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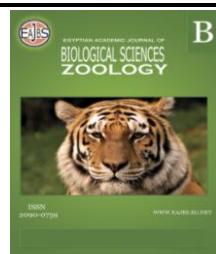


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Possible Short-Term Biological Effects of Kefir: II: Protective Role and Therapeutic Efficacy of Kefir Beverage on The Cell Biological, Histochemical, Histopathological and Biochemical Changes in Liver of High-Fat-Fed STZ- Induced Diabetic Male Wistar Rat

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ABSTRACT

This study was designed to investigate the protective role of kefir and insulin consumption on cellular activities, histochemical components, histological architecture, and integrity of the liver by measuring IL-6 and TNF- α in high fat-fed STZ- induced diabetic male Wistar rats.

From a cell biological and histochemical point of view, both kefir and insulin have beneficial various effects on cellular activities and the histochemical components such as DNA, RNA, total protein, collagen, polysaccharides, and phospholipids materials synthesis in the examined normal and diabetic hepatocytes of the male rats. Biochemically, kefir and insulin have various biochemical effects on the serum IL-6 and TNF- α levels.

From a pathological point of view, we can say the beneficial various cell biological and histochemical effects of kefir, pathologically may make kefir, to some extent, completely repair all the pathological side effects of type1 diabetes on the hepatocytes in diabetic rats. Also, insulin repair some of the pathological side effects of diabetes on the hepatocytes.

The positive results of using kefir and insulin in treating the pathogen effects of diabetes make it possible to obtain positive clinical applications/implications. Therefore, these positive results encourage us to continue working and complete the various long-term pre-clinical trials and clinical trials phases.

INTRODUCTION

Obesity is considered the leading risk factor for various metabolic disorders and chronic diseases, including type 2 diabetes, hypertension, non-alcoholic fatty liver disease, stroke, coronary heart disease, and diverse cancers (Nyberg *et al.*, 2018).

Diabetes mellitus, defined by elevated glycemic markers, is a major risk factor for cardiovascular disease (CVD), which is the most common cause of death among adults with diabetes mellitus (Go *et al.*, 2013).

There are improvements that dairy foods and the important nutrients they contain—namely, calcium, vitamin D, and potassium are linked to reduced risk of heart disease; type2diabetes; and metabolic syndrome, which is led to obesity and diabetes (Lichtenstein *et al.*, 2006).

Millions of cases could be prevented by including dietary modification to functional nutrition, which is a primary option for preventing metabolic disturbances and for reducing undesirable outcomes in DM (Pasin and Comerford, 2015; Astrup, 2014).

The probiotic kefir has been associated with a range of health benefits, which have been reviewed by others (De Oliveira Leite *et al.*, 2013).

Besides drug treatment for diabetes; in recent years, many efforts have been made on traditional medicines as complementary therapy in the treatment of diabetes. In this regard, probiotics have been considered in diabetic patients (Guarner *et al.*, 2005).

Kefir consumption has been associated with several health-promoting properties, such as antimicrobial (Rodrigues *et al.*, 2005), anti-inflammatory (Lee *et al.*, 2007), reduction of cholesterol and triglycerides plasma levels (Huang *et al.*, 2013), and has also been shown to exert a beneficial effect on gut health (Urdaneta *et al.*, 2007).

Aref *et al.* (2021) showed that from the cell biological, histochemical, and histopathological point of view; we can say that logically and biologically, any biological stress factor affects the size of cells nuclei that change the cellular activities such as metabolism and cellular membranes permeability and then lead to quantitatively and qualitatively changes in the chemical components in cells and tissues such as nucleic acids, protein, carbohydrates, and lipids, then these changes are reflected in a form of pathological manifestations and then finally dysfunction of the organ occurs in the organism.

While the kefir mitigates oxidative stress, lowers blood glucose and hyperglycemia, we do not fully understand how it acts against diabetic side effects in the liver. Therefore, we cell biological, histochemical, pathological, and biochemical analyzed the liver to answer this question.

MATERIALS AND METHODS

Experimental Animals:

From: ENVIGO Company, USA. IACUC Protocol Number (ORA use only): 2017-17, we got the experimental animals in this work. We used white male albino rats (Wistar rat) (*Rattus norvegicus*) from the order Rodentia and family Muridae. Adult rats were kept in Lab Animal Research Facility (LARF) building, University of Idaho, USA, under observation for 1 week before experimentation to exclude any intercurrent infection and to acclimatize the animals to the new conditions. The rats marked and housed (2-3) rats per cage from 30 polypropylene cages with softwood chips as bedding with good ventilation. The rats were kept at constant experimental conditions at a temperature of light /dark cycle (12 hr), $23 \pm 2^{\circ}\text{C}$, and humidity of $50 \pm 5\%$ during the experiment duration. The animals fed on a standard rodent pellet diet or high-fat diet (Sirrivasan *et al.*, 2004) with drinking ad libitum.

Generally, the protocol followed the general guidelines of animal care. All efforts were made to minimize the number used and their suffering.

Composition of HFD:

Ingredients Diet	(g/kg)
Powdered NPD	365
Lard	310
Casein	250
Cholesterol	10
Vitamin and mineral mix	60
dl-Methionine	03
Yeast powder	01
Sodium chloride	01

Induction of Diabetes Mellitus:

Diabetes mellitus was experimentally induced in male animals by streptozotocin, STZ was dissolved in cold 0.01 M citrate buffer, pH 4.5, and always prepared freshly for immediate use within 5 minutes. Rats were fasted for overnight to induce diabetes by intraperitoneal (IP) injection of streptozotocin (STZ) at the dose of 45 mg/kg body weight (Judiono *et al.*, 2011; Suharyo *et al.*, 2012; Giovana *et al.*, 2014). The normal control group was given citrate buffer without STZ. The development of diabetes was confirmed after 48 hours – 7 days of STZ injection. The animals with fasting blood glucose levels of more than 200 mg/dl were considered as diabetic and included in this study. For glucose assay, blood samples were collected from the tail-tip or tail-vein of the rats and measured using a glucometer.

Animal Grouping:

The experimental animals were divided into six groups 10 animals each, 3 non-diabetic, and 3 diabetic groups:

Experiment I:

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water at a dose of 0.7 ml/animal/day.

Group 2: Animals were fed a standard diet and received oral administration of kefir (0.7 ml/animal/day).

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir (0.7 ml/animal/day).

Experiment II:

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water (0.7 ml/animal/day).

Group B: Diabetic animals received HFD plus oral administration of kefir (0.7 ml/animal/day).

Group C: Diabetic group, fed HFD, was subcutaneously injected insulin (0.76 UI/200 mg BW/day).

After 5 weeks, all animals fasted 4-6 hour, weighted, collected their blood, anesthetized, and then they sacrificed to extract the livers for the histological investigations which were fixed using Formal saline 4% for routine paraffin sections. The collected blood from the rats was used to get serum and stored at -80°C to do a different analysis later. The kidney was immediately excised, were prepared and fixed in 4% neutral buffered formalin, then transferred to Washington State University, Veterinary School, Pathological lab, Pullman, WA, USA, for complete tissue process, 5 µm sections were stained in specific dyes such as Hematoxylin and eosin stain. Tissues were completed preparation for studies under the supervision of Dr. Abdel-baset Aref Mohamed Aref in the Autoradiographic lab. of Cell

Biology and Immunology studies, Faculty of Science, South Valley University, Egypt. The liver sections were stained according to the different examinations and techniques as follows:

Experimental Studies:

1- Cell Biological Studies.

Karyometric studies were applied to the liver sections stained with Hematoxylin and eosin stain. The volume of cell nuclei was performed using a camera program (LAS ZA). A total number of (200) nuclei were measured/animal. The measurements were carried out according to shape nucleus (rounded nuclei) and the following equation was applied: $V = \frac{4}{3}\pi r^3$. Where: **V= volume of nucleus, r = semi diameter** (Lewinski *et al.* 1984).

