Low carbohydrate ketogenic diet prevents the induction of diabetes using streptozotocin in rats

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ABSTRACT

Diabetes continues to be an overwhelmingly prevalent endocrine disorder that leads to several micro- and macrocomplications. It has been widely accepted that changes in dietary habits could induce or prevent the onset of diabetes. It is shown that low carbohydrate ketogenic diet (LCKD) is effective in the amelioration of many of the deleterious consequences of diabetes. However, its role in preventing the onset of diabetes is not understood. Therefore, this study is focused on the effect of LCKD in preventing the induction of diabetes using streptozotocin (STZ) in rats by biochemical and histological methods. Forty-two Wistar rats weighing 150–250 g were used in this study. The animals were divided into three groups: normal diet (ND), low carbohydrate ketogenic diet (LCKD), and high carbohydrate diet (HCD). Specific diets *ad libitum* were given to each group of animals for a period of 8 weeks. Each group was further subdivided into normal control, sham control and diabetic groups. Animals in the diabetic group were given a single intraperitoneal injection of STZ (55 mg/kg). All the animals were sacrificed 4 weeks after the injection of STZ. Daily measurements of food and water intake as well as weekly measurement of body weight were taken during the whole 12 weeks of the experiment. After injecting with STZ, the blood glucose level of all the groups increased significantly except for the group fed on LCKD (p value < 0.01). Also, food intake, water intake and urine output were significantly increased in all groups except for the LCKD group (p value < 0.01). There was also a significant decrease in the weight gain of the animals that were fed on a LCKD as compared to other groups (p value < 0.05). Although, substantial decrease in the number of β cells was noticed in diabetic rats, there were no change in the number of β cells in the LCKD treated diabetic animals as compared to LCKD control group. The results presented in this study, therefore, suggests that LCKD prevents the development of diabetes using streptozotocin in rats.

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1. Introduction

Diabetes mellitus (DM) is characterized by hyperglycemia which results from the defects in insulin secretion and/or insulin action. It is one of principal cause of morbidity and mortality in human population due to the development and progression of micro- and macrovascular complications including neuropathy, nephropathy, cardiovascular and cerebrovascular diseases (Altan, 2003). It has been estimated that diabetic patients exceed over 200 million worldwide and its prevalence is rapidly increasing (Malecki, 2004). Similarly, in the Gulf region, especially in Kuwait, diabetes is widely spreading and is considered as a major health problem (Abdella et al., 1999).

Several chemical agents can alter β-cell function leading to diabetes. One important chemical agent which can cause diabetes in experimental animals is streptozotocin (STZ). STZ is produced by *Streptomycetes achromogenes* and was originally identified as an antibiotic (Lewis and Barbiers, 1960). It is composed of a glucose molecule with a nitrosourea side chain. Upjohn Laboratories accidentally discovered that STZ could produce hyperglycemia, but the exact mechanism was not known until it was described how a single dose of streptozotocin (STZ) in dogs and rats can lead to β-cell death and results in the diabetic state (McNeill, 1999).

The glucose moiety of STZ binds to the glucose transporter GLUT2 present on the pancreatic β-cell membrane and enters the cell while the nitrosourea moiety is the part responsible for its cytotoxic effects. After STZ enters the β cells, cell death can occur

through methylation, the release of free radicals, or by the formation of nitric oxide (McNeill, 1999; Szkudelski, 2001). Due to these mechanisms, STZ is selectively cytotoxic to the β cells of pancreatic islets and a single intravenous or intraperitoneal injection of STZ (40–60 mg/kg body weight) can lead to type 1 diabetes in experimental animals (Bolzán and Bianchi, 2002). Using this molecule, hyperglycemia and insulin deficiency is provoked within 48 h of administration.

Although, a wide variety of drugs are used for diabetes management, there is still no satisfactory/effective therapy available for its cure. Furthermore, these drugs are either unaffordable due to socio-economic conditions or have undesirable side effects (Pari and Satheesh, 2004). It has been shown that changes in the dietary habits, especially a reduction in the carbohydrate content is quite effective and safer in diabetes management. The carbohydrate content of the diet is the most important factor that influences the glycemic level. Low carbohydrate diets appear to improve glycemic control and lessen the need for exogenous insulin and hypoglycemic medication (Arora and McFarlane, 2005). These diets have been found to significantly improve insulin sensitivity by up to 75% (Boden et al., 2005).

Low carbohydrate ketogenic diet (LCKD) is a diet that is low in carbohydrates (<100 g/day) causing ketosis, mimicking the physiological state of fasting (Freeman et al., 2006). It was first introduced as an epilepsy treatment at the beginning of the 20th century and with time LCKD became important in the treatment of several other conditions such as obesity, cardiovascular disease, and type 2 diabetes (Dashti et al., 2003, 2004, 2006, 2007; Freeman et al., 2006). This study, therefore, is aimed at investigating the protective role of LCKD in the development of diabetes in an experimental animal model of diabetes.

2. Materials and methods

2.1. Animals and experimental design

A total of 63 male Wistar rats, weighing 150–200 g, were used in this study. Animals were housed singly, under controlled environmental conditions of temperature $22.3 \pm 0.3\,^{\circ}$C, $31.2 \pm 0.8\%$ humidity, and a 12-h light/dark cycle in the Animal Care Facility at Kuwait University. This study was approved by the Animal Protection Ethical Committee of Kuwait University. The animals were divided into three groups: normal diet (ND) of regular commercial rat food (Mathew et al., 2006), Low carbohydrate ketogenic diet of 30% fat, 10% carbohydrate and 60% protein (LCKD), and high carbohydrate diet of 70% carbohydrate, 10% fat and 20% protein (HCD) as described previously (Al-Khalifa et al., 2009). Specific diets *ad libitum* were given to each group of animals for a period of 8 weeks. Each group was subdivided into normal control, sham control and diabetic groups. After 8 weeks, diabetes was induced using an intraperitoneal injection of STZ (S-0130, Sigma, Ronkonkoma, NY, USA), 55 mg/kg in saline, while the animals in the sham control group were given only saline.

On the day of injection, the blood glucose level was measured from the tail using a glucometer (One touch ultra, Lifescan, Tokyo, Japan; Ugochukwu and Figgers, 2006). For the first 48 h after STZ injection, the animals were kept in metabolic cages and development of diabetes was confirmed using Keto-Diabur test strips (Accu-chek, Roche, Selangor Darul Ehsan, Malaysia; McNeill, 1999). Daily measurements of food and water intake as well as weekly measurements of body weight were taken during the whole experiment. In addition, blood glucose level (diabetic $\geq$ 250 mg/dL; Ugochukwu and Figgers, 2006) and urine output were taken once a week starting from the 8th week till the end of the experiment at the 12th week.

3. Preparation of specimen

At the end of the 12th week, animals were sacrificed and the rat's abdomen was opened with a midline incision, where the pancreas was taken for histological analysis by routine H&E and Gomori's Chrome Alum Haematoxylin–Phloxine staining methods.

Gomori's Chrome Alum Haematoxylin–Phloxine stain was used to distinguish endocrine cells of the pancreas and to highlight insulin producing cells (β cells) from α and δ cells (Drury and Wallington, 1980) as previously described (Al-Khalifa et al., 2009). Briefly, the staining method is as follows. Sections fixed in 10% formalin were treated with Bouin's fluid for 16–24 h. The slides were then washed in tap water to remove picric acid and then treated for 1 min with and an equal mixture of 0.3% potassium permanganate and 0.3% sulphuric acid. The tissues were decolourized with 2–5% solution of sodium bisulphate and washed well in running water. The slides were then stained in haematoxylin solution for 15 min until β cells become deep blue and rinsed in water and differentiated in acid alcohol for about 1 minute to remove background staining. They were washed well for about 10 min in running tap water until the sections were clear blue. After that, the slides were stained in 0.5% aqueous phloxine for 5 min and rinsed in water. The slides were then treated with 5% phosphotungstic acid for 1 min, washed in running tap water for 5 min so that sections become red colour and differentiated in 95% alcohol. Finally, the slides were dehydrated, cleared and mounted with a cover slip using a mixture of distyrene (a polystyrene), a plasticizer (tricresyl phosphate), and xylen (DPX). The tissue sections were examined using a light microscope (Zeiss, Hamburg, Germany) and images were captured with a Zeiss digital camera using Axiovision software (Zeiss, Hamburg, Germany).

3.1. Statistical analysis

For statistical analysis of the data Student's *t*-test and analysis of variance with Bonferroni correction were performed. A value of $p < 0.05$ was considered to be significant for the comparisons between the animals fed the normal, high carbohydrate, and low carbohydrate ketogenic diets.

4. Results

4.1. Effect of different diets on body weight

In the first 8 weeks, the body weight increased gradually in all groups, but significantly higher in ND and HCD compared to LCKD groups. After administration of STZ, there was significant drop in body weight in both groups ND-D and HCD-D as a characteristic feature of diabetic status. While in the LCKD-D group the body weight remained increasing constantly (Fig. 1).

4.2. Effect of different diets on blood glucose level

The blood glucose level was measured weekly using a glucometer with the blood collected from the rat's tail. After the administration of STZ the blood glucose level in ND-D and HCD-D groups were increasing from 105 mg/dL upto 650 mg/dL at the end of study except in the LCKD-D group where the blood glucose level remained within the normal range of 100 mg/dL (Fig. 2).

4.3. Changes in food intake in different groups

The food intake was almost constant in ND and HCD groups in the first 8 weeks. After that, the diabetic groups ND and HCD were in a state of polyphagia. The LCKD groups showed the least food
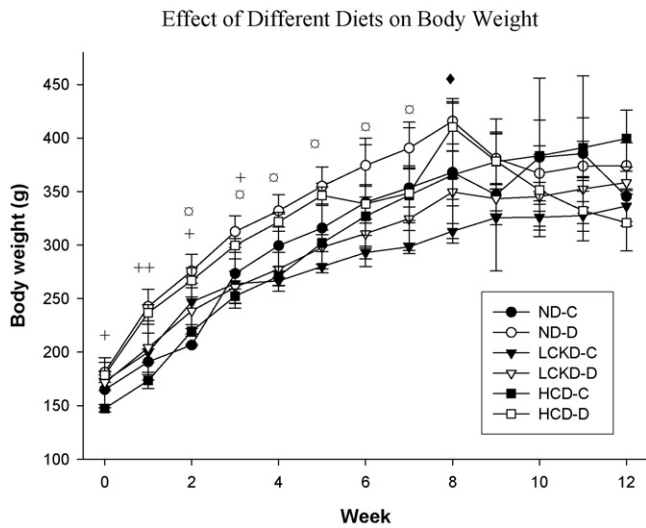
Fig. 1. Effect of different diets: normal diet (ND), high carbohydrate diet (HCD), and low carbohydrate ketogenic diet (LCKD) on body weight (g) in control (C) and diabetic rats (D). The values are mean ± SEM ($n = 42$). ¤ = $p < 0.05$, ND-D compared to LCKD-D. ♦ = $p < 0.05$, HCD-D compared to LCKD-D.

intake comparing to the other groups. There was little increase in the food intake within the first 4 weeks, after that it decreased and remained constant during the whole experiment (Fig. 3).

4.4. Calories intake of different diet

The caloric content of the different diets was calculated form the combustion values for CHO, fats, and proteins, which are 4, 9 and 4 kcal/g, respectively. This amounted to 4.65 kcal/g in the ND, 4.5 kcal/g for HCD, and 5.5 kcal/g for LCKD. All the groups ingested about the same number of calories in the first few weeks until week 5. There was a significant increase in the caloric intake for ND and HCD compared to LCKD. This difference increased sharply with $p < 0.001$ after STZ administration in the diabetic groups of ND and HCD compared to LCKD-D as calorie intake was ranging between 600 and 800 kcal/g to reach up to 1600 kcal/g. This result was with-
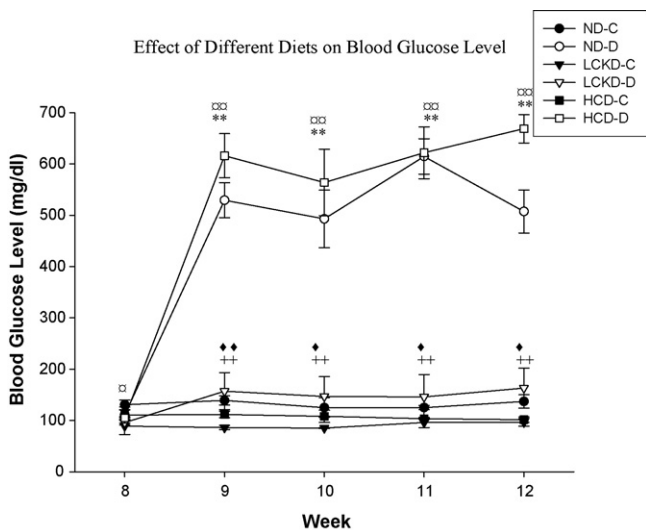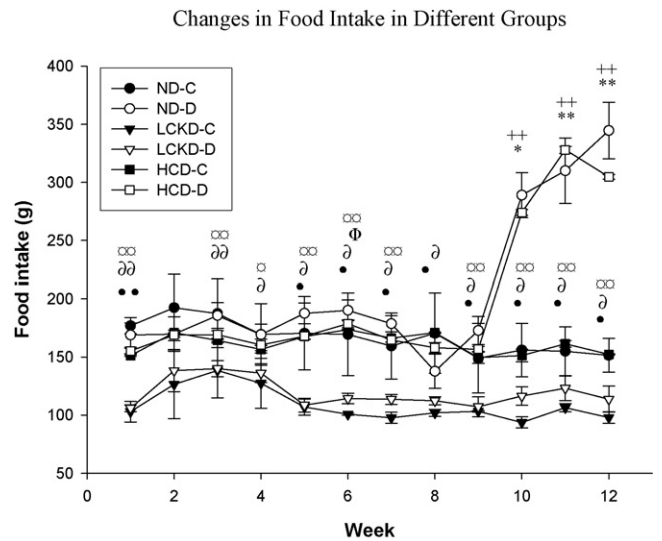

Fig. 3. The effect of different diets: normal diet (ND), high carbohydrate diet (HCD), and low carbohydrate ketogenic diet (LCKD) on food intake (g/week) in control (C) and diabetic rats (D). The values are mean ± SEM ($n = 42$). * = $p < 0.05$, ** = $p < 0.01$, ND-C compared to ND-D. + = $p < 0.05$, ++ = $p < 0.01$, HCD-C compared to HCD-D. ∞ = $p < 0.05$, ∞∞ = $p < 0.01$, HCD-C compared to LCKD-C. ∂ = $p < 0.05$, ∂∂ = $p < 0.01$, HCD-D compared to LCKD-D. ¤ = $p < 0.05$, ¤¤ = $p < 0.01$, ND-D compared to LCKD-D.

out subtracting the energy lost as urinary glucose or ketone bodies (Fig. 4).

4.5. Changes in water intake in different groups

In the first 8 weeks, there was no difference in water intake between the groups. However, after 8 weeks, as polydipsia is a characteristic feature of diabetes, the HD-D and HCD-D groups showed an increase in water intake. The LCKD-D group on the other hand showed a constant water intake throughout the experimental period (Fig. 5).

4.6. Effect of different diets on urine output

The amount of urine excreted was monitored weekly and it was between the range of 15 and 35 mL/day in all the groups throughout
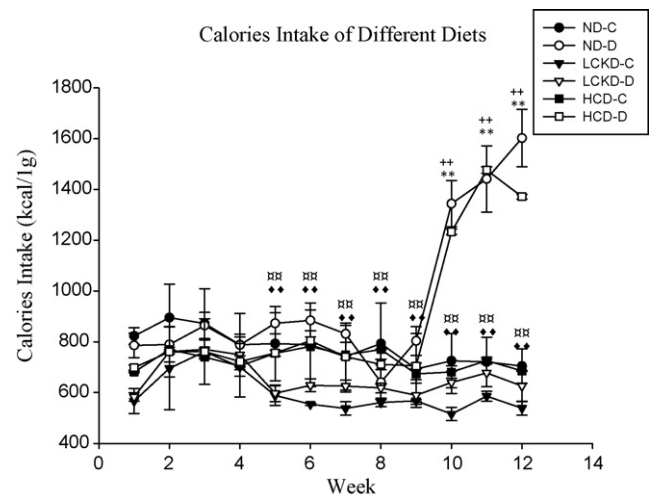

Fig. 2. The effect of different diets: normal diet (ND), high carbohydrate diet (HCD), and low carbohydrate ketogenic diet (LCKD) on blood glucose level (mg/dL) in control (C) and diabetic rats (D). The values are mean ± SEM ($n = 42$). ** = $p < 0.01$, ND-C compared to ND-D. ++ = $p < 0.01$, ND-D compared to LCKD-D. ¤¤ = $p < 0.01$, HCD-C compared to HCD-D. ♦♦ = $p < 0.01$, HCD-D compared to LCKD-D.

Fig. 4. The calories intake of different diets: normal diet (ND), high carbohydrate diet (HCD), and low carbohydrate ketogenic diet (LCKD) in control (C) and diabetic rats (D). The values are mean ± SEM ($n = 42$). ** = $p < 0.001$, ND-C compared to ND-D. ++ = $p < 0.001$, HCD-C compared to HCD-D. ¤¤ = $p < 0.001$, ND-D compared to LCKD-D. ♦♦ = $p < 0.001$, HCD-D compared to LCKD-D.
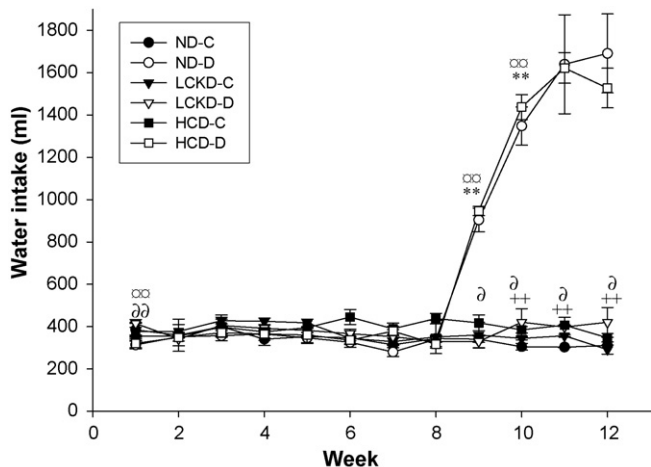
Fig. 5. The effect of different diet: normal diet (ND), high carbohydrate diet (HCD), and low carbohydrate ketogenic diet (LCKD) on water intake (mL) of control (C) and diabetic rats (D). The values are mean ± SEM ($n = 42$). ** = $p < 0.01$, ND-C compared to ND-D. ++ = $p < 0.01$, ND-D compared to LCKD-D. $\partial\partial$ = $p < 0.01$, HCD-D compared to LCKD-D. ⋈ = $p < 0.01$, HCD-C compared to HCD-D.
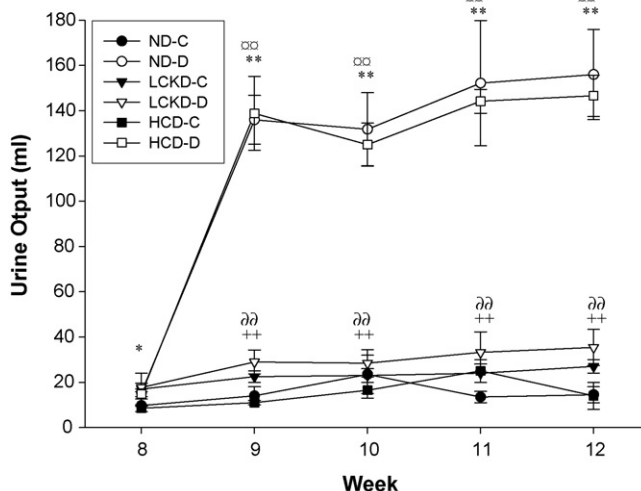
Fig. 6. The effect of different diet: normal diet (ND), high carbohydrate diet (HCD), and low carbohydrate ketogenic diet (LCKD) on urine output (mL) of control (C) and diabetic rats (D). The values are mean ± SEM ($n = 42$). ** = $p < 0.01$, ND-C compared to ND-D. ++ = $p < 0.01$, ND-D compared to LCKD-D. $\partial\partial$ = $p < 0.01$, HCD-D compared to LCKD-D. ⋈ = $p < 0.01$, HCD-C compared to HCD-D.

the experiment except for the diabetic ones (ND-D and HCD-D). There was a sudden increase in the urine output (polyurea) in the ND-D and HCD-D groups reaching about 165 mL/day at the end of the study (Fig. 6).

4.7. Effect of different diets on urine glucose level

The urine was tested for the presence of glucose, which is usually negative in normal conditions. Before week 8, all the groups showed negative glucosuria. After STZ injection, there was a sig-

nificant appearance of glucose in the diabetic ND and HCD only, above 1000 mg/dL (past stage 4) indicating the development of a diabetic state. However, the diabetic group of LCKD continued to show negative glucosuria.

4.8. Histological assessment of islets of Langerhans

H&E staining showed the presence of several round to elongated islets distributed throughout the pancreas in all the control groups
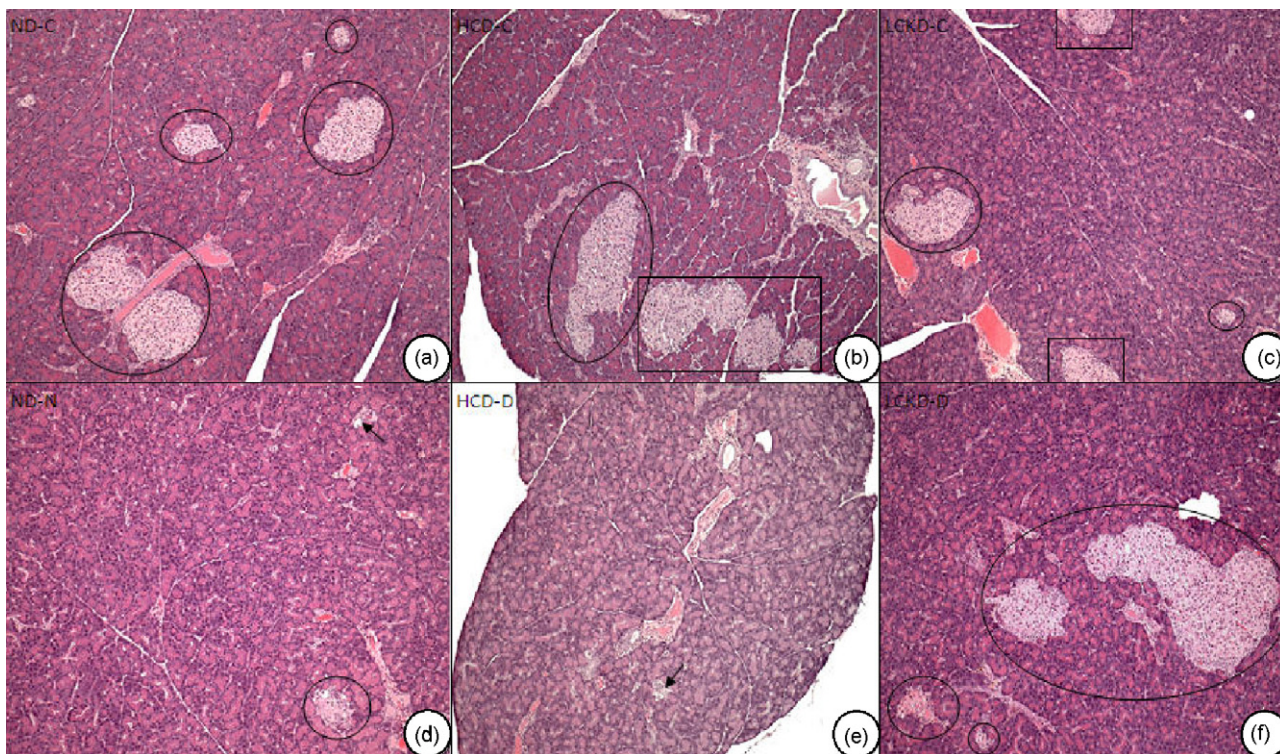

Fig. 7. Sections of the pancreas from control and diabetic rats of pre-fed experiment stained with H&E. Circles shows islets of Langerhans. Arrows shows vacuoles. Magnification 10×.
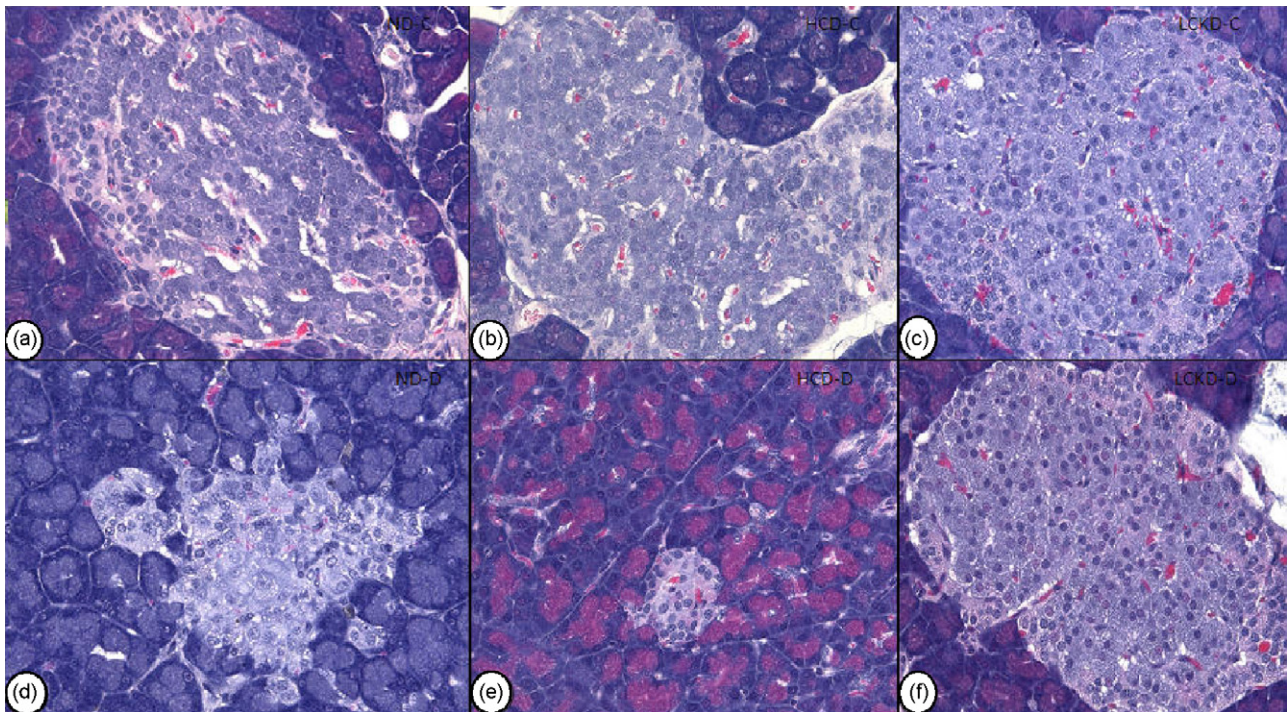
Fig. 8. Sections of the pancreas from control and diabetic rats of pre-fed experiment stained with Gomori's Chrome Alum Haematoxylin–Phloxine stain. β cells with blue and located interior, α cells with red found at periphery and δ cells pink to red located among α cells. Magnification 40×. (For interpretation of the references to color in the figure caption, the reader is referred to the web version of the article.)

(Fig. 7a and b). In the diabetic groups of ND and HCD, the islet morphology was altered with vacuoles and only a few islets were seen in the tissue sections (Fig. 7d and e). On the other hand, the islets were normal and intact in both the control and the diabetic group of LCKD (Fig. 7c and f). Gomori's Chrome Alum Haematoxylin–Phloxine stain was used to distinguish the endocrine cells of pancreas and to highlight the blue stained insulin producing β cells from the red α and the pink to red δ cells (Fig. 8). The data showed a decrease in the number of β cells in diabetic rats of ND and HCD with $p = 0.022$ and $p = 0.012$, respectively, compared to their control groups. There was no difference, however, between LCKD control and diabetic groups ($p = 0.981$) as well as between LCKD control and control of ND and HCD (Fig. 9).

5. Discussion

Diabetes mellitus (DM) is a common endocrine disorder worldwide and its occurrence continues to increase. It has been widely accepted that dietary components have a significant role in the clinical management of DM. Various studies from our laboratory have shown that ketogenic diet is effective in achieving weight loss and may have beneficial effects on glycemic control, triglyceride levels, and high-density lipoprotein cholesterol levels in diabetic patients. (Dashti et al., 2003, 2004, 2006, 2007). The results of the animal experiments presented in this study clearly shows that the rats fed on LCKD had remarkable tolerance to STZ and did not develop diabetes.

In this study, diabetes was induced in rats using STZ injection. STZ selectively destroys pancreatic β cells (insulin producing cells) by inducing DNA methylation and DNA damage, causing activation of poly(ADP-ribose) polymerase (PARP). This results in the reduction of cellular NAD^+, severe ATP depletion and eventually cell death (Bolzán and Bianchi, 2002). The rats on LCKD showed greater resistance to the diabetogenic action of the drug. This was proven by measuring the metabolic parameters, including changes

in body weight, blood glucose, food and water intake and urine output.

The tendency to put on weight are higher in the normal and high carbohydrate diets fed animals as compared to LCKD. The mechanisms by which ND and HCD increase and LCKD reduce body weight were explained in several studies (Ludwig, 2002; Ebbeling et al., 2003). ND and HCD with high carbohydrate (CHO) contents, have high glycemic index. CHO is considered as the major stimulus for insulin secretion. This induces a more rapid insulin response which leads to a hypoglycemic postprandial period, that causes appetite stimulation and increase in caloric intake. Secondly, insulin is an anabolic hormone and with continued hyperinsulinemia there is

