#]	16.59±1.83[b #]
	(6.14-7.58)	(15.06-18.12)
Diabetic + KM (20 ml/ kg)		
95% CI	5.84±0.61[b#,c*]	14.41±1.42[b#,c*]
	(5.33-6.34)	(13.23-15.60)

All values are offered as Mean±SD (n = 8). CI: Confidence interval, [a]: Significant versus the control, [b]: Significant versus the diabetic, [c]: Significant versus the diabetic + KM (10 ml/kg) group, (*p≤0.05, [#]p≤0.001)

Impact of Kefir Milk (KM) on Hepatic Histopathology in Diabetic Rats

Figure 2 represents the histopathological findings within the different groups. Control rats showed normal hepatic histological appearance with normal hepatocytes and normal biliary and vascular tissues (Figure 2A). Diabetic rats' hepatic

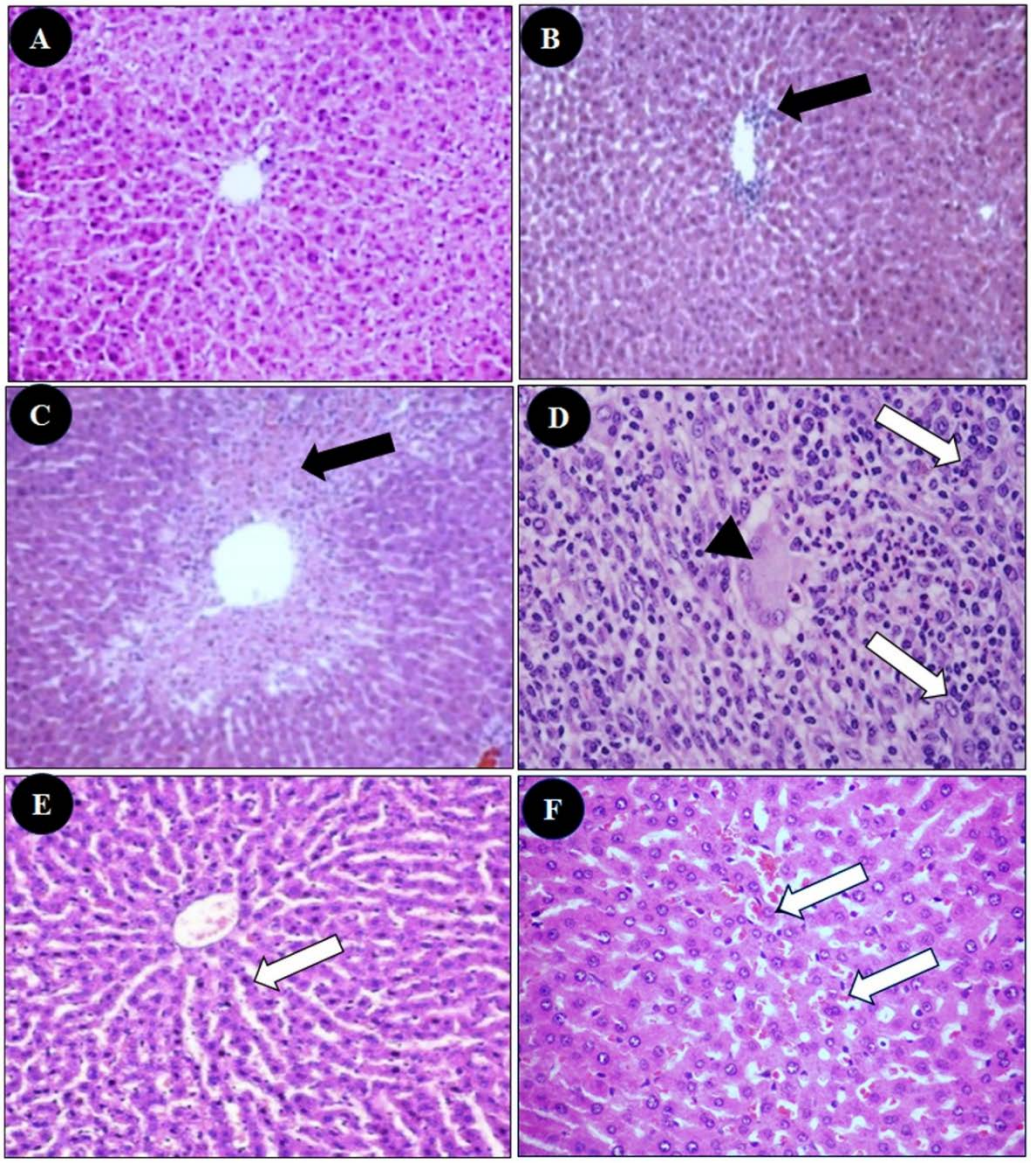

Figure 2: Impact of KM on hepatocytes histopathological changes. The hepatic of the control rats showing normal hepatic histological appearance (A). Hepatic tissue sections of diabetic rats (B-D) revealing marked periportal mononuclear inflammatory cells infiltration (black arrow) (B), centrolobular necrosis (arrowhead) and features of chronic hepatitis associated with mononuclear inflammatory cells (white arrows) and giant cells infiltration (black arrows). Diabetic rats treated with KM (10 ml/kg) showing mild degenerative changes associated with nuclear pyknosis (white arrow) (E). Diabetic rats treated with KM (20 ml/kg) showing marked improvement in hepatocytes unless mild congestion within the hepatic sinusoids (white arrows) (F). H and E stain. Bars equal 100 µm except Figure D bar equals 50 µm

tissue sections revealed the centrolobular and periportal hepatic necrosis associated with severe degree of hepatic degeneration, mononuclear inflammatory cells including monocytes and macrophages, as well as giant cells (Figures 2B-D). Diabetic rats treated with KM (10 ml/kg) showed a remarkable decrease in the hepatic necrosis and inflammation within the hepatic tissues (Figure 2E). Diabetic rats treated with KM (20 ml/kg) showed marked improvement of hepatocytes consistent with the normal control unless mild congestion of the central vein and blood sinusoids (Figure 2F).

