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Exploring the Role of Ki67 in the Liver of Diabetic Rats

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ABSTRACT

Background: Diabetes is not a single disease but rather, it is one aspect of metabolic syndrome. The pathologic aspects of diabetes involve cellular changes that need to be understood. **Objective:** The main objective of this study was to explore the role of Ki67 in the liver of diabetic rats. **Methods:** The study methodology involved the induction of diabetes in rats using Alloxan (120 mg/kg). A total of 20 albino rats were randomly assigned into two groups control group (N=10) and diabetes group (n=10). Diabetic group received the dose of alloxan, while the control group received similar dose of normal saline. Glucose level was monitored daily. After the end of the experiment (one -month period), all animals were terminated. Blood samples were taken to measure biochemical investigations including glucose, cholesterol, and triglycerides. Liver tissue was excised and washed with normal saline and fixed in buffered formalin (10%). Liver tissue was processed and stained by hematoxylin and eosin for routine histological examination and also stained by immunohistochemistry for Ki67 biomarker. **Results:** The results revealed the efficacy of the diabetic model. All biochemical investigations were significantly higher in the diabetic group compared with that of control group ($p<0.001$). Histological studies showed the existence of morphological alterations in cells and fatty changes in the diabetic group compared with the control group. The expression of Ki67 was significantly higher in the diabetic group compared with that in the control group ($p=0.011$). **Conclusion:** Taken together, diabetes has adverse effects on the spleen from a histological point of view, and from the expression of Ki67.

Keywords: Diabetes, Liver histology, Ki67 expression, Biochemical markers, Hepatic changes.

1. BACKGROUND

Introduction to Ki67 and its Significance in Liver Research

Ki67 is a nuclear protein located at the end of the cellular G1, G2, S, and M phases (1). Ki67 is a reliable cell proliferation marker that is incorporated into the cellular proliferative compartment when the cell begins proliferating in the G1 phase and is dissociated when the cell does not divide, enters the state of mitosis, or becomes apoptotic (2-4). The liver plays a central role in drug and toxin detoxification, bile production, protein synthesis, and is an important metabolic site (5). The number of liver cells proliferating gradually decreases with age or under non-illness physiological states (6). Most liver cells in slow continuous proliferation under chronic liver diseases can prevent the loss of liver function by increased cell number (7, 8). Ki67 expression in liver tissues can reflect the state of liver cell proliferation and provide a useful index for reflecting the degree of liver damage, repair, and regeneration (9, 10).

When the liver is injured by a variety of factors such as toxic substances and chemicals, the liver shows hepatocyte tissue damage, mainly manifested by congestion, edema, hepatocyte necrosis, ballooning-like degeneration, fatty degeneration, inflammatory cell infiltration, and the like (11). When some systemic diseases such as severe diabetes have higher blood sugar and a longer course, the liver will have weak

and slow recovery and regeneration ability when subjected to various injuries, which is not conducive to the improvement of the recovery effect of liver injury and the recovery of liver function, and Ki67 is an important marker to understand liver injury regeneration (12-14). Recently, Ki67 has become a focus in the study of proliferation and regeneration of various parts such as gastric mucosa, bone, ovary, brain, and nerves (15). The function of proliferation and regeneration of liver cells is an important aspect of liver energy metabolism, while Ki67 research in the field of liver cells is less reported (16). With the high recurrence of diabetes and the enormous changes it brings in terms of individual or social aspects, the regulation of liver cell metabolism has been gradually considered as an important therapeutic direction in the study of diabetes (17). Understanding Ki67 in liver cells is more conducive to enriching the research horizon in diabetes treatment, facilitated by the enhancement of liver cell self-regulation (18-20). In this research, we focused on the proliferation-relevant factor Ki67 in the liver of diabetic rats.