2-Histochemical Examinations Include:

For histochemical studies, the following histochemical techniques were applied to liver sections.

- I. DNA content changes (Feulgen reaction).
- II. RNA materials content changes (toluidine blue technique).
- III. Protein contents changes (bromophenol blue technique).
- IV. Collagen contents changes (Masson's trichrome method)
- V. Polysaccharides content changes (Periodic acid-Schiff reaction).
- VI. Phospholipid's materials content changes (Sudan Black B technique).

3-Histopathological Examination of Liver Tissue:

The liver sections were stained in specific dyes such as Hematoxylin and eosin stain for pathological studies. All histochemical and pathological methods were applied according to Carleton *et al.* (1980).

4-Gross Morphology of Liver Tissue:

The liver was dissected out and dried on filter paper. The absolute weight of the organ was determined, and its relative weight was calculated.

5- Biochemical Examination: Determination IL-6 and TNF- α :

Collected blood sera from each group were frozen after the sacrificed process in -80° C, then send to Metabolism Core Laboratory/ Human Physiology Core Laboratory, University of Alabama (USA).

- **IL-6 and TNF- α** was measured in duplicate with two Mesoscale Discovery (Rockville, Maryland 20850-3173, USA) Rat Proinflammatory Panel 2 kits using chemiluminescence. assays on 2/20/18 & 2/22/18 (Divakar *et al.*, 2017).

Statistical Analysis:

Variables with a normal distribution were expressed as mean \pm standard deviation. Variables with no normal distribution were expressed as median (25th -75th percentile). One-Way ANOVA test was used for comparing the mean of variables that were normally distributed between groups. Multiple comparisons between different groups were done using the Post hoc Tukey test. For variables that were not normally distributed, Kruskal- Wallis 1-way ANOVA test was used. Data were analyzed by using SPSS (Statistical Package for Social Science) version 24 software. P value < 0.05 was considered significant.

RESULTS

Cell Biological Studies:

Cell biological changes in hepatocytes liver (Karyometric studies):

In experiment I:

In the liver of male rats of groups 1, group 2, and group 3, the values of mean volume nuclei of the hepatocytes were $286.6 \pm 42 \mu\text{m}$, $191.2 \pm 14 \mu\text{m}$, and $247.6 \pm 44 \mu\text{m}$ (Fig. 1).

From the quantitative point of view, the daily receiving of kefir and standard food for 35 days decreased by 33.3% the value of mean volume nuclei of the hepatocytes in the liver of male rats (group 2) versus those of control rats (group 1). While the daily receiving of kefir and high diet food for 35 days increased by 29.5% the value of mean volume nuclei of hepatocytes in the liver of male rats (group 3) versus those of rats who daily received kefir and standard diet food for 35 days (group 2).

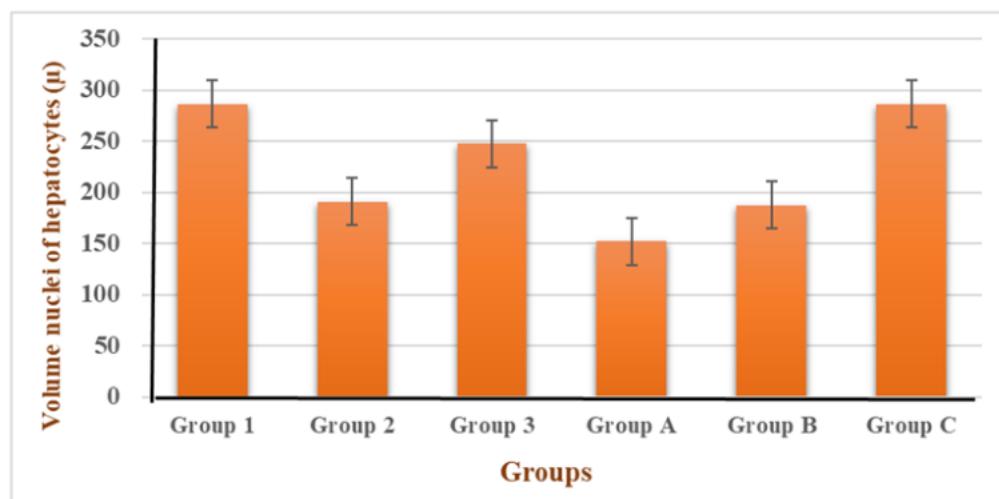


Fig. 1: Volume of nuclei in hepatocytes in liver tissue of two experiments I (group 1, 2 & 3) and experiment II. (A, B & C), Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water, Group 2: Animals were fed a standard diet and received oral administration of kefir, Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir, Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water, Group B: Diabetic animals received HFD plus oral administration of kefir and Group C: Diabetic group, fed HFD, was injected insulin

In experiment II:

The liver of diabetic male rats (group A) showed value $152.3 \pm 38 \mu\text{m}$ of the mean volume nuclei of hepatocytes, while these values were $187.5 \pm 58 \mu\text{m}$ and $286.3 \pm 58 \mu\text{m}$ in hepatocytes of diabetic male rats which treated with kefir and insulin (group B and group C) respectively (Fig. 1).

From the quantitative point of view, the daily treatment with kefir and insulin separately for 35 days increased by 23.1 % and 87.9 %, respectively, the value of mean volume nuclei of the hepatocytes in the liver of male rats of group B and group C versus those of (group A). The liver of male rats which were treated with insulin (group C) showed the value of mean volume nuclei of the hepatocytes higher by 52.7% than those of the rats treated with kefir (group B).

From the histochemical point of view, both Kefir and insulin have an inhibitory effect and stimulatory effect on the cellular activities in the examined normal and diabetic hepatocytes respectively.

Histochemical Examinations Include:

1-DNA Content Changes (Feulgen reaction):

Experiment I:

The liver of rat in C-N (group1) and C-N + Kefir (group 2) exhibited deeply stained coloration in the nuclei of cells with high DNA content, while the liver of rat in CHFD+

Kefir (group 3) showed very deeply stained coloration with very high DNA content (Table 1 & Fig. 2). From the quantitative point of view, DNA content revealed a slight increase in group 2 than those of group 1 while group 3 revealed a slight increase in DNA content than group 2.

Experiment II:

The liver of rats in Diab-HFD (group A), Diab-HFD+Kefir (group B), and Diab-HFD+ Insulin (group C) nearly showed very deeply stained coloration with very high DNA content. DNA content in both group B and group C was slightly less than group A (Table 1 & Fig. 2).

From the histochemical conception, both kefir and insulin have a slight efficacy on the DNA synthesis in the examined normal and diabetic hepatocytes.

Table 1: The histochemical score of liver of rat of groups (1, 2 and 3) in experiment I and groups (A, B and C) in experiment II stained with Feulgen reaction for DNA, toluidine blue for RNA, bromophenol blue for total protein, Mallory trichrome technique for collagen, PAS for polysaccharides and Sudan black B for lipoprotein. Results severity were classified according to number of (+).

