Abnormalities of liver cells in streptozotocin induced diabetic rats

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Background : *Diabetes is a major health problem that affects population worldwide. Prevalence of diabetes in Thai population dramatically increases every year. Diabetes mellitus can cause structural changes and dysfunctions of the liver cells as well as affecting the pathway of protein synthesizes in the liver associated with DNA synthesis.*

Objective : *This study aimed to evaluate structural changes and protein and DNA contents in liver cell (hepatocyte) of diabetic rats.*

Methods : *Ma le Sprague Dawley rats induced diabetes by receiving streptozotocin (STZ) were studied for histopathology by Hematoxylin & Eosin staining and counted by ImageJ program. Studies of protein damage were performed by Bromophenol blue staining and DNA damage by Feulgen staining. Cell intensities were measured and calculated in comparison with the control.*

Results : *Qualitative study demonstrated abnormal morphologies in hepatocyte such as unclear cell boundaries and destroyed cytoplasm in diabetic-induced rats. In this quantitative study, the percentage of the number of normal hepatocytes was significantly decreased in diabetic-induced rats when compared with the control. Numerically, the relative optical density of protein contents was slightly increased, while DNA contents was decreased in hepatocytes of diabetic-induced rats but these just failed to reach significant when compared to the control.*

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Conclusion   **:**   *This study demonstrates structural and functional changes in hepatocytes of diabetic rats induced by streptozotocin (STZ). These could also reflect abnormal liver function in human with diabetes.*

Keywords   **:**   *Diabetes mellitus, hepatocyte, streptozotocin, bromophenol blue, Feulgen stain.*

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เหตุผลของการทำวิจัย : *ปัจจุบันภาวะเบาหวานเป็นปัญหาทางด้านสุขภาพที่สำคัญที่เกิดขึ้นกับประชากรของทุกประเทศทั่วโลก ความชุกของเบาหวานในประชาชนไทยเพิ่มขึ้นทุก ๆ ปี ซึ่งในผู้ป่วยเบาหวานนั้นมักพบภาวะแทรกซ้อนต่าง ๆ มากมาย จากการศึกษาข้อมูลที่ผ่านมายังพบอีกว่าภาวะเบาหวานยังส่งผลต่อการเกิดความผิดปกติในตับ โดยภาวะเบาหวานทำให้โครงสร้างและหน้าที่ของเซลล์ตับ (hepatocyte) เกิดการเปลี่ยนแปลงไป หรือเกิดภาวะตับโต (hepatomegaly) อีกทั้งภาวะเบาหวานยังส่งผลทำให้เกิดการเปลี่ยนแปลงในวิถีการสังเคราะห์โปรตีนในตับและยังสัมพันธ์กับการสังเคราะห์ดีเอ็นเออีกด้วย ดังนั้นผู้วิจัยจึงมีจุดมุ่งเน้นไปที่การศึกษาถึงความเสียหายของเซลล์ตับ โดยทำการศึกษาถึงการเปลี่ยนแปลงรูปร่างของเซลล์ตับ รวมทั้งโปรตีนและดีเอ็นเอของเซลล์ตับในภาวะเบาหวาน*

วัตถุประสงค์ : *เพื่อศึกษาความเสียหายของโปรตีนและดีเอ็นเอในตับของหนูที่ถูกเหนี่ยวนำให้เกิดภาวะเบาหวาน*

วิธีการทำวิจัย : *ใช้เนื้อเยื่อตับจากหนูขาว เพศผู้สายพันธุ์ Sprague Dawley rats ที่ได้รับ streptozotocin (STZ) โดยนำมาศึกษา morphology โดยทำการย้อมด้วย Hematoxylin and Eosin และทำการนับเซลล์ด้วย Image J ทำการศึกษาดูความเสียหายของโปรตีนด้วยการย้อม bromophenol blue และศึกษาความเสียหายของดีเอ็นเอด้วยการย้อม Feulgen stain และนำมาวัดค่า intensity*