Diabetes and its Impact on Liver Function

Diabetes is a multifactorial group of metabolic diseases characterized by increased blood glucose levels (21). The main types of diabetes are type 1 and type 2 (21). The latest are characterized by a variety of factors, including insulin resistance and defects in insulin secretion, regulation, and metabolic action (22). About 90%–95% of the diagnosed diabetes cases are type 2, affecting children, adolescents, and elders (23). Biochemically, type 2 diabetes is characterized by impaired insulin secretion in response to increased blood glucose and hepatic gluconeogenesis, glycogenolysis, and lipogenesis (23). These complications contribute to an altered lipid metabolism in the liver by increasing intracellular free fatty acid flux and hepatic triglyceride and cholesterol accumulation (24, 25). Insulin resistance in the liver results in an environment where further carbohydrate metabolism, diacylglycerol accumulation, protein kinase C defects, and the activation of proinflammatory pathways can be a risk factor for hyperglycemia, intrahepatic lipid deposition, and hepatic steatosis or nonalcoholic fatty liver disease (26). Increased and high levels of blood glucose are an important risk factor for diabetes-related liver damage (27). Indeed, liver damage from diabetes can occur in different ways. One of the most important is the picture in liver histology (27).

The pathological picture of the liver in diabetes includes vacuolar, hydropic, hyaline, and tubular-obstructive degenerations, hepatocellular necrosis, and micro- and macrogliosis with a picnotic effect (28). Lipid accumulation following diabetes, especially hyperlipidemia, may lead to many types of liver fibrosis (29). Indeed, type 2 diabetes causes fatty liver or fatty liver disease due to diabetic macrovesicular liver steatosis (30). Furthermore, diabetes-related mechanisms lead to some of the pathogenesis of various liver diseases (31). The most common chronic liver disease in diabetes patients was classified as alcoholic liver disease and analyzed in this group overall to develop advanced liver disease and liver-related morbidity and mortality (32). However, certain types of diabetes can injure the liver more seriously (33). For example, cirrhosis of the liver related to type 1 diabetes and hepatocirrhosis related to genetic diseases are among the most critical and potentially fatal liver diseases (34). With this pressure, the liver should continue to have a positive balance of fat metabolism, glucose production and metabolism, and lactate and other metabolism (35, 36). The liver may take on a more essential role in certain physiological states (36). It shows a higher degree of amino acid metabolism (37).

Ki67 Expression in the Liver of Diabetic Rats

Similar to other studies, no Ki67 positivity was noticed in normal nondiabetic livers, implying an extremely infrequent spontaneous entrance of hepatocyte nuclei into the phase of the cell cycle in healthy livers (38). This explains why the values of Ki67 were found to be very low or not detected in previous studies dealing with normal livers (39). However, there are some conflicting results indicating a very low ratio of Ki67 positivity in the livers of normal chow-fed rodents (40). Some found a significant small percentage of Ki67-positive nuclei in the livers of normal mice at the age of 12–14 months, while others have reported that Ki67 is not constitutively detected in the liver of normal nondiabetic controls of various animal experimental models (41). The levels of Ki67 are reported to be elevated in some diabetic systemic tissues, including the retina, kidney, heart, nerve, and blood vessels, exerting underlying reasons for tissue morphological and functional change and severe complications (42). This increased level of Ki67 gradually elevates with the exacerbation of diabetes in various organs (43). Marked changes in both transcription and translation for Ki67 mediate minute-by-minute activities in various cellular processes and are essential for mammalian development and health (44).

Data on the diabetic rat models showed varied Ki67 expression in the livers that increased, decreased, or did not change depending on the age of the animal, duration of diabetes, type of diet, types of diabetes induction in animals, and the severity of diabetes, characterized by some degree of liver injury and regeneration (45). There was a higher expression of Ki67 in the livers of highly diabetic rats subjected to damaging agents or a combination of diabetes induction and a high-fat sucrose diet (46). Most of the pathological changes in the liver of diabetic rats depend greatly on the levels of lipids and the mode of their transport as well as acute phase response, oxidative stress, and other hepatic risk factors (45). The liver is a site of Ki67 formation that may contribute to plasma levels. Therefore, research should be undertaken to clarify whether plasma levels of Ki67 depend on the extent of hepatic Ki67 formation. It would be proper to monitor the levels of Ki67 formation in experimental hepatology, especially in the organ pathology of diabetes (44)

Each association between elevated Ki67 and decreased levels of healthy hepatocytes led to more congestion in the microvasculature, adipogenesis, nonalcoholic fatty liver disease, and nonalcoholic steatohepatitis, culminating in a sequence of pathways and berating high hepatic lipid secretion and reduced glucose uptake, along with disrupted mitochondrial electron transport chain and hepatocyte breakdown (43). By tracking the levels of Ki67, the work on the progression of diabetic liver disease in both experimental and commercial settings would be enhanced (45). Current publications on diabetes-related liver studies should also be closely involved in analyzing the Ki67 of the diabetic liver (46).