Experiments	Experiment I			Experiment II			
	Group	G1	G2	G3	A	B	C
Feulgen reaction for DNA content in liver							
Red stained coloration		+++	++++	+++++	+++++	+++	+++
DNA contents		+++	++++	+++++	+++++	+++	+++
Toluidine blue for RNA content in liver							
Blue stained coloration		+++	+++	++	++	++	++
RNA contents		+++	+++	++	++	++	++
Bromophenol technique for protein contents in liver							
Blue stained coloration of protein content		+++	++	+	+	++	+
Protein distribution inside cells		+++	++	+	+	++	+
Masson's trichrome technique for collagen contents in liver							
Blue stained coloration of dense collagen fibers		+	++	+	++	++	++
Interstitial fibrosis		+	++	+	++	++	++
Perivascular fibrosis		+	++	+	++	++	++
PAS for polysaccharides in liver							
The red-stained coloration of glycogen content		++	+++	++	++	+++	++++
Polysaccharides precipitation inside cells		++	+++	++	++	+++	++++
Sudan Black B for phospholipids in liver							
Black stained coloration		+++	++	+++	+	++++	+
Lipoproteins contents		+++	++	+++	+	++++	+

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 2: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

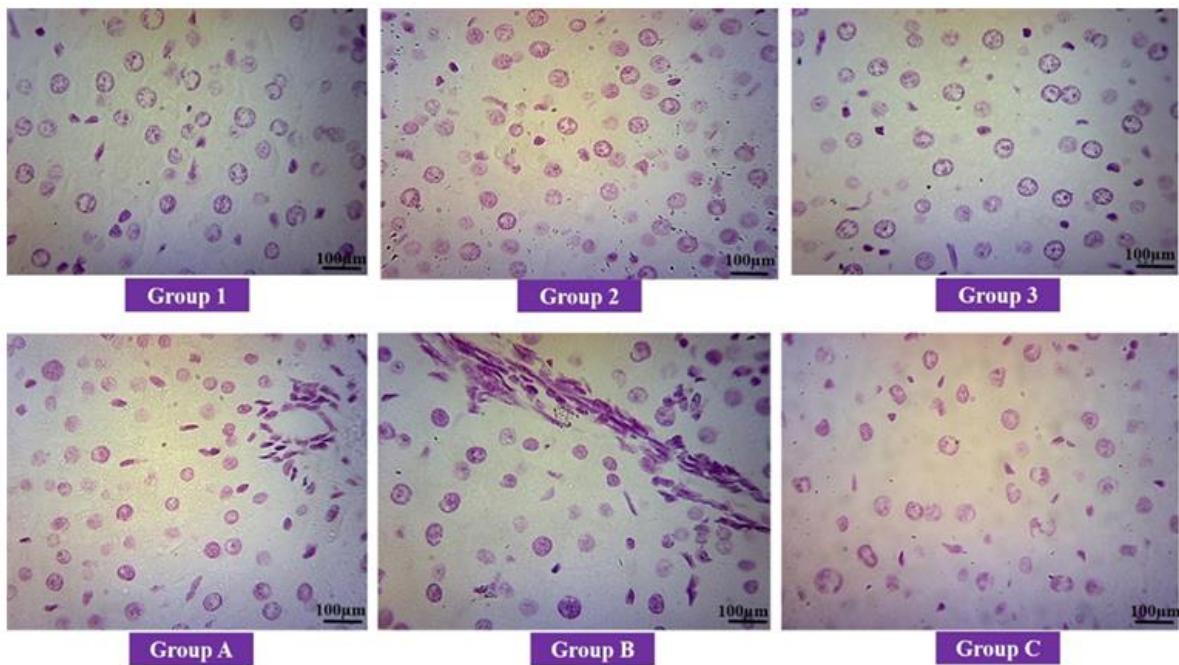


Fig.2: Photomicrograph of the liver in the six rat groups in experiment I (group 1, 2, and 3) and experiment II (group A, B, and C) stained with Feulgen method, bar = 100 μ m.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

2-RNA Materials Content Changes (toluidine blue):

Experiment I:

The liver of rats in both group 1 and group 2 showed deeply stained coloration with high RNA materials content in cytoplasm and nucleolus of the hepatocytes, while the liver of rats (group 3) showed moderately stained coloration with moderate RNA materials content (Table 1 & Fig. 3). The comparison between the animals in all groups showed no change in RNA materials content between groups 2 and 1 and a slight decrease in group 3 than group 2.

Experiment II:

The liver of rats in group A, group B, and Group C showed moderately stained coloration with moderate RNA materials content (Table 1 & Fig 3). Qualitatively, RNA content materials revealed no change between the three groups.

From the histochemical point of view, both kefir and insulin have no efficacy on RNA material contents in the examined normal and diabetic hepatocytes.

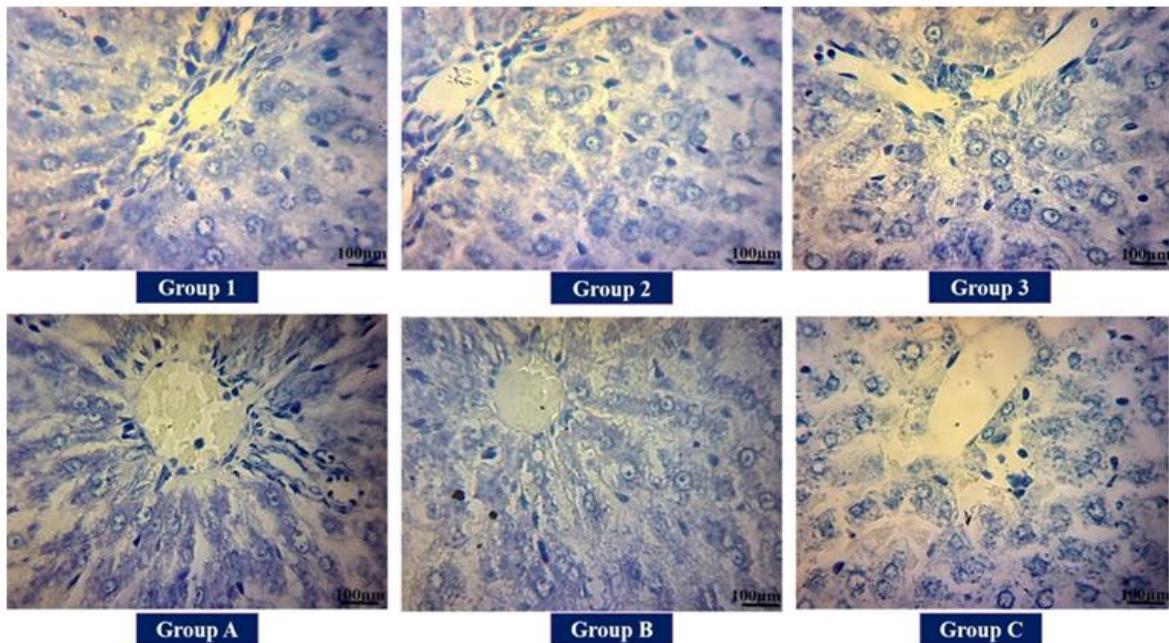


Fig. 3: Photomicrograph of the liver in the six rat groups in experiment I (group 1, 2, and 3) and experiment II (group A, B, and C) stained with Toluidine blue method, bar = 100x.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

3-Protein Contents Changes (bromophenol blue technique):

Experiment I:

The liver of rats in group 1 showed deeply dark blue spotted granules of highly protein materials equally distributed in the hepatocytes. The liver of rats in group 2 revealed moderate dark blue granules with moderate protein-stained materials equally distributed in the hepatocytes (Table 1 & Fig 4). The animals in group 2 showed a slight decrease in the total protein contents than those of the animals in group 1. While the liver of rats in group 3 revealed faint blue color with pronounced few protein-stained materials of the hepatocytes (Table 1 & Fig 4). The animals of group 3 revealed a slight decrease in the total protein contents than the animals of group 2.

Experiment II:

The liver of rats in group A and group C revealed a slightly blue color with pronounced few protein-stained materials in the hepatocytes, while the liver of rat in group B revealed moderate deep dark blue granules with sharp protein-stained materials, which is nearly equally distributed (Table 1 & Fig 4). Qualitatively, the total protein contents in the liver of animals of group B highly increased compared with those of groups A and C.

From the histochemical conception, kefir has strong stimulatory efficacy on protein synthesis in diabetic hepatocytes.

