Hypoglycemic effects of date seed extract

Possible mechanism of action, and potential therapeutic implications

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ABSTRACT

الأهداف: بحث الآلية الممكنة التي تمكن مستخرج من أنوية التمر من خفض نسبة الجلوكوز بالدم.

الطريقة: أجريت الدراسة في قسم التشريح، كلية الطب، جامعة الملك سعود خلال الفترة من من مايو إلى ديسمبر عام2012م. و قد تم تقسيم 80 فأراً على 4 مجموعات، حيث استخدمت المجموعة الأولى كمجموعة ضابطة بدون أي علاج، وأعطيت فئران المجموعة الثانية جرعة يومية من مستخرج أنوية التمر قدرها 10 ملليلتر بالفم، وقد تم إصابة فئران المجموعتين الثالثة والرابعة بداء السكري بواسطة حقنها بمادة «ستربتوزوتوسين»، كما تم إخضاع هاتين المجموعتين للعلاج بالأنسولين بجرعة قدرها 3 وحدات عالمية يومياً لمدة 8 أسابيع بالإضافة إلى إعطاء فئران المجموعة الرابعة نفس الجرعة اليومية من المستخرج المعطاة للمجموعة الثانية. و في نهاية التجربة، تم قتل الفئران و فصل مصل الدم من كل منها على حدة لقياس مستوى الببتيد سي. كما تم إعداد البنكرياس للدراسة النسيجية لجزر لانجرهانز و للدراسة النسيجية المناعية للأنسولين، كما تم إجراء دراسة تحليلية للصور المجهرية الضوئية لتقدير كمية الأنسولين.

النتائج: ولقد أظهرت النتائج زيادة ملحوظة في متوسط مستوى الببتيد سي للمجموعة الرابعة مقارنة بالثالثة، كما أظهرت جزر لانجرهانز ببنكرياس فئران المجموعة الثالثة ضعفاً في التفاعل المناعي للأنسولين، بينما أظهرت تفاعلاً قوياً لبعض خلايا بيتا المتضخمة بالمجموعة الرابعة، كما وجدت خلايا إيجابية التفاعل للأنسولين بجدار القنوات بين الفصوص و بالخلايا العنيبية المركزية ببنكرياس فئران المجموعة الرابعة فقط. و قد أظهر التقدير الكمي المناعي للأنسولين انكماشاً واضحاً بحجم الجزر و بمدى التفاعل المناعي للأنسولين بالمجموعتين المصابتين بداء السكري مقارنة بالمجموعتين الضابطتين.

خاتمة: أن مستخرج أنوية التمر قد يحفز إفراز الأنسولين الذاتي من خلال مصادر من خارج الجزر.

Objectives: To investigate the possible mechanism, by which an extract from date seeds exert its hypoglycemic effect.

Methods: This study was performed at the Anatomy Department, College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia from May to December 2012. Eighty rats were divided into 4 groups. Group 1 received no treatment. Group 2 received daily ingestions of 10 ml of date seed extract for 8 weeks. Animals of groups 3 and 4 were made diabetic by streptozotocin injection, and were given daily subcutaneous injections of 3 IU/day of insulin for 8 weeks. Group 4 received, in addition, daily ingestions of 10 ml of seed extracts. Rats were sacrificed, and the sera were separated for estimation of serum C-peptide levels. Pancreatic tissues were processed for histological study of the islet cells, immunohistochemical study for insulin secretion and image analysis for insulin quantification.

Results: Mean serum C-peptide level was significantly higher in group 4 compared to group 3. Pancreatic islets from rats of group 3 showed weak immunoreactivity for insulin, while those of group 4 showed strong immunoreactivity in some hypertrophied beta cells. Immunopositive cells were detected in the wall of interlobular ducts and in centroacinar cells of pancreas only in group 4. Quantification of insulin immunoreactivity showed a marked reduction in islet size and extent of insulin immunoreactivity in diabetic compared to control groups.

Conclusion: Date seed extracts may stimulate endogenous insulin secretion through extra-islet sources.