ผลการศึกษา : *หนูที่ถูกเหนี่ยวนำให้เกิดภาวะเบาหวานมีลักษณะจุลกายวิภาคที่เปลี่ยนแปลงไป ขอบเขตเซลล์ไม่ชัดเจน ไซโตพลาสซึมภายในเซลล์มีความเสียหาย และเมื่อนำมานับเซลล์พบว่าจำนวนเซลล์ hepatocyte ที่ปกติมีจำนวนลดลง นอกจากนี้ยังพบว่าปริมาณของโปรตีนเพิ่มขึ้นและปริมาณดีเอนเอลดลงเมื่อเทียบกับหนูกลุ่มปกติ*

สรุป : *จากผลการทดลอง แสดงให้เห็นว่าภาวะเบาหวานการศึกษาวิจัยในครั้งนี้พบว่าภาวะเบาหวานส่งผลกระทบต่อตับ ทั้งโครงสร้างภายในตับ เซลล์ hepatocyte ที่ผิดปกติไป และอาจทำให้การทำงานของตับผิดปกติ แสดงให้เห็นถึงความเสียหายที่เกิดจากภาวะเบาหวานที่ส่งผลต่อตับที่เป็นอวัยวะที่สำคัญในกระบวนการต่าง ๆ ของร่างกาย*

คำสำคัญ : *ภาวะเบาหวาน, hepatocyte, streptozotocin, bromophenol blue, Feulgen stain.*

Diabetes mellitus is a major disease affecting a large number of population worldwide. According to the latest data from the International Diabetes Federation in 2015, there were 415 million people with diabetes and expecting to increase to 642 million people in 2040. [1] In Thailand, there were about 20,000 people died from diabetes each year. The prevalence of diabetes in Thai population gradually increases every year. Currently 6.4% of the population or 3.2 million people were diagnosed diabetics, and predicted to reach 1.1 million people in the next 20 years. [2]

Diabetes is caused by abnormal blood sugar levels associated with insufficiency of insulin or insulin resistance. [3] Most patients with diabetes have been reported many complications including retinopathy, cataracts, arteriosclerosis, peripheral neuropathy, kidney disease and abnormalities in wound repairing process. However, there appeared to be very few report in the effect of diabetes to liver functions. Diabetes has been reported to induce structural and functional changes in the liver cells. [4] Additionally, diabetes can also cause the changes in the pathway of protein and DNA syntheses in the liver. [5, 6] However, the previous studies did not show the deficits of proteins and DNA contents in cytoplasm and nucleus of liver cells. Therefore, this study aimed to evaluate structural changes and the abnormalities of protein and DNA contents in the hepatocytes of diabetic rats. Moreover, a quantitative study was included by counting the number of normal and abnormal hepatocytes.

Methods

Eight male Sprague Dawley rats, weighing 250 - 320 g were carried out in compliance with Center for Animal Research Naresuan University, Phitsanulok, Thailand (58 01 002). Rats were divided into two groups as: control group (C) received normal saline daily by intraperitoneal injection for 48 days, and diabetes mellitus group (DM) that received a single dose of streptozotocin (STZ) 60 mg/kg on day eight, and rats with fasting blood glucose level $\geq$ 250 mg/dL were monitored and used for the study. After 7 - 12 weeks of induction, the rats were sacrificed by carbon dioxide gas. Liver tissues were immediately removed and immersed into 10% neutral buffer formalin to preserve the tissue structure. Then, the tissues were cut into small pieces, followed by tissue processing and embedding. The tissue blocks were kept at $4^{O}C$ until section. The tissue sections were cut at 5 μm thickness, followed by mounting onto coated slides. The sections were then used for morphological study, and protein and DNA intensity studies.