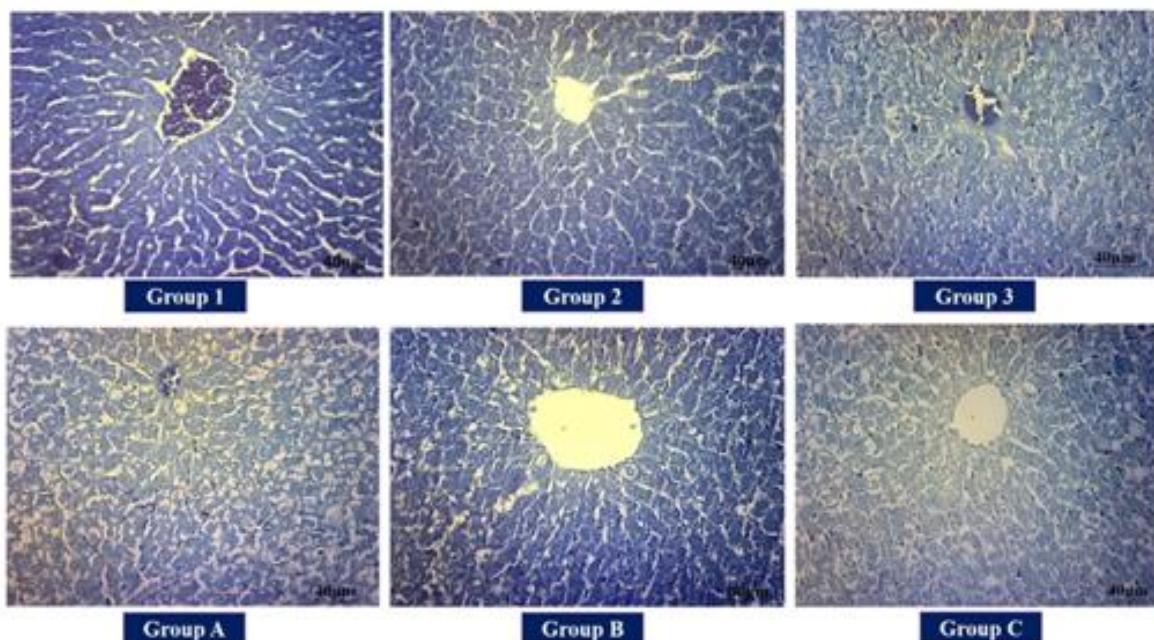


Fig.4: Photomicrograph of the liver in the six rat groups in experiment I (group 1, 2, and 3) and experiment II (group A, B, and C) stained with Mercuric bromophenol blue method, bar = 40x.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

4-Collagen Contents Changes (Masson's trichrome):

Experiment I:

The liver of rats in group 1 and group 3 showed a slightly blue color with few normally distributed collagen fibers within the hepatocytes, while the liver of rats in group 2 revealed moderate sharp dark blue with high interstitial collagen fibrosis. Also, a marked degree of perivascular fibrosis was detected (Table 1 & Fig 5). Qualitatively, the collagen fibrosis in group 2 showed a high increase than those of the animals in group 1 and group 3.

Experiment II:

The liver of animals in all groups: A, B, and C revealed moderate dark blue with moderate interstitial collagen fibrosis and perivascular fibrosis, therefore, the comparison between the groups showed no change in collagen contents (Table 1 & Fig 5).

From the histochemical conception, kefir and insulin have strong stimulatory efficacy on collagen synthesis and no effect in the examined normal and diabetic hepatocytes.

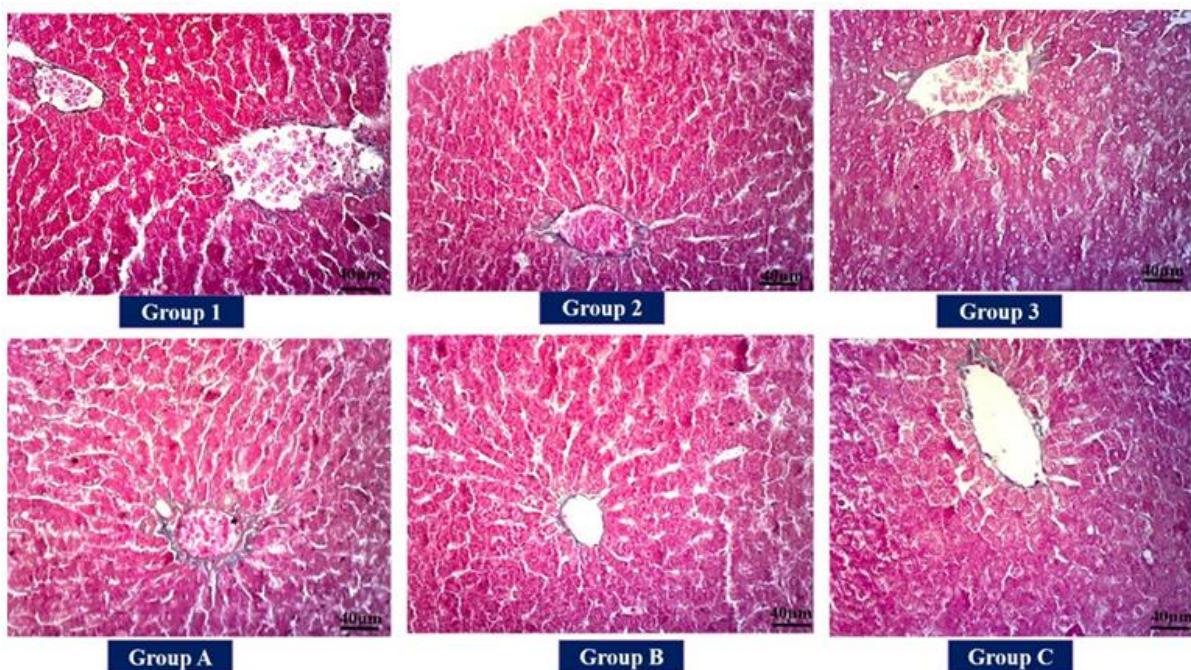


Fig.5: Photomicrograph of the liver in the six rat groups in experiment I (group 1, 2, and 3) and experiment II (group A, B, and C) stained with Masson trichrome method, bar = 40x. Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

5-Polysaccharide's Content Changes (Periodic acid-Schiff reagent) (PAS): Experiment I:

The liver of rats in groups 1 and 3 exhibited moderate red coloration with medium polysaccharides content materials in hepatocytes, while the liver of rats in group 2 revealed deeply intense coloration with high polysaccharides content (Table 1& Fig. 6). Quantitatively, the Polysaccharides content in hepatocytes of the animals in group 2 increased remarkably than those of the animals in group 1, while it was low remarkably in group 3 than group 2.

Experiment II:

The liver of rats in group A exhibited moderate red coloration with moderate Polysaccharide's content materials in hepatocytes. The animal's liver of group B revealed deeply intense coloration with highly polysaccharides content materials, while the animal's liver in group C revealed very deeply coloration with very highly polysaccharides content materials in hepatocytes (Table 1 & Fig. 6). Quantitatively, the polysaccharides content materials in hepatocytes nearly highly increased in the rats of group B and group C versus those of the animals in group A.

From the conception of histochemistry, both kefir and insulin have high stimulatory efficacy on polysaccharides material contents in the examined normal and diabetic hepatocytes.