Quantitative scoring of the hepatic lesions in different groups demonstrated a marked increase in the lesions score in diabetic untreated group, there was a significant difference relative to the control rats ($p \leq 0.001$). Diabetic rats treated with KM exhibited decrease of the hepatic lesions score. The

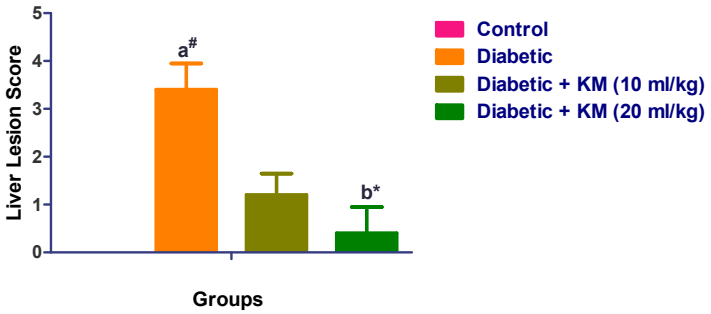

Figure 3: Impact of KM on hepatic histopathological lesions measured in STZ-induced hyperglycemic rats. All values are offered as Mean±SD, : Significant versus the control, [b]: Significant versus the diabetic. (*p≤0.05, [#]p≤0.001), 95% CI (confidence interval) [0.00-0.00; 2.72-4.08; 0.64-1.76; and -028-1.08, for control; diabetic; diabetic+KM (10 ml/kg); and diabetic+ KM (20 ml/kg), respectively]

effectiveness of KM was dependent on the dose administered. There was a significant difference between diabetic rats treated with 20 ml/kg KM relative to the diabetic untreated group (Figure 3).

DISCUSSION

Key Findings

Diabetes drugs can be effective in treating diabetes, but they often have side effects. As a result, many people turn to natural foods and products to help manage their diabetes. This research studied the influence of KM on the serum levels of glucose, HbA1c, insulin, markers of hyperlipidemia and inflammation, as well as hepatic histopathology in diabetic rats caused by STZ. Based on our findings, KM was effective in reducing blood glucose, HbA1c, TG, TC, LDL-C and VLDL-C in rats with diabetes. Besides, KM markedly raised serum insulin and HDL-C concentrations in the hyperglycemic rats. These effects were most pronounced in the group that received the highest dose of KM. The concentrations of two inflammatory mediators (TNF-α and IL-6) were reduced in the blood of the KM diabetic groups.

Strengths and Limitations

The major strength of the study is that, this study examines how probiotics may help reduce hyperglycemia, hyperlipidemia and inflammation, highlighting their potential for therapeutic use in conditions beyond diabetes. One main limitation was that the specific active components of KM responsible for its effects, such as probiotics or peptides, were not thoroughly analyzed, limiting insight into the precise mechanisms involved.