Morphological study

The morphological changes of hepatocyte were analyzed from routine hematoxylin & eosin (H&E) staining technique. Each H&E sections was investigated under a light microscope (Olympus BX51) at 20X magnification and all pictures were taken with ZEISS Oxia cam 105 color. The total number of normal hepatocytes were analyzed by image J software according to the criteria used by Suphakhong K, et al. [7] The quantitative data were showed as the percentages of normal hepatocytes.

Protein and DNA intensity study

Total of proteins in hepatocytes were analyzed by bromophenol blue staining technique. Briefly, two sections from each sample were deparaffinized with xylene followed by passing through decreasing concentration of alcohol and distilled water for 3 minutes. The sections were dipped with bromophenol blue for 5 minutes, followed by dehydrated with 70% alcohol, 95% alcohol, 100% alcohol and finally with xylene. All sections were mounted with mounting media. The total of proteins were evaluated under a light microscope (Olympus BX51) at 20X magnification and all pictures were taken with ZEISS Oxia cam 105 color. Ten pictures per two sections from each sample were captured for data analysis. The total protein intensities were analyzed by image J software. [8] Relative optical density (ROD) of protein contents was evaluated and calculated according to the following formula.

$$ROD = \log (256/\text{Intensity mean})$$

DNA intensity in hepatocytes was demonstrated by Feulgen staining technique. After deparaffinization, all sections were transferred to the hydrolysis process by warming all section at $60^{\circ}C$ hydrochloric acid 1 N for 1 hour, followed by distilled water for 3 minutes. Then, the sections were immersed in Schiff's reagent for 1 hour, followed by dipping with bisulfite solution for 3 times. The sections were washed by tap water for 5 - 10 minutes and counter stained for 1 - 3 minutes, followed by dehydrated with 70% alcohol, 95% alcohol, 100% alcohol twice and finally with xylene. Then, the sections were mounted with mounting media. The total DNA contents in the nucleus of hepatocyte were evaluated under a light microscope (Olympus BX51) at 20X magnification and all pictures were taken with ZEISS Oxia cam 105 color. Ten pictures per two sections form each sample were captured for data analysis. The total DNA intensities were analyzed by image J software. [8] Relative optical density)ROD) of protein content was evaluated and calculated according to the formula previously.

All statistical analyses were carried out using SPSS statistical software (IBM SPSS Statistics for Window, version 17.0).

Results

The morphological changes of liver cells in diabetic rats in comparison with the control group were shown in figure 1. In diabetic rats, the liver cells demonstrated unclear cell boundaries and empty spaces in the cytoplasm (Figure 1B), while the liver cells in the control were generally in the normal form (Figure 1A). Interestingly, most liver cells in the control group showed euchromatic nucleus with prominent nucleolus (Figure 1C), while the cell nuclei in the diabetic rats seemed to be more heterochromatic (Figure 1D).

Quantitative study demonstrated that percentage of number of normal hepatocytes was significantly decreased in DM group when compared with control group ($P < 0.05$) (Figure 2).