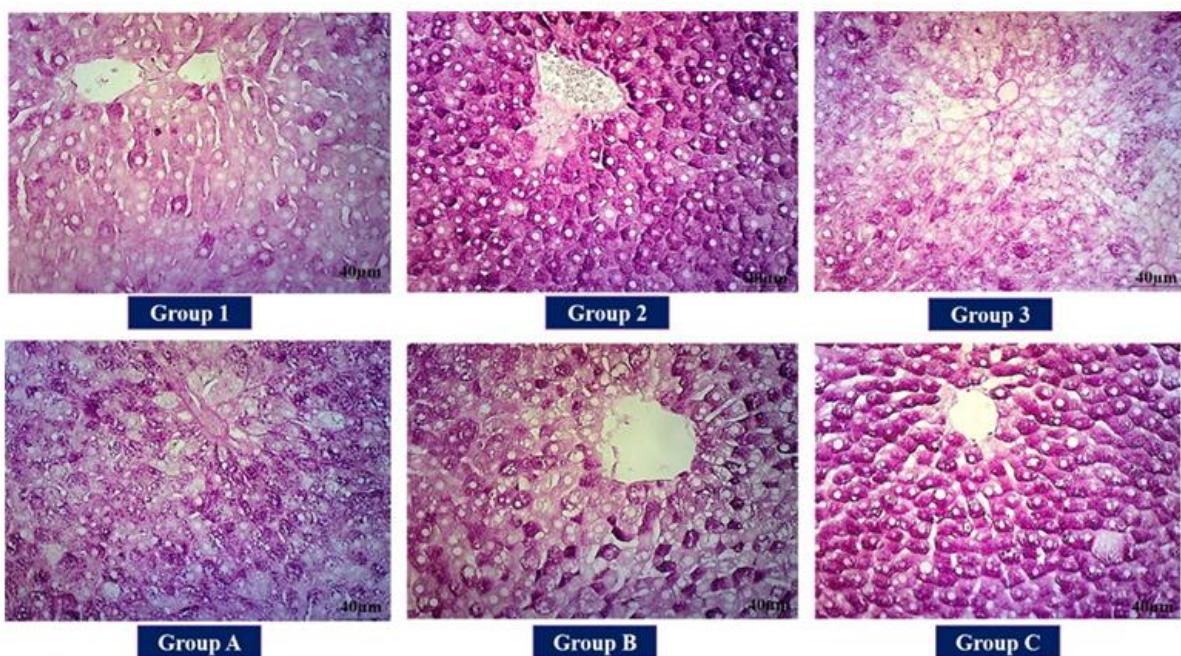


Fig. 6: The carbohydrates distribution and condenses in the liver tissue in the different rat groups; PAS, bar= 50μm.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

6-Phospholipid's Materials Content Changes (Sudan Black B technique):

Experiment I:

The liver of rats in group 1 and group 3 exhibited deeply black blue coloration with highly phospholipids materials content in hepatocytes. While the liver of rats in group 2 revealed moderate black blue coloration with moderate phospholipids content (Table 1 & Fig. 7). Quantitatively, the Phospholipids materials content in group 2 was lower medullary than those of the rats in group 1 and group 3.

Experiment II:

The liver of rats in group A and group C exhibited faint black blue coloration with few phospholipids materials content in hepatocytes, however, the liver of rats in group B revealed very deeply black blue coloration with very highly phospholipids content in hepatocytes (Table 1 & Fig. 7). Quantitatively, the Phospholipids content in group B very highly increased than those of the rats in groups A and C.

From the histochemical point of view, kefir has an inhibitory and very highly stimulatory effect on the phospholipid's materials synthesis in the normal and diabetic hepatocytes respectively.

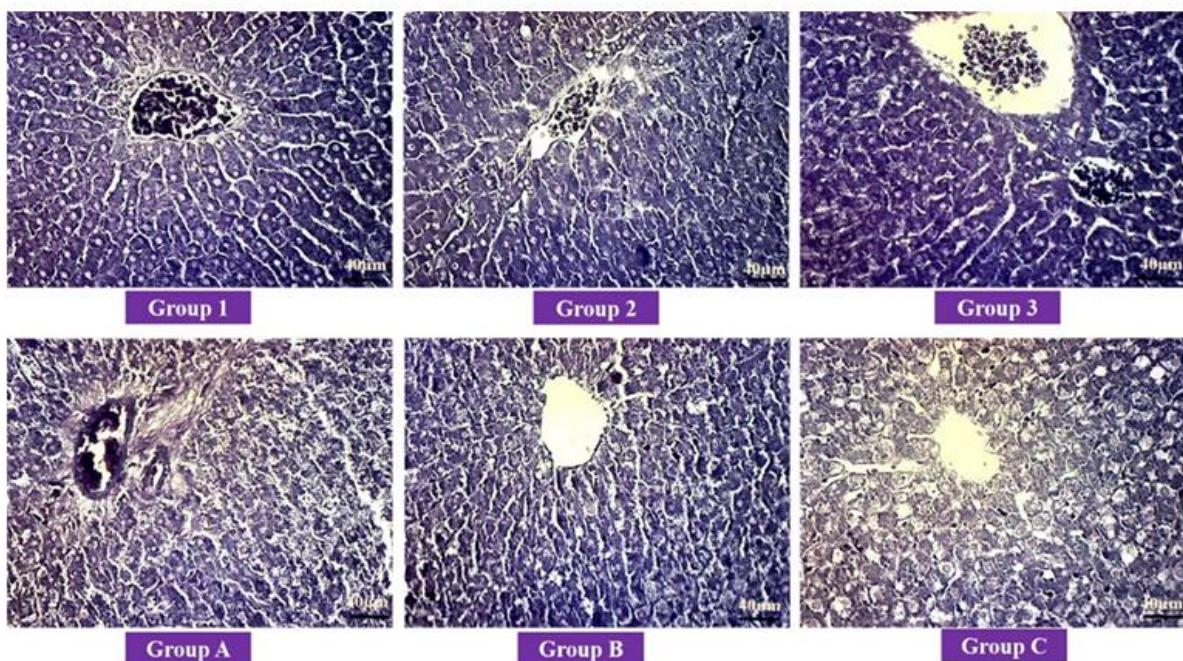


Fig.7: Photomicrograph of the liver in the six rat groups in experiment I (group 1, 2, and 3) and experiment II (group A, B, and C) stained with Sudan black B method, bar = 40x.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

3-Histopathological Examination Of Liver Tissue:

Experiment I:

Figures (8) and (9) showed the pathological changes that occurred in the liver in the six rat groups using different magnifications. The liver of rats in C-N (group 1) revealed a radial arrangement of hepatocytes around the central vein. The liver of rats in C-N + Kefir (group 2) revealed normal histological hepatic lobules composed of radially arranged hepatocytes around a central vein with a normal portal triad containing artery, vein, and bile ducts.

The liver of rat CHFD+ Kefir (group 3) revealed the almost normal architecture of hepatic lobules and hepatic tissue compared to the control group except for minimal hepatic vacuolation especially relate to the portal triad and mild fatty degeneration in the periportal hepatic zone (circled); higher magnification revealed cytoplasmic circumscribed vacuolation of fat droplets in the periportal zone of the hepatic lobule and distinct cytoplasmic vacuoles of fat droplets in periportal hepatocytes.

From the pathological point of view, kefir maintains a normal histological structure of the liver in normal rats.