Comparison with Similar Researches

Previous research has shown that probiotic fermented milk can improve fasting blood glucose levels, HbA1c levels and lipid profiles in subjects with type 2 diabetes [21]. Alihosseini *et al.* [22] showed that administering probiotic KM had a decreased HOMA-IR mean value. Their outcomes, however, contradicted our findings since they demonstrated that probiotic KM intake had no effect on insulin levels. In line with our results, both *Bifidobacterium bifidum* (*B. bifidum*) and *Lactobacillus casei* (*L. casei*), when taken alone or together, improved blood sugar levels, cholesterol levels and cellular oxidative damage markers in rats with hyperglycemia caused by STZ [23]. The probiotic bacteria *L. casei* and *B. bifidum* modulate the levels of insulin in the serum by raising the synthesis of glucagon-like peptide 1 (GLP-1), a chemical that increases the body's sensitivity to insulin and controls its release [24].

Explanations of Findings

High levels of pro-inflammatory factors may play a role in the advancement of insulin resistance in people with type II diabetes. The chemical byproducts of fermented foods can activate or alter immunity, boost the initiation of pro-inflammatory and anti-inflammatory markers and overall modify the signaling networks involved in oxidative harm and inflammatory reactions [25]. Research has demonstrated that food containing probiotics can lower the blood sugar measurements by reducing the generation of pro-inflammatory cytokines and reactive oxygen metabolites (ROM). Both ROM and cytokines can play a part in the damage of beta cells, which are the part of the pancreas that produce insulin [26]. Probiotics can improve insulin sensitivity by reducing inflammation [27]. This is because high blood sugar levels, insulin resistance and hyperlipedemia are all associated with inflammation [28].

Probiotics may lower cholesterol levels by using cholesterol for their own metabolism. They binding to cholesterol and converting it into waste products. Cholesterol levels decreased by deconjugating cholesterol by bile-salt hydrolase of probiotics to the bile acids [29]. Probiotics can lower cholesterol levels by binding onto their cellular surfaces [30]. In addition, cholesterol can be broken down into a substance called coprostanol in the intestines, which is

then eliminated from the body in the stool [24]. This can help to reduce the total amount of cholesterol in the body.

Diabetes is linked to numerous liver problems, including acute liver disease, non-alcoholic fatty liver disease (NAFLD) and elevated liver enzymes [31]. The uncontrolled buildup of glycogen in the liver can worsen insulin resistance. Insulin resistance and hyperglycemia can damage the liver [32]. In this study, KM treatment improved hepatic inflammation, vacuolization and necrosis of STZ diabetes rats. The present study is one of the first to show that KM can help with diabetes-induced hepatic histopathological changes. These findings run parallel with many prior research. Chen *et al.* [33] showed that kefir dramatically declined the number of large lipid droplets in the hepatic tissue of mice with NAFLD by inhibiting the lipogenesis pathway. Additionally, administering kefir peptides to mice with NAFLD for eight weeks significantly lowered the buildup of fat particles, the generation of 4-HNE, an indicator of cellular oxidation and the concentrations of certain inflammatory mediators (IL-6, IL-1β and TNF-α) in their hepatocytes [34]. Oral administration of kefir to animals that had been exposed to gamma radiation significantly reduced the damage to their hepatic tissue. This was likely due to kefir's ability to reduce the cellular oxidative and the inflammatory markers and in the hepatocytes [35]. The benefits of fermented foods go beyond just the probiotics they contain. The modified proteins in foods that are fermented can assist the immune system regulation through reducing the production of inflammatory markers (IL-1β and INF-γ) in the hepatocytes. They can also improve the gut microbiome and gene expression patterns of adhesion molecules [36].

Implications and Actions Needed

The findings of this study highlighted the broader advantages of probiotics, including lipid profiles reduction, regulation of oxidative stress and inflammation, enhancement of insulin secretion and ameliorate hepatic lesions which could positively impact diabetes, cardiovascular health and immune system regulation. Clinical trials must be conducted to confirm the efficacy and safety of KM in diabetic patients, with an emphasis on long-term outcomes and possible adverse effects.

CONCLUSION

In conclusion, KM administration successfully decreased STZ-induced hyperglycemia with enhanced insulin secretion. Furthermore, KM administration declined the pro-inflammation indices and plasma markers of dyslipidemia. Moreover, KM consumption appears to ameliorate hepatic lesions in STZ-induced diabetic rats.

Acknowledgment

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Conflict of Interest

The authors declare no conflict of interest.

Ethical Statement

The experiment was conducted entirely in compliance with the standards for the handling and use of experimental animals established by the experimental animal care ethical committee, Faculty of Medicine at KAU, with reference No "PH-1444-9".

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