The protein distribution in hepatocytes was analyzed by bromophenol blue staining technique. Blue staining represented protein contents inside the cell. Qualitatively, the appearance of blue staining was slightly dominant in the hepatocytes in the control group (Figure 3A) when compared to the diabetic group (Figure 3B). Quantitatively, the protein intensity measured from relative optical density (ROD) in DM group was slightly higher but failed to reach level of significance when compared with the control group (Figure 4).
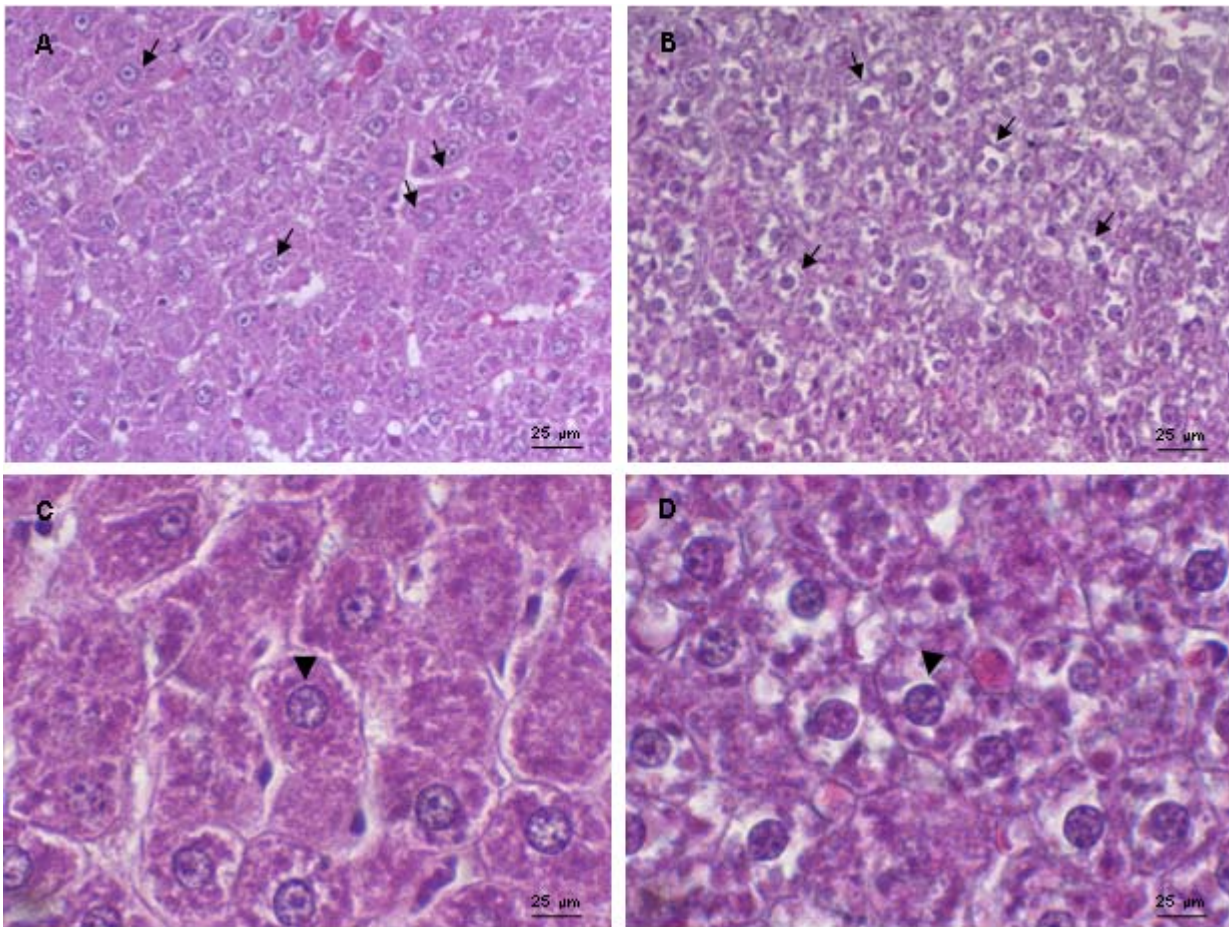
Figure 1. Hematoxylin-eosin staining for revealing morphological changes of liver cells; control (A, C) and DM (B, D). Arrow pointed hepatocytes and arrow head pointed nucleus.
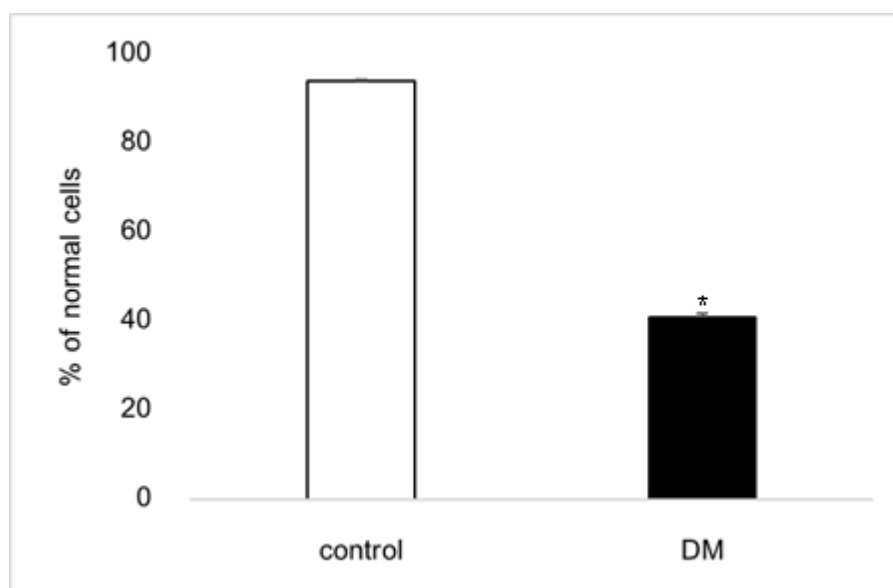