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Diabetes is a predominant public health concern affecting a large population in the whole world. The disease causes substantial morbidity, mortality and long-term complications.[1,2] Considering insulin as the only drug available for type 1 diabetes mellitus (T1DM), its disadvantages were discussed in previous studies.[3,4] There is an increasing use of complementary and alternative medicine among general public.[5] In a previous study, we tested successfully the efficacy of an aqueous extract from date seeds on the glycemic control of T1DM in rats.[6] In another study, we also demonstrated the safety of the date seed extract administration on liver and kidney of rats and showed that date seed extract-insulin combination minimizes the diabetic toxic effects on liver and kidney of rats, compared with insulin administration as a single drug.[7] We proposed a potential mechanism by which date seed extract exerts its hypoglycemic effect through stimulation of certain cells to differentiate into insulin-secreting cells. We detected a lag period of approximately 2 weeks between time of administration of the date seed extract and manifestation of its hypoglycemic effect, and suggested that such period might correspond to the period taken by the cells to differentiate and secrete insulin.[6] The C-peptide or connecting peptide is a 31 amino-acid polypeptide present in the proinsulin molecule. During insulin secretion, it is enzymatically cleaved off and co-secreted in equimolar proportion with mature insulin molecules. Because synthetic insulin does not have such peptide, the level of C-peptide indicates how much insulin is being secreted in the body.[8] The present study was designed to investigate the mechanism, by which date seed extract exerts its hypoglycemic effect through measurements of C-peptide levels in serum of diabetic rats treated with date seed extract, as well as, quantitative evaluation of insulin secreted by beta cells of pancreas, if any, of those rats.

Methods. This study was performed at the Department of Anatomy, College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia (KSA) from May to December 2012. The present study was an extension of 2 previous reports on the effects of an extract of date seeds on diabetic rats.[6,7]

Disclosure. This study was funded by the Research Center, College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia.

Animals. Eighty male adult Sprague Dawley albino rats weighing 250-300 g, obtained from the Animal House, Faculty of Medicine, King Saud University, Riyadh, KSA were used in this study. Principles of laboratory animal care were followed, as well as specific national laws were applicable.

Study design. Rats were divided into 4 groups of 20 each. Group 1 - was used as a control group that did not receive any treatment. Group2 - was used as a control group that was given a daily ingestion, through gavages of 10 ml of the date seed extract for 8 weeks. Group 3 - was treated with a daily subcutaneous injection of insulin. Group 4 - was treated with a daily subcutaneous injection of insulin immediately followed by a daily ingestion, through gavages of 10 ml of the date seed extract for 8 weeks. Diabetes mellitus was induced in animals of groups 3 and 4 by a single intravenous injection of freshly prepared streptozotocin (Sigma Chemical Co, St. Louis, Missouri, USA) at a dose of 60 mg/kg body weight dissolved in 0.1 mol/l citrate buffer (pH 4.5).[9] Rats in groups 1 and 2 received an equivalent dose of the buffer. Only animals with a blood glucose level higher than 300 mg/dl, 3 days after streptozotocin injection were included in the experiment.[10] Diabetic rats in groups 3 and 4were treated with a daily subcutaneous injection of insulin glargine (Lantus, 100 IU/ml; Sanofi Aventis, Frankfurt, Germany). The dose of insulin was 3 IU/day/rat in 2 divided doses for 8 weeks. The dose of insulin was adjusted to maintain the life of animals (not to return blood glucose to normal).[6]

Determination of serum C-peptide levels. At the end of experiment, animals were anesthetized with ether and then sacrificed. Blood of each rat was collected into an appropriately labeled sterile tube. Blood was allowed to clot and left for 10 minutes at room temperature for serum formation. The tubes were centrifuged at 3750 g for 5 minutes. The sera were collected in appropriately labeled tubes, kept frozen at -20°C and used for determination of C-peptide levels. Serum C-peptide concentrations were measured by radio immunoassay according to the manufacturer's instructions (Rat C-Peptide RIA KIT, Cat. # RCP-21K; Millipore, St. Charles, Missouri, USA).

Histological and immunohistochemical studies. Rat pancreatic tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin, sectioned at 5 μm thickness, and then stained with either Hematoxylin and Eosin (H&E) for histological study, or immunohistochemically for insulin using a rabbit polyclonal anti-insulin antibody (H-86, Cat. # SC-9168; Santa Cruz Biotechnology, USA) at a dilution of 1:250 for one hour at room temperature.

Immunohistochemical sections were incubated with Novolink polymer detection system (product # RE7280-K; Leica Biosystems Newcastle Ltd, Newcastle, United Kingdom) for 30 minutes. Sections were further incubated with substrate/diaminobenzidine (DAB). Immunostained sections were then counterstained with Mayer's hematoxylin. Negative control sections for immunostaining were performed by omission of the primary antibody. These negative control sections showed no immunostaining. Both H&E and immunostained sections were evaluated and photographed by means of an Olympus DP72 camera fitted on an Olympus BX51 bright field microscope (Olympus Corporation, Japan).