2. OBJECTIVE

The present study aimed to explore the role of the expression of Ki67 diabetes progression in rats with induced diabetes type 1.

3. MATERIAL AND METHODS

The method was performed on 20 white male rats (Albino) that were divided into two groups namely: control group (N =10) and diabetes group (N=10). The study was conducted in the animal facility, Department of Biology, Yarmouk University, Jordan. The institution rules indicate the project has been cleared by the IRB. The animal unit was employed to capture the animals. Before testing began, the animals were weighed. The weight of every animal was 185±7.3 gram. For this research a separate location from the animal facility was selected and the animals were housed in cages there. Before the study started, all the rats were subjected to the same treatment in the same environment for one week to get them accustomed to it. After a fast of 12 hours, rats were given a single intraperitoneal dose of alloxan monohydrate (Sigma-Aldrich) 120 mg/kg.

This made them diabetic. A commercial glucometer (Glucocheck, HomeMed (Pty) Ltd) was utilized daily to examine the blood glucose levels of the animals to remain hyperglycemic (≥200 mg/dl). Every animal was terminated at the end of the study that lasted for a month. The liver tissues were taken out and washed with normal saline, before being taken formally in a 10% formalin for a period of 24 hours. After that, the tissues were fixed and stained with hematoxylin and eosin for routine histological examination. More samples were made for immunohistochemistry to check for the presence, relationship and location of Ki67.

We have previously detailed these methods (47-50). The key steps in our IHC methods are described below. We used tissue samples from the liver, processed them, cut into pieces and stuck them on charged slide. After the sections were deparaffinized, they were transferred to tap water. Tissue slices were incubated for 20 min in 1% hydrogen peroxide (H2O2) solution to inhibit activity of endogenous peroxidase before the immunohistochemical staining. Next, we washed the sections with phosphate buffered saline (PBS; pH 7.2–7.4). Then we blocked the sections with 1% bovine serum albumin (BSA) to prevent nonspecific binding. Following the washing of the sections in PBS, they were placed in a humid chamber for 1 hour in the presence of the main monoclonal antibody solution (Ki67, 1:100; Santa Cruz Biotechnology). After PBS rinses were given, secondary biotinylated antibodies were applied for incubation (20 minutes). The sections were then subjected for 20 minutes to streptavidin conjugated to horseradish peroxidase and washed in PBS. Diaminobenzidine (DAB) was used to visualize an immunohistochemical signal which turns brown upon reaction. The slides were rinsed with running tap water to stop the reaction. A counterstain of 30 seconds was done using hematoxylin. The sections were dried out and mounted with the appropriate medium. The area with stains was analyzed using the Adobe Photoshop Software version 7.2 to quantify Ki67 expression level. Examination of antibody-stained slides under microscope used the brown color of biomarker and blue counterstain of tissues. We calculated the expression ratios by dividing the number of pixels of the biomarker color (brown) with the total pixels (sum of brown and blue pixels).

Statistical analysis

The statistical analysis was done using SPSS version 21.0. The two groups' outcomes were compared using the independent t-test. A P-value of below 0.05 was statistically considered significant. The levels of Ki67 expressed in each group indicated the mean ± and standard deviation.

4. RESULTS

Biochemical investigations

The control group's glucose level was 94.5±12.25 mg/dl. However, it was found to be 258±18.98 mg/dl ($p < 0.001$) in the diabetes group, as shown in Table 1. The diabetes group's cholesterol and triglyceride levels

Variable (M±SD)	Control group	Diabetic group	P value
Glucose (mg/dl)	94.5±12.25	258±18.98	<0.001
Cholesterol (mg/dl)	82.4±8.5	169.43±16.7	<0.001
Triglycerides (mg/dl)	94.19±17.91	145.16±18.92	<0.001