Figure 2. Percentages of the number of normal hepatocytes in control and DM groups. Data are presented as mean ± SEM. * $P < 0.05$ versus control group.
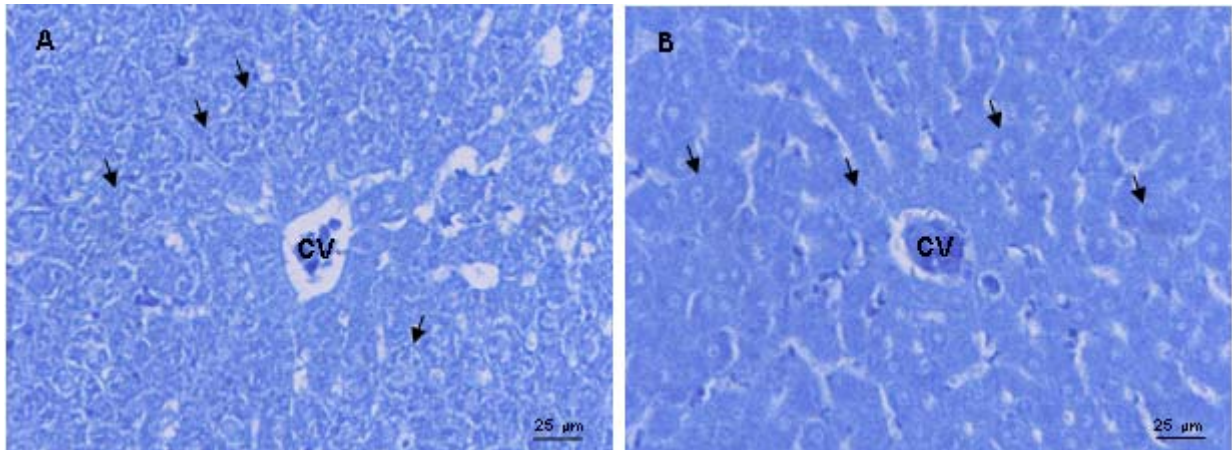
Figure 3. Bromophenol blue staining for evaluating protein contents in hepatocyte: control (A) and DM (B).
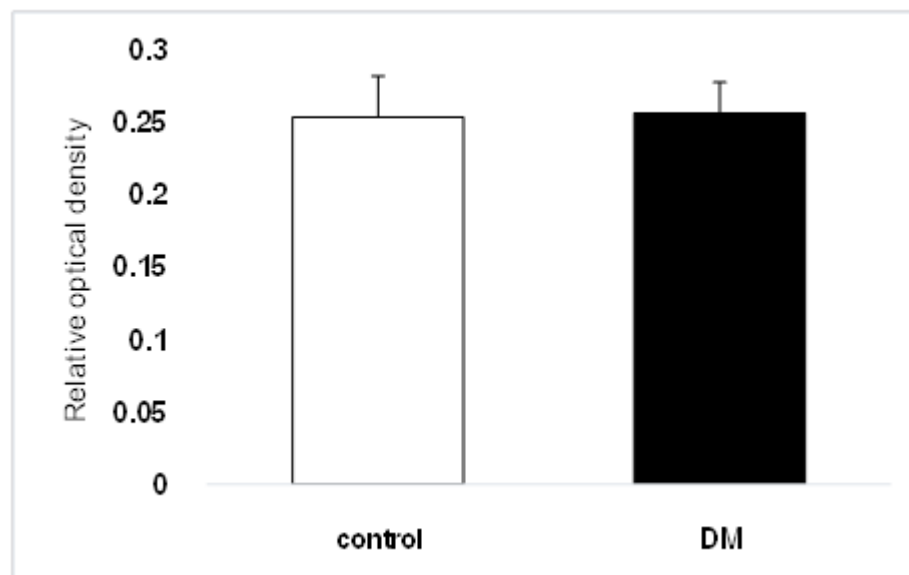