Image analysis. High-resolution, whole-slide digital scans of all anti-insulin immunostained pancreatic tissue sections were created with a ScanScope scanner (Aperio Technologies Inc). The digital slide images were then viewed and analyzed, using the viewing and image analysis tools of Aperio's Image Scope software. To quantify the insulin-secreting ability of pancreatic islets, a total of 80 islets per group were randomly selected and outlined by freehand tracing of their margins. The color deconvolution algorithm (Aperio Technologies Inc) was then run to measure the area percent (relative to islet area), and the average optical density of pancreatic islets insulin immunopositivity (brown coloration). The algorithm output also included a score of islet insulin immunopositivity calculated by a simple formula involving the positive percentages; Score = (1.0 × [%weak positive]) + (2.0 × [%medium positive]) + (3.0 × [%strong positive]).

Preparation of the date seed extract.[6,7] Seeds obtained from "Sukkary" dates were washed with tap water, left to dry, roasted, and crushed. Crushed seed powder was added to distilled water to make a mixture of 50 g/l. The mixture was boiled until it becomes brownish in color then finally filtered.

Statistical analysis. Data collected were subjected to statistical analysis using the Statistical Package for Social Sciences (PASW Statistics 18, Chicago, IL, USA) software where the analysis of variance (ANOVA) was used for an overall comparison between the study groups, and post hoc Tukey HSD test was used for pairwise comparisons. Differences were considered significant when $p \leq 0.05$. A 95% confidence level was used to calculate a confidence interval, which is a range of values around the mean where the "true" (population) mean can be expected to be located with 95% certainty.

Results. *Biochemical results.* No significant differences ($p>0.05$) in serum C-peptide level were observed between group 1 (control group) and group 2 (date seed extract-treated control group). Group 3 (insulin-treated diabetic group) and group 4 (date seed extract-insulin-treated diabetic group) showed a statistically significant reduction in serum C-peptide level ($p<0.05$) compared to group 1 and group 2. The serum C-peptide level was significantly higher in group 4 compared to group 3 ($p<0.05$) (**Table 1**).

Histological results. Histological examination of H&E stained pancreatic islets from rats of group 1 (**Figure 1A**) and those of group 2 (**Figure 1B**) showed normal architecture. The islets were variable in size, oval, or rounded in shape, and with regular outlines. Each islet was formed of anastomosing cords of polygonal cells separated by blood capillaries. Pancreatic islets from rats of group 3 showed reduction in size of almost all pancreatic islets with irregularity of their outlines. These islets demonstrated a decrease in number and size of their cells, and showed also multiple areas of loss of cellular details and necrosis (**Figure 1C**). Pancreatic islets from rats of group 4 showed reduction in size of almost all pancreatic islets with irregularity of their outlines. Many of these islets showed reduction in the number and size of their cells. However, few islet cells (probably beta cells) were hypertrophied (**Figure 1D**).

Immunohistochemical results. Histological examination of anti-insulin immunostained pancreatic islets from rats of group 1 (**Figure 2A**) and those of group 2 (**Figure 2B**) showed numerous and strongly immunoreactive beta cells. Pancreatic islets from rats of group 3 showed weak immunoreactivity in very few beta cells (**Figure 2C**). Pancreatic islets from rats of group 4 showed strong immunoreactivity in some beta cells, which were hypertrophied (**Figure 2D**). In addition, immunoreactivity was detected in some cells in the wall of interlobular ducts (**Figures 3A & 3B**), and in some centroacinar cells (**Figure 3C**). Sections from other groups showed no duct cells with insulin immunoreactivity.

Image analysis results. Groups 3 and 4 (diabetic groups) showed a statistically significant reduction compared to group 1 and group 2 (control groups) in all parameters examined by image analysis (area percent of insulin immunopositivity relative to area of islet, average optical density, and score of immunopositivity). No significant differences were observed between the 2 control groups, as well as, between the 2 diabetic groups regarding all parameters examined by image analysis (**Table 2**). Quantification of insulin immunopositivity in pancreatic islets showed a marked reduction in islet size and extent of insulin immunopositivity in the diabetic groups compared to control groups (**Figure 4**).