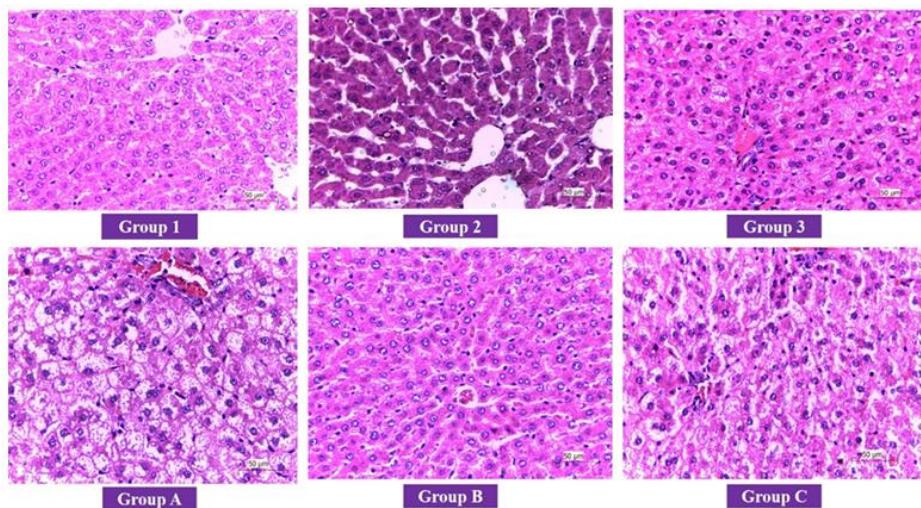


Fig.8: Pathological changes in the liver in the six rat groups, in experiment I (group 1, 2, and 3) and experiment II (group A, B, and C) H&E, bar = 50 μ m.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

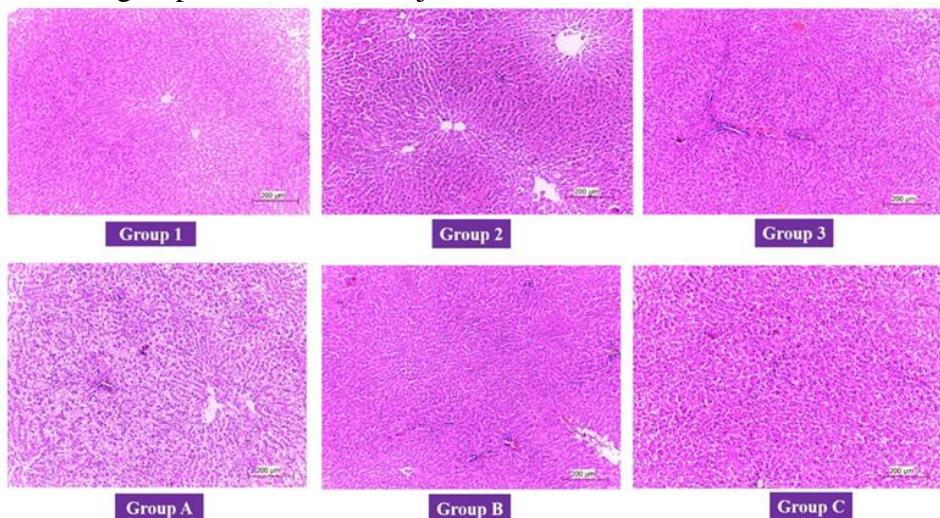


Figure (9): Pathological changes in the liver in the six rat groups, in experiment I (group 1, 2, and 3) and experiment II (group A, B, and C) H&E, bar = 200 μ m.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

Experiment II:

The liver of rat in Diab-HFD (group A) revealed extensive cytoplasmic vacuolation of hepatocytes and smallness of hepatic lobules (circled) due to necrosis of hepatocytes between the portal areas (black arrows), higher magnification revealed extensive cytoplasmic vacuolation of hepatocytes including hydropic (indistinct vacuoles) and fatty (distinct vacuoles) degeneration, also necrotic changes of hepatocytes including pyknotic, karyorrhectic and karyolytic nuclear changes; we could notice smallness of hepatic lobules and narrowing the distance between portal triads, hepatocytes were vacuolated with hydropic and fatty degeneration.

The liver of rat in Diab-HFD+Kefir (group B) revealed almost similar histological picture to the control group unless mild hydropic and fatty degeneration in (one rat of the group), higher magnification revealed almost similar histological picture to control group with mild hydropic and fatty degeneration in hepatocytes related to the portal area in one rat. The liver of rats in Diab-HFD+ Insulin (group C) revealed diffuse vacuolar degeneration in hepatocytes (3 cases) with mild vacuolization of hepatocytes, higher magnification displayed vacuolated hepatocytes around the central vein (CV).

From a pathological point of view, kefir, to some extent, completely repairs all the side effects of type1 diabetes on the hepatocytes in diabetic rats.

From the pathological point of view, kefir, to some extent, maintains a normal histological structure of the liver in normal rats and completely repairs all the side effects of type1 diabetes on the hepatocytes in diabetic rats.

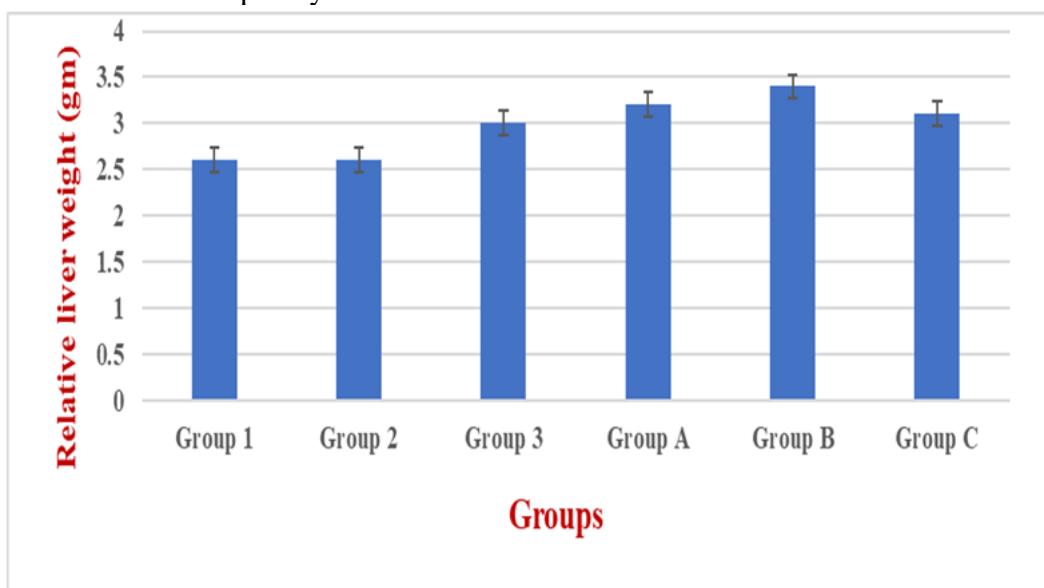


Fig.10: Relative weight of liver of two experiments (I and II) groups at the end of the experiment.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

4-Gross Morphology of Liver Tissue:

The mean relative weight of the liver was showed in figure 10 which indicated non-significance appearance in both experiments I and II.

5-Biochemical examination: Determination of IL-6 and TNF- α :

-Serum IL-6 (pg/ml):

After analyzed serum IL-6 marker in both experiments, in experiment I, it was found increase significance between the three groups, similarity in experiment II was significantly increased between group B and A; group C and B, the results represented in figure 11. The fewer data were in groups one and two while the diabetic ones were with high levels, in the meantime, diabetic ones treated with kefir and insulin showed slight improvement.

-Serum TNF- α (pg/ml):

Regarding the measurement of the serum TNF- α marker and calculated the mean, data showed in figure 12. There was non-significance and the values were less in experiment I (the normal animals) while higher in the experiment II (the diabetic animals) with noticeable improvement in diabetic groups treated with kefir and insulin.

Biochemically, kefir has a significant effect on serum IL-6 and Serum TNF- α levels in the examined normal and diabetic rats.

Biochemically, kefir has a significant effect on serum IL-6 and Serum TNF- α levels in the examined normal and diabetic rats.