Figure 4. Protein intensity measured by relative optical density (ROD) in control and DM groups. Data are presented as mean $\pm$ SEM.

The total of DNA in hepatocyte was analyzed by Feulgen staining technique. Light blue or green staining represented DNA inside the cell. Qualitatively, Feulgen staining appeared prominently in the hepatocytes of DM group (Figure 5B) when compared to the control (Figure 5A). However, quantitative data on relative optical density (ROD) of DNA intensity showed no statistic difference between two groups (Figure 6).
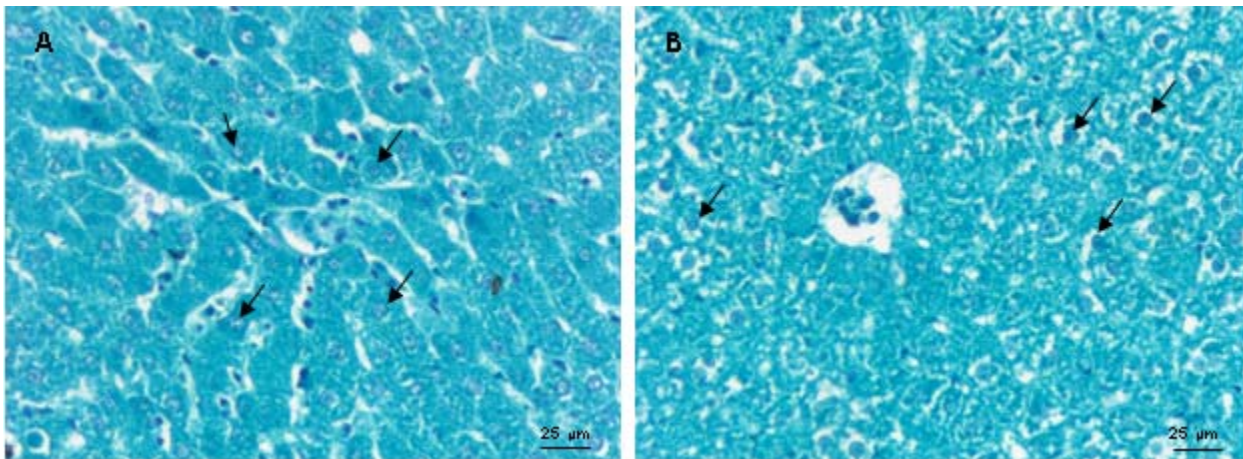
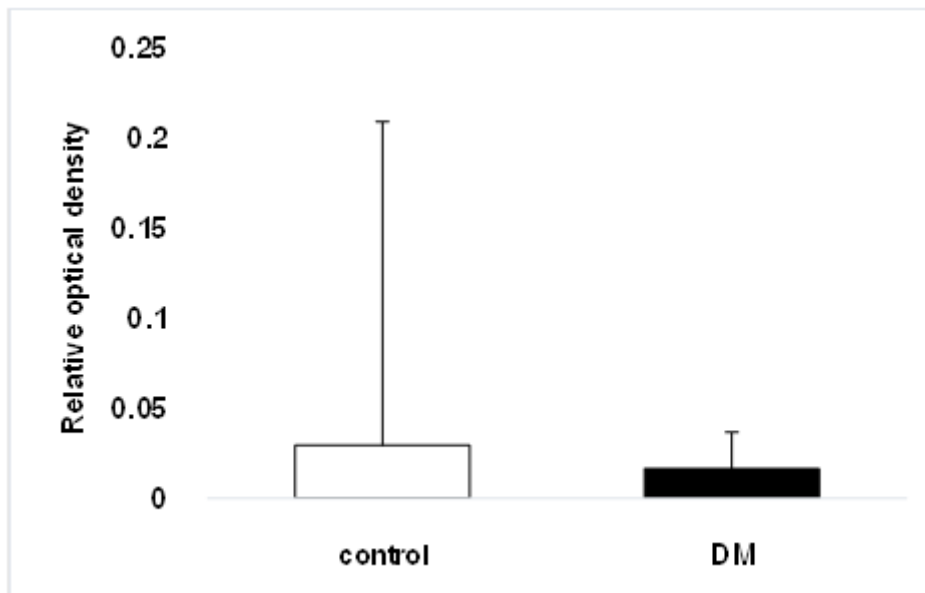**Figure 5.** Feulgen staining technique for evaluating DNA contents in hepatocytes; control (A) and DM (B).