Table 1 - Serum C-peptide level (pM) in all groups of rats included in a study performed at the Department of Anatomy, College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia.

Groups	Group 1		Group 2		Group 3		Group 4	
	Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	95% CI
C-peptide	107.75 ± 9.36	96.128 to 119.372	104.50 ± 11.24	90.544 to 118.456	12.45 ± 1.70	10.339 to 14.561	61.50 ± 8.06	51.492 to 71.508
P1			0.9455		0.0002*		0.0002*	
P2	0.9455				0.0002*		0.0002*	
P3	0.0002*		0.0002*				0.0002*	

SD - standard deviation, CI - confidence interval, P1 - versus group 1, P2 - versus group 2, P3 - versus group 3, *significant difference - $p \leq 0.05$

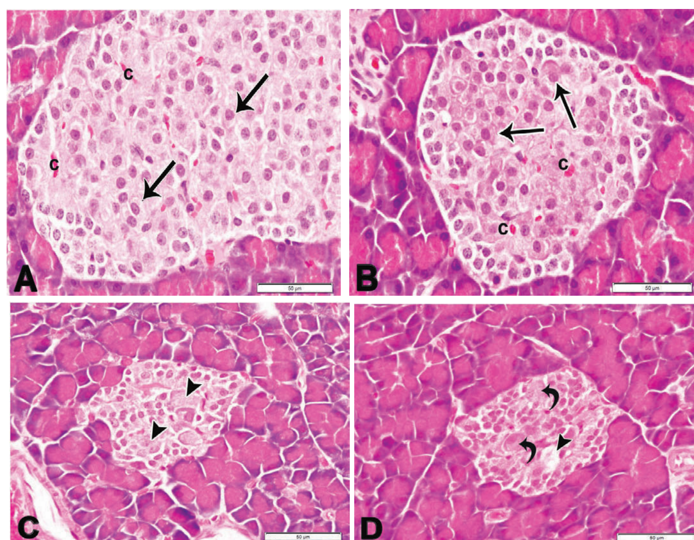

Figure 1 - Photomicrographs of pancreatic islets stained with Hematoxylin and Eosin showing: **A**) group 1 rats with cords of polygonal islet cells (arrows) separated by blood capillaries (c); **B**) group 2 rats with the same normal architecture shown in (**A**); **C**) group 3 rats with reduction of islet size with irregular outlines. Islet cells are decreased in number and size with multiple areas of loss of cellular details and necrosis (arrowheads). **D**) group 4 rats with reduction of islet size with irregular outlines. Islet cells are decreased in number and size with areas of loss of cellular details and necrosis (arrowhead). Some cells (probably surviving beta cells) are hypertrophied (curved arrows). Scale bars = 50 µm

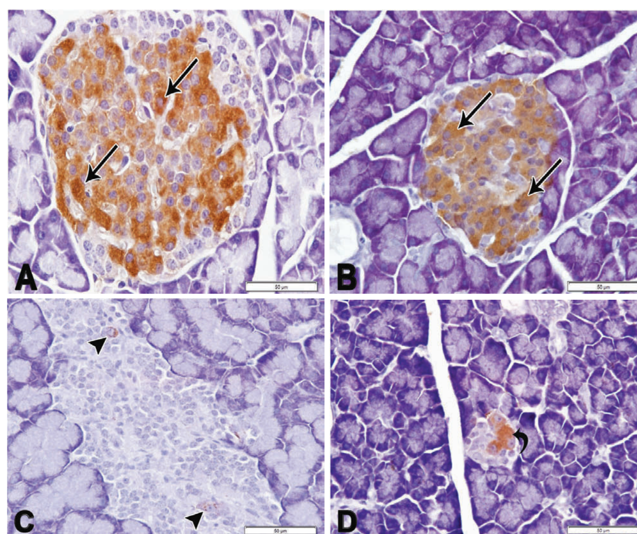

Figure 2 - Photomicrographs of pancreatic islets immunostained for insulin showing: **A**) group 1 rats showing numerous and strongly immunoreactive beta cells (arrows); **B**) group 2 rats showing numerous and strongly immunoreactive beta cells (arrows); **C**) group 3 rats showing weak immunoreactivity in very few beta cells (arrowheads); **D**) group 4 rats showing strong immunoreactivity in some beta cells, which are hypertrophied (curved arrow). Scale bars = 50 µm