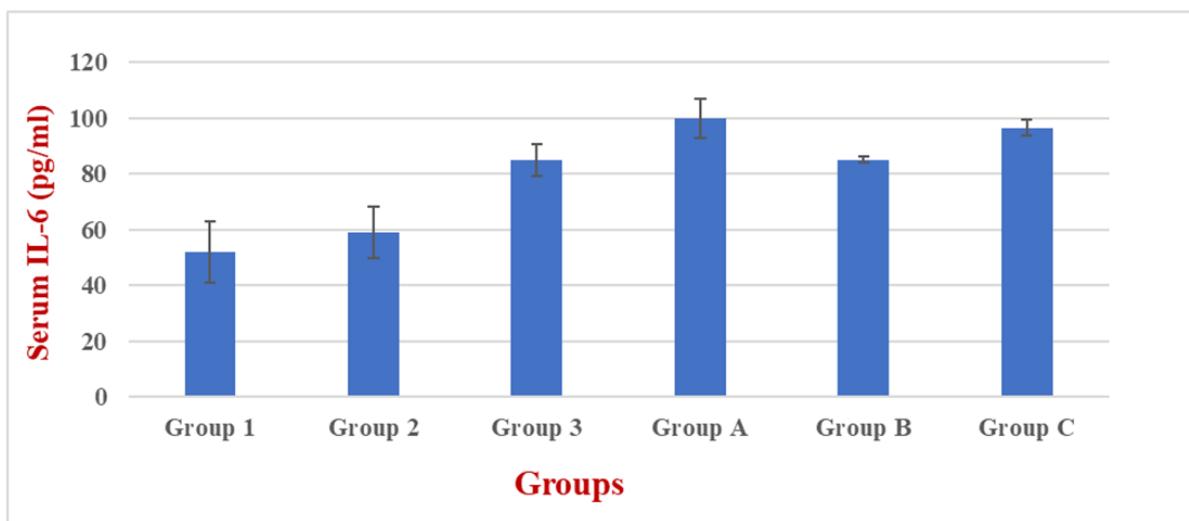


Figure 11: Serum IL-6 (pg/ml) for two experiments (I and II) groups at the end of the experiment.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water.

Group 2: Animals were fed a standard diet and received oral administration of kefir.

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir.

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water.

Group B: Diabetic animals received HFD plus oral administration of kefir.

Group C: Diabetic group, fed HFD, was injected insulin.

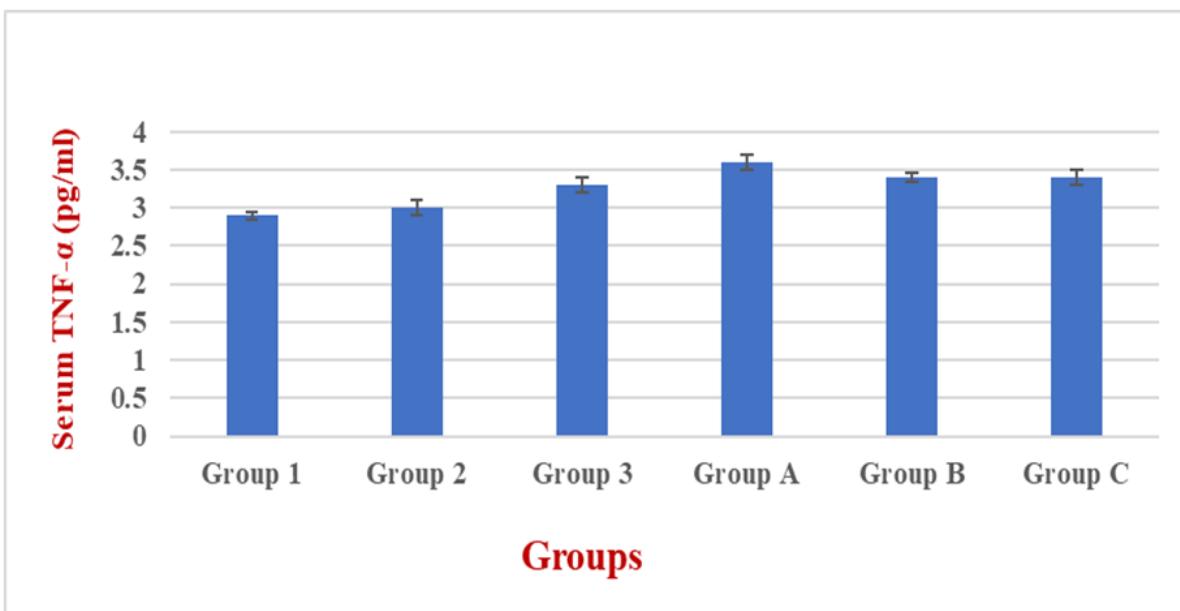


Fig. 12: Serum TNF- α (pg/ml) for two experiments (I and II) groups at the end of the experiment.

Group 1: Control animals, (negative group) were fed a standard diet plus oral administration of distilled water

Group 2: Animals were fed a standard diet and received oral administration of kefir

Group 3: Animals received a high-fat diet (HFD) and additionally oral administration of kefir

Group A: Diabetic group (positive group), was fed HFD and received oral administration of distilled water

Group B: Diabetic animals received HFD plus oral administration of kefir

Group C: Diabetic group, fed HFD, was injected insulin.

DISCUSSION

In the present work, the cell biological, histochemical, and pathological data showed that:

Both kefir and insulin revealed beneficial various changes in the volume of nuclei and the histochemical components such as DNA, RNA, total protein, collagen, polysaccharides, and phospholipids materials in the examined normal and diabetic hepatocytes of the male rats. Kefir, to some extent, maintained the normal histological architecture of the liver in normal rats and completely repaired all the side effects of type1 diabetes on the hepatocytes in diabetic rats.

The death rate in DM adults is 2-4 times higher than for non-DM adults, with cardiovascular diseases (CVD) being the most common cause of death (Roger *et al.*, 2012).

The consumption of probiotics may decrease the serum level of glucose and glucose tolerance in diabetes (Davari *et al.*, 2013; Zhang *et al.*, 2014).

Kefir is one of the fermented kinds of milk derived from various species of lactobacilli. It is known that kefir has a lot of biological activities including antitumor, immunostimulating effect, an antioxidant action by reducing the lipid peroxidation, an antidiabetic effect, antibacterial and antifungal properties (Quiro's *et al.*, 2005).

Therefore, our present study tends to investigate the effect of consuming kefir as a common probiotic product that is highly spread all over the world as a healthy drink.

Several reports have been published on the effect of streptozotocin on the glycemic state of different animal species. (Gunnarson *et al.* 1974; Annamala and Augusti, 1980;

Yamamoto *et al.*, 1981; Uchigata *et al.*, 1983; Okamoto 1984; Al-Awadi *et al.*, 1985; Noreen *et al.*, 1988, 1992; El-Seifi *et al.*, 1993 a, b; Rawi 1995; Rawi *et al.*, 1996; Abdel-Moneim *et al.*, 1997, 1999; Judiono *et al.*, 2011; Suharyo *et al.*, 2012; Giovana *et al.*, 2014).

Animals with chemically induced diabetes have been used to study either insulin-dependent diabetes mellitus (IDDM) (Wilson *et al.*, 1986; O'Brien *et al.*, 1993; Mathe 1995; Ulicna *et al.*, 1996; Rawi *et al.*, 1996; Ohno *et al.*, 1998; Abdel-Moneim *et al.*, 1999) or non-insulin-dependent diabetes mellitus (NIDDM) (Ostenson *et al.* 1989; Ali *et al.* 1993; Masiello *et al.*, 1998).

"From a biological point of view, the chemistry of cellular structure and function is well established. Therefore, studying the chemical components in their natural locations in the cells and tissues, and tracking the changes that occur to them under abnormal conditions, whether pathological or experimental, is very important, as any change that occurs to these substances is often accompanied by some pathological manifestations" (Aref *et al.* 2021).

Through the previously published works, it is clear that there is little or no research that concerning the effect of Kefir or HFD, or insulin on the volume of nuclei and histochemical contents, such as DNA, RNA, proteins, collagen, polysaccharides, and lipoproteins components in the liver of both control and HFD-diabetic male rats.

The basis on these data and reviewing the deficiencies relative to the effect of Kefir and/or insulin on the cellular activities and histochemical material contents in the liver of both normal and HFD-diabetic male rats, we discussed the results study of investigated liver without comparing it to publications of others authors dealing with the same study /organ.