Table 1. Biochemical profiles of glucose and lipids in study groups

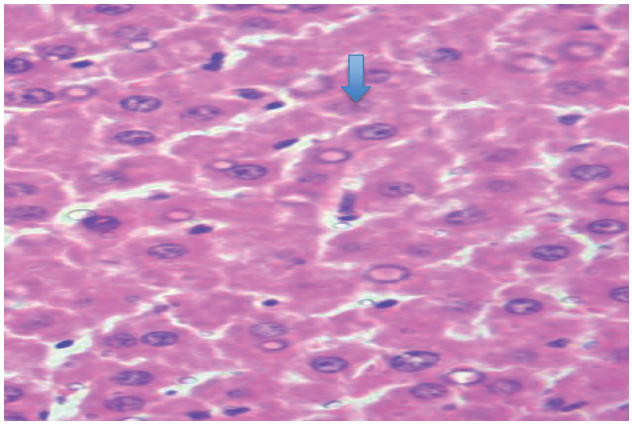

Figure 1. Liver section of the control group, 40X. Normal hepatocytes exist in their normal features.

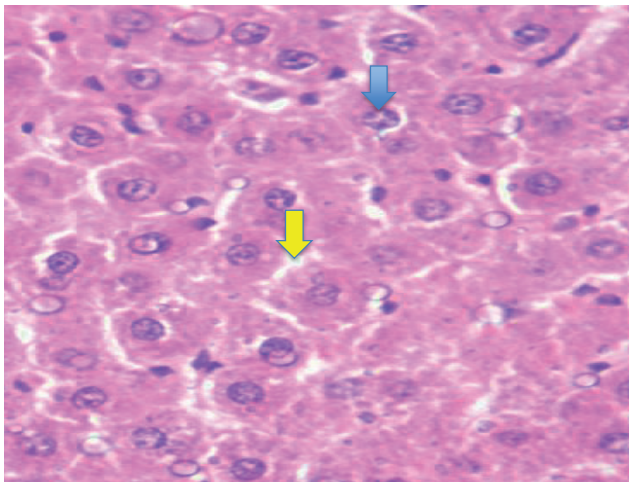

Figure 2. Liver section of the diabetic group, 40X. Enlargement of hepatocytes and nucleoli are demonstrated by blue arrow. Fatty changes are demonstrated by yellow arrow.

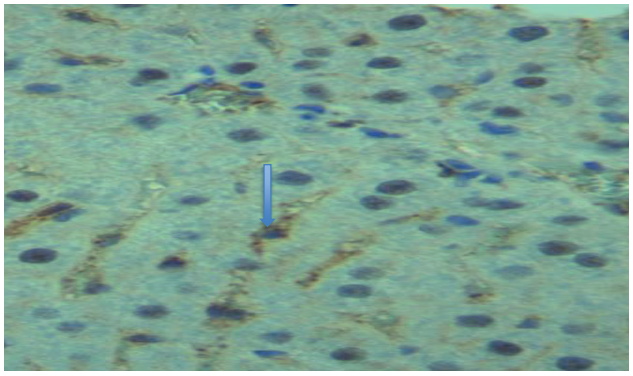

Figure 3. Immunohistochemistry staining of Ki67 in the liver tissue of the control group. Slight expression of Ki67 in the cytoplasm of hepatocytes (blue arrow).

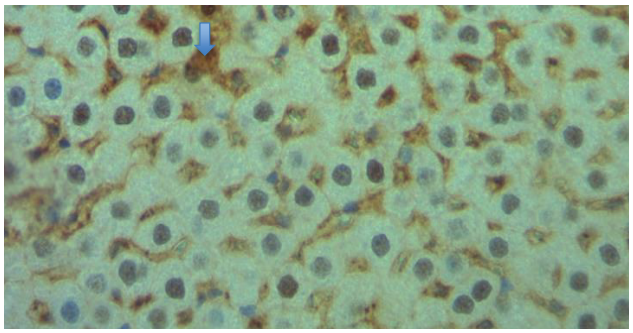

Figure 4. Immunohistochemistry of Ki67 in the liver tissue of the diabetic group, 40X. The expression of Ki67 was intense in the nucleoli of hepatocytes (blue arrow).

were much more than in the control group while the mean differences between the two groups were also significant at $p < 0.001$.

Histological findings

As demonstrated in Figure 1, the normal histological features of the liver are retained. Normal hepatocytes are connected with each other (arrow).

Figure 2 illustrates the liver tissue of the diabetic group. Morphological changes are observed including enlargement of cells and nucleoli. Mitotic activities are also observed in the nucleoli of hepatocytes (blue arrow). Fatty changes are also demonstrated (yellow arrow).