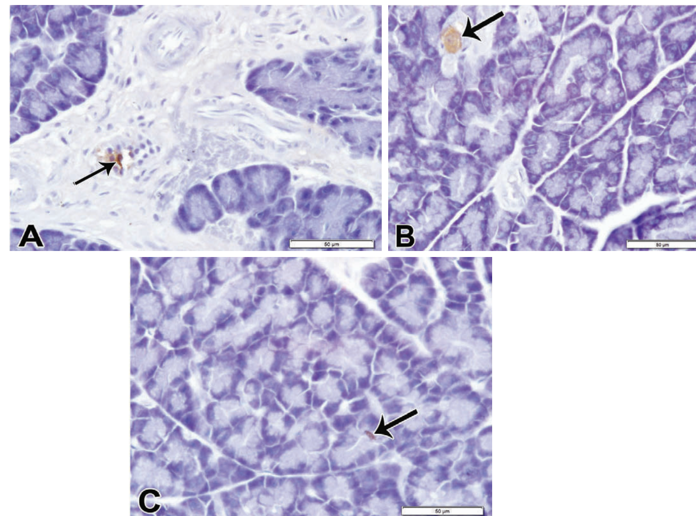

Figure 3 - Photomicrographs of section of pancreas of a rat from group 4 showing: **A**) insulin immunoreactivity in few cells (arrow) in the wall of the interlobular ducts; **B**) immunoreactivity in one cell (arrow) in the wall of interlobular duct; **C**) immunoreactivity in a centroacinar cell (arrow) of a pancreatic acinus. Scale bars = 50 µm