Cell Biological Changes in Hepatocytes (Karyometric studies):

In experiment I:

From the cytological point of view, the present results showed that the kefir has an inhibitory effect on the cellular activities of hepatocytes in the liver of normal male rats. Also, high diet food has a stimulatory effect on the cellular activities of hepatocytes in the liver of kefir-received male rats.

In experiment II:

From the cytological point of view, although both kefir and insulin have a stimulatory effect on the cellular activities of hepatocytes in the liver of diabetic male rats insulin stimulatory effect is higher than that of kefir.

From the histochemical point of view, both Kefir and insulin have an inhibitory effect and stimulatory effect on the cellular activities in the examined normal and diabetic hepatocytes respectively.

The histochemical examination methods used for the determination of DNA content, RNA materials content, total proteins content, collagen fibers, polysaccharides, and phospholipids content in the liver revealed variable differences between the treated groups.

In the liver tissue, feeding the normal animals with kefir or HFD+kefir increased the DNA content slightly in experiment I, while the DNA content was nearly the same in all diabetic groups in experiment II.

From the histochemical conception, both kefir and insulin have a slight efficacy on the DNA synthesis in the examined normal and diabetic hepatocytes.

The RNA material contents nearly had no differences in both the experiment I and II between the compared groups.

From the histochemical point of view, both kefir and insulin have no efficacy on RNA material contents in the examined normal and diabetic hepatocytes.

The total protein contents in compared animal groups were gradually decreased cause of feeding with kefir or HFD+ kefir in experiment I, otherwise, the treated diabetic animals with kefir showed a slight increase in their total proteins content than the others.

From the histochemical conception, kefir has strong stimulatory efficacy on protein synthesis in diabetic hepatocytes.

In experiment I; the animal group fed on SD+ kefir showed a slight increase in collagen fibrosis than other groups, while in the diabetic animal groups, there was nearly the collagen fibrosis between them.

From the histochemical conception, both kefir and insulin have strong stimulatory efficacy on collagen synthesis and no effect in the examined normal and diabetic hepatocytes.

Regarding the polysaccharides content in the experiment, I, the normal animals fed on standard diet+ kefir showed a slight increase in their polysaccharides content than the others may be due to feeding daily kefir. while the diabetic animals treated with kefir or insulin in experiment II showed a noticeable increase in the amount of polysaccharide than the untreated diabetic ones.

From the conception of histochemistry, both kefir and insulin have high_stimulatory efficacy on polysaccharides material contents in the examined normal and diabetic hepatocytes.

In the experiment, I the normal animal group fed on SD+kefir showed less decrease in the phospholipid materials content, while in experiment II, the diabetic animals treated with kefir showed highly increase in their phospholipid materials content comparing with the other groups.

From the histochemical point of view, kefir has an inhibitory and very highly stimulatory effect on the phospholipid's materials synthesis in the normal and diabetic hepatocytes respectively.

From the pathological point of view, our results revealed kefir, to some extent, maintains a normal histological structure of the liver in normal rats and completely repairs all the pathological side effects of type1 diabetes on the hepatocytes in diabetic rats. Also, insulin repair some pathological side effects of diabetes.

Scientifically, the histochemical and pathological results are cumulative results; Therefore, some of the present weak and slight results are maybe due to the short term of the experiments.

Histopathological findings in some studies supported that the liver of the kefir-added group, close to the normal histological structure was observed; it concluded that consumption of kefir beverage would be beneficial against T2DM which cause serious damage to the liver (Bülent *et al.*, 2017).

In diabetic liver degenerative changes in hepatocytes (Benjamin *et al.*, 2006; Mir and Darzi 2009), severe lipids in and vacuolization in bile dutepitelyum can be noticed Charlton (2004).

Our data go with several studies, the antioxidant, hepatoprotective of the kefir (Hoolihan, 2001; Ozsoy, 2016).

The mean relative weight of the liver was calculated in each group at the end of the experiment, P value showed non-significance between the six groups; the values were nearly the same in the untreated and treated rat males, these results agree with Urdaneta *et al.* (2007); Sahin and Yardimci (2009) showed that using kefir supplemented diet had no significant differences the weight of the body organs examined and agree with that kefir has the efficacy to prevent the loss in body weight of diabetic male rats, while neither affects abdominal circumference nor relative fat weight (Aref *et. al.*, 2020).

Several studies reported that using probiotics did not increase liver weight (Jin *et al.*, 1998; Ozcan E 2003). Similarly, Mohan-Kumar and Christopher (1988) reported a significant decrease in liver weight due to lactobacillus and other beneficial microorganisms, which are present in probiotics, which prevent pathogens from colonizing the gastrointestinal tract with

the decrease in harmful microflora of the intestine, so that the liver would be under a less pressure for detoxifying these byproducts (Mohan-Kumar and Christopher 1988).

Cenesiz *et al.* (2008) reported that no changes have been observed in liver weight in response to the various doses of Kefir treatment.

Also, overall, of the seven studies reporting changes in fat mass and different organs showed a difference with non-significant because effects sizes were small (Borgeraas *et al.*, 2018).

Diabetes mellitus is a disease with acute and chronic complications, the most serious complications include pancreatic necrotic and degenerative changes in the liver (Mir and Darzi 2009; Sağkan *et al.*, 2015).

Biochemically, kefir has a significant effect on serum IL-6 and Serum TNF- α levels in the examined normal and diabetic rats.

The biochemical markers that were analyzed in the blood serum, it was included IL-6 and TNF- α ; The results showed significance between all the group trials, it was found significantly decreased in groups treated with kefir beverage and insulin.

Kefir treatment of type 1 DM rats led to a decrease in the pro-inflammatory cytokines IL-1 and IL-6 as well as an increase of anti-inflammatory IL-10 compared to control groups (Aune *et al.*, 2013). Kefir-induced reduction in the inflammatory response (Firouzi *et al.*, 2016).

In agreement with these results, it has been shown that kefir reduced pro-inflammatory cytokines, including TNF α in DM (Hadisaputro *et al.*, 2012; Tonucci *et al.*, 2015).

Prebiotics and probiotics have a great effect on insulin sensitivity, inflammatory markers (Musso *et al.*, 2010).

Consumption of kefir has been associated with several health-promoting properties, such as antimicrobial (Rodrigues *et al.*, 2005) and anti-inflammatory (Lee *et al.*, 2007).

There is evidence that kefir and its polysaccharide extract possess anti-inflammatory activity (Rodrigues *et al.*, 2005).

An anti-inflammatory effect was also observed when L. Plantarum isolated from kefir was administered orally in the mouse model (Lee *et al.*, 2007).

From the cell biological and histochemical point of view, both kefir and insulin have beneficial various effects on cellular activities and the histochemical components such as DNA, RNA, total protein, collagen, polysaccharides, and phospholipids materials synthesis in the examined normal and diabetic hepatocytes of the male rats. From the pathological point of view, these various effects may make kefir, to some extent, maintain the normal histological architecture of the liver in normal rats and completely repair all the side effects of type1 diabetes on the hepatocytes in diabetic rats. Also, insulin repair some of the pathological side effects of diabetes type 1 on the hepatocytes.

The histochemical and pathological results are accumulated results; therefore, the present week and slight results are maybe due to the shortest term of the experiments.

Conclusion

We can say that the beneficial various cell biological and histochemical effects of kefir pathologically may make kefir, to some extent, completely repair all the pathological side effects of type1 diabetes on the hepatocytes in diabetic rats.

The positive results of using kefir and insulin in treating the Pathogen effects of type 1 diabetes on the liver of male rats make it possible to obtain positive clinical applications/implications. Therefore, these positive results encourage us to continue working and complete the various long-term pre-clinical trials and clinical trials phases.

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Competing Interests:

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