Figure 6. DNA intensity measured by relative optical density (ROD) in control and DM groups. Data are presented as mean ± SEM.

Discussion

In the present study, we demonstrate structural changes of hepatocytes in diabetic rats induced by streptozotocin (STZ) including unclear cell boundaries, empty cytoplasm and inactive nuclei which similar to the study of Herrman CE, et al [4] who reported abnormalities of the central vein, enlarged hepatocytes, the boundary of the cells was unclear and cytoplasmic destroyed. Moreover, significant decrease in the number of hepatocytes with normal morphology may reflect the effect of diabetes mellitus in development of hepatocytes. [7]

The present study demonstrated that protein contents in hepatocytes seem to be slightly increased in diabetic rats. These may reflect proteins storage inside the cell. Conversely, the distribution of DNA in the nucleus of hepatocyte in diabetic rats was numerically decreased when compared with normal

rats. These results may reflect abnormal nuclear functions in hepatocyte caused by diabetes as there have been reported that diabetes mellitus results in higher glycation and higher advance glycation endproducts (AGEs) [3] resulting in oxidative stress and damage to DNA. Moreover, diabetes mellitus results in reduced DNA synthesis. [9]

Although there are no differences of protein and DNA contents in hepatocytes between control and diabetic rats, the abnormalities of protein and DNA in liver cells are obviously seen in the morphological studies. It is possible that there are specific proteins that are changed but the bromophenol blue staining techniques are restricted for overall proteins measurement. In the same way, the feulgen staining is not specified to measure the DNA of hepatocytes, the DNA contents of all cells are included. Further studies are needed to employ the advance techniques for identifying the abnormalities of specific proteins in hepatocytes. However, the results of this study can be preliminarily data to support the effects of diabetes on abnormalities of protein and DNA contents in hepatocytes.

Conclusion

In summary, the present study shows that diabetes mellitus can induce morphological change of hepatocyte and the decrease in the percentage of hepatocyte with normal morphology. Even though, the changes of protein and DNA intensities in hepatocytes of diabetic rats did not showed significant differences to the control but numerical changes were found in this study that may reflect the effects of diabetes to the contents in the hepatocytes if the disease has been carried out for a longer period. Therefore, the results from the present study demonstrate structural changes of hepatocytes that might consequently result to abnormal functions of hepatocyte caused by diabetes.

Acknowledgement

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