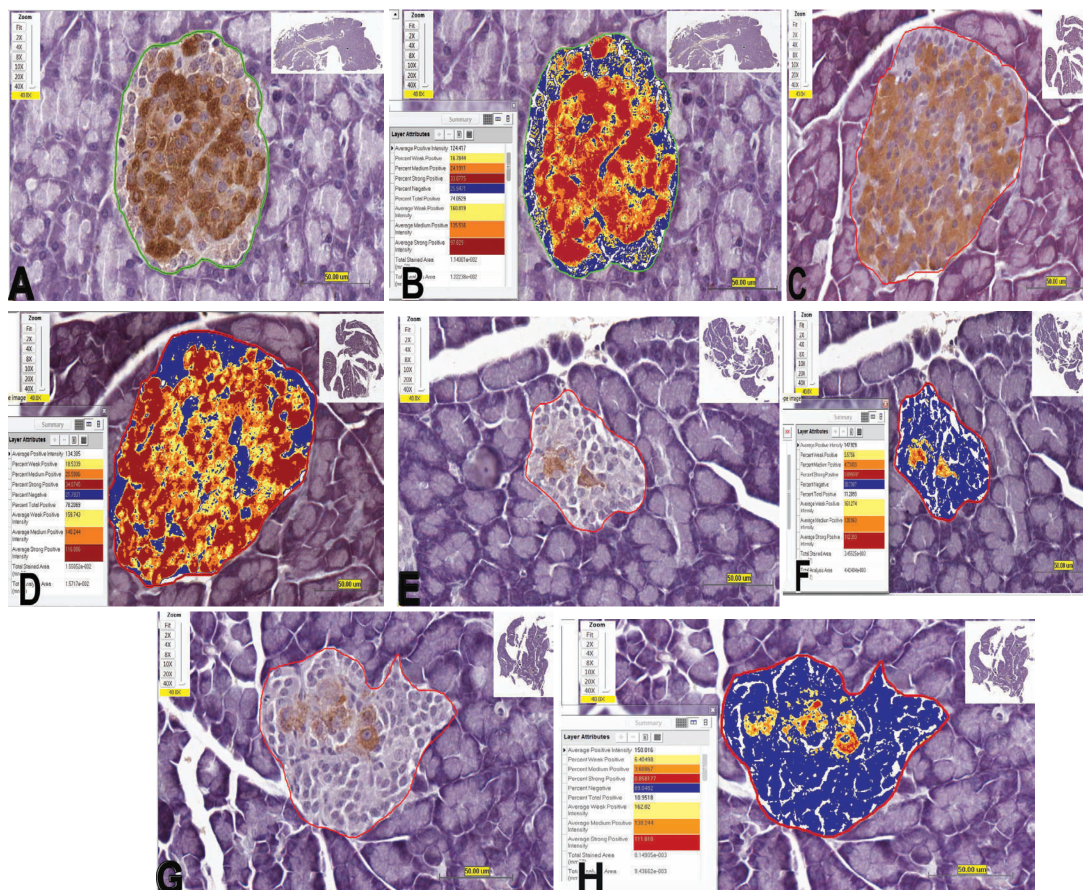

Figure 4 - Quantification of insulin immunopositivity in the pancreatic islets from: group 1 (**A**), group 2 (**C**), group 3 (**E**) and group 4 (**G**) are shown outlined. The color markup overlay produced by the color deconvolution algorithm reflecting the intensity ranges of the 3,3'-diaminobenzidine (DAB) (brown) stain are shown in picture **B**, **D**, **F**, and **H**, such as: blue - negative; yellow - weak positive; orange - medium positive; and red - strong positive. Notice the marked reduction in islet size and extent of insulin immunopositivity in the diabetic groups (groups 3 & 4) compared to the control groups (groups 1 & 2). Scale bars = 50 µm

Table 2 - Percent insulin immunopositivity, islet area, optical density (OD) and score of immunopositivity in all studied groups of rats included in a study performed at the Department of Anatomy, College of Medicine, King Saud University, Riyadh, Kingdom of Saudi Arabia.

Variables	Group 1		Group 2		Group 3		Group 4	
	Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	95% CI	Mean ± SD	95% CI
Percent positive								
Mean ± SD	65.781 ± 8.409	61.124 to 70.438	67.423 ± 9.640	60.527 to 74.319	5.327 ± 6.419	1.249 to 9.405	6.315 ± 9.294	2.561 to 10.069
P1			0.9667		0.0002*		0.0002*	
P2	0.9667				0.0002*		0.0002*	
P3	0.0002*		0.0002*				0.9879	
Islet area (mm²)								
Mean ± SD	0.243 ± 0.031	0.226 to 0.260	0.231 ± 0.023	0.215 to 0.248	0.185 ± 0.027	0.168 to 0.202	0.191 ± 0.027	0.180 to 0.202
P1			0.6955		0.0002*		0.0002*	
P2	0.6955				0.0016*		0.0017*	
P3	0.0002*		0.0016*				0.9222	
Average OD								
Mean ± SD	0.018 ± 0.015	0.010 to 0.026	0.018 ± 0.008	0.0123 to 0.024	0.007 ± 0.005	0.004 to 0.010	0.006 ± 0.005	0.004 to 0.008
P1			0.9995		0.0052*		0.0003*	
P2	0.9995				0.0181*		0.0019*	
P3	0.0052*		0.0181*				0.9862	
Scores								
Mean ± SD	127.16 ± 27.45	111.959 to 142.361	137.50 ± 31.65	114.859 to 160.141	8.42 ± 10.43	1.793 to 15.047	10.23 ± 16.63	3.513 to 16.947
P1			0.6481		0.0002*		0.0002*	
P2	0.6481				0.0002*		0.0002*	
P3	0.0002*		0.0002*				0.9952	

SD - standard deviation, CI - confidence interval, P1 - versus group 1, P2 - versus group 2, P3 - versus group 3, *significant difference - $p \leq 0.05$

Discussion. The efficacy of an aqueous extract from date seeds has been tested successfully on the glycemic control of T1DM in rats.[6] The mean blood glucose levels of diabetic rats treated with date seed extract-insulin combination were found significantly lower compared to those of diabetic rats treated with insulin as a single drug. Glycosylated hemoglobin (HbA1C), a more reliable index of glycemic control in the management of diabetes mellitus than fasting blood glucose levels[11] was significantly lower in date seeds extract-insulin-treated diabetic rats.[6] The safety of the date seed extract administration on liver and kidney of rats has also been demonstrated. In addition, date seed extract-insulin combination was found to minimize the toxic effects of diabetes on these organs.[7] The previous results stimulated the authors to conduct the present study, aiming to investigate the potential mechanism(s), by which date seed extract exert its hypoglycemic effect.