Variable	M±SD	P value
Ki67-Control Ki67-Diabetes	6.85%±1.5% 13%±4%	0.011

Table 2. The expression rate of Ki67 in Study groups

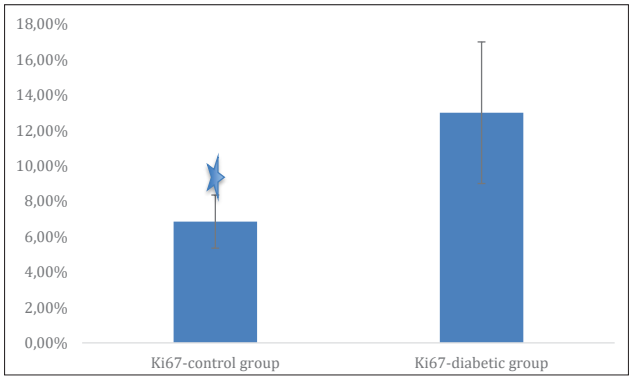

Figure 5. Quantitative expression of Ki67 in the study groups

Immunohistochemistry results of Ki67 staining in the study groups

Figure 3 demonstrates the expression of Ki67 in the liver of the control group. Slight expression of Ki67 is observed. The expression is mainly seen in the cytoplasm of hepatocytes (blue arrow).

Figure 4 shows the immunohistochemistry of Ki67 in the liver of the diabetic group. The expression of Ki67 is demonstrated in the nucleoli of hepatocytes (blue arrow).

Quantitative expression rates of Ki67 in the study groups

As demonstrated in Table 2 and Figure 5, the expression rate of Ki67 in the liver of the control group is 6.85%±1.5%, and this was significantly increased in the diabetic group 13%±4% ($p=0.011$).

5. DISCUSSION

The biochemical, histological, and immunohistochemical results show that control and diabetic differed significantly, showing the changes in metabolism and cellular environment due to diabetes. These findings are consistent with previously reported data in the literature and add to our knowledge of diabetes-related liver changes.

In the group with diabetes, glucose level was increased significantly (258±18.98 mg/dl) as compared to the control group (94.5±12.25 mg/dl $p < 0.001$). When you have diabetes, the amount of glucose in your blood is much higher than the healthy range. The cholesterol and triglycerides levels of the diabetic group were found to be higher and showed significant difference ($p < 0.001$) with the control group. Modifications in lipid profiles are associated with a higher risk of NAFLD and cardiovascular disease (51).

Histological Findings

The histological analysis showed noteworthy changes in the liver tissue of the diabetic rats. The control group did not show any change in hepatocytes, while the diabetic group showed hepatocyte enlargement with nucleolar mitotic activity and fatty change. According to earlier studies that focused on the dia-

betic model, the changes in the hepatic histopathology occurred due to accumulation of lipids and oxidative stress and inflammation (52).

The occurrence of fatty changes (yellow arrow, Figure 2) indicates steatosis (fatty liver), commonly seen in the diabetic liver, occurring due to fat movement and insulin resistance (53)

Immunohistochemical Assessment

Ki67 is a cell proliferative marker; its expression provides insight into regenerative and pathological responses (11, 38). In this study, Ki67 expression was significantly higher in the diabetic group (13%±4%) than in control group (6.85%±1.5%, p = 0.011). As diabetes damages the liver, there is a consequent increase in the proliferation of the hepatocytes. Although these regenerative processes can initially compensate for the injury and may even stabilize liver function, a long-term activation of this type may give rise to fibrosis and worsening liver dysfunction in the future (54). In diabetic groups, Ki67 localized in the nucleolus (Figure 4) unlike its cytoplasmic expression in the control group (Figure 3). It shows altered cellular dynamics and stress response in diabetic hepatocytes.

6. CONCLUSION

The diabetes effect on liver biochemistry, histopathology and cellular profile discussed in detail in these results. When glucose and lipids are high it stresses the liver, causing histological changes and increased proliferation of liver cells. This means that if we can improve the metabolic conditions of a diabetic person, liver damage might not happen. Future research should examine therapeutic strategies that prevent the progression or halting of diabetes-associated liver disease.

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