A biochemical study was performed to measure the serum C-peptide levels in rats of all groups. Evaluation of C-peptide level is a useful parameter to indicate the amount of endogenous insulin secreted in the body.[12] No significant differences in serum C-peptide level were observed between control groups (group 1 and group 2). The present results were in accordance with those of our previous study,[6] in which no significant differences in mean blood glucose levels were detected between the control groups. The results of both studies indicated the absence of hypoglycemic effect for the date seed extracts when administered to normoglycemic animals. The serum C-peptide level was significantly higher in date seed extract-insulin-treated diabetic rats (group 4) compared to insulin-treated diabetic rats (group 3). Biochemical results suggested an increase in endogenous insulin secretion in case of diabetic rats treated with date seed extract which might be the cause of the significant reduction of mean blood glucose levels demonstrated in those rats. The effect of date seed extract on the control of hyperglycemia is further supported by our previous report,[7] indicating that the combination of date seed extract and insulin minimizes the histopathological effects of diabetes on liver and kidney of diabetic rats when compared to insulin administration for the same period.

In order to investigate the source of endogenously secreted insulin, histological and immunohistochemical studies were performed on pancreatic islets of all groups. Control groups showed normal pancreatic islets architecture and strong insulin immunoreactivity of beta cells. On the other hand, pancreatic islets of both diabetic groups showed reduction in size and irregularity of their outlines. However, some hypertrophied islet cells with strong insulin immunoreactivity were only detected in pancreatic islets of date seed extract-insulin-treated diabetic rats. The present observations might suggest that, in date seed extract-insulin-treated diabetic rats, there is a compensatory hypertrophy of some beta

cells, which secrete more insulin than normal cells do. More importantly, immunoreactivity was detected in some cells in the wall of interlobular ducts, and in some centroacinar cells in date seed extract-insulin-treated diabetic group. Sections from other groups showed no ductal cells with insulin immunoreactivity. Both biochemical and immunohistochemical results supported our hypothesis that date seed extract might stimulate certain cells to differentiate into insulin-secreting cells. It is well known that, early in the fetal period, pancreatic acini and islets bud from primordial pancreatic ducts; islets separate, and come to lie between the acini.[13] As unlimited islet formation could result in life-threatening hypoglycemia, there may be an evolutionary advantage to suppress duct proliferation and, thus a limitation of beta cells neogenesis.[14] Being normally restrained by unidentified local factors, such neogenicability could be triggered in vivo by certain stimulants; date seed extract might be one of them. In accordance with the present hypothesis, Bonner-Weir et al,[14] reported that mature pancreatic ductal epithelium, in all species including human, can transiently regain a less differentiated state, and thus, serves as a potential pool of progenitors for both islet and acinar tissues. Also, studies performed on non-obese type 1 diabetic mice reported islet regeneration once the autoimmune process is arrested.[15,16]

Image analysis study showed a statistically significant reduction in all parameters examined in case of diabetic groups compared to control groups. Although that there was a reduction in all parameters in case of insulin-treated compared to date seed extract-insulin-treated diabetic group, the reduction was not significant. The results of image analysis, showing a small amount of insulin secreted by the pancreatic islets in both diabetic groups were not in accordance with biochemical results indicating a significant increase in serum C-peptide level in date seed extract-insulin-treated group compared to insulin-treated diabetic group. Although it seems that date seed extract stimulates insulin secretion from certain extra-islet cells as those detected in the interlobular ducts of the pancreas, the possibility of stimulation of insulin secretion from extrapancreatic sources should not be ignored. Studies reported the presence of insulin-producing cells in the liver,[17,18] adipose tissue,[19] spleen,[20] bone marrow,[21,22] and thymus[19] of diabetic rodents, and speculated that the regulation of extrapancreatic insulin expression may be entirely different from that in pancreatic beta cells.

We did not test the effect of multiple dosages of the extract, as well as the effect of extract for longer durations, and this limits our study. In addition, the study was initially designed to include a fifth group of diabetic rats taking daily ingestion of 10 ml of seed extract alone. However, most animals died at the beginning of the experiment. The lag period between the administration of the extract and the manifestation of its effect might be the possible cause of high mortality rate in group 5. Animals of such group suffered from high blood glucose levels for a relatively long period without effective treatment.

In conclusion, the present study suggested a mechanism by which date seed extract exerts its hypoglycemic effect on type 1 diabetic rats; by stimulation of endogenous insulin secretion through extra-islet sources. The present results would stimulate further studies to investigate the presence, if any, of insulin-producing cells in extrapancreatic tissues of date seed extract-treated diabetic rats. Investigation of the effect of dosage variations and duration of administration of the extract on the structure and function of vital organs, as well as performance of an in-vitro study for analysis of the constituents and determination of the active ingredient in the date seed extract would also be essential. The results would encourage testing the efficacy of the date seed extract for treatment of T1DM in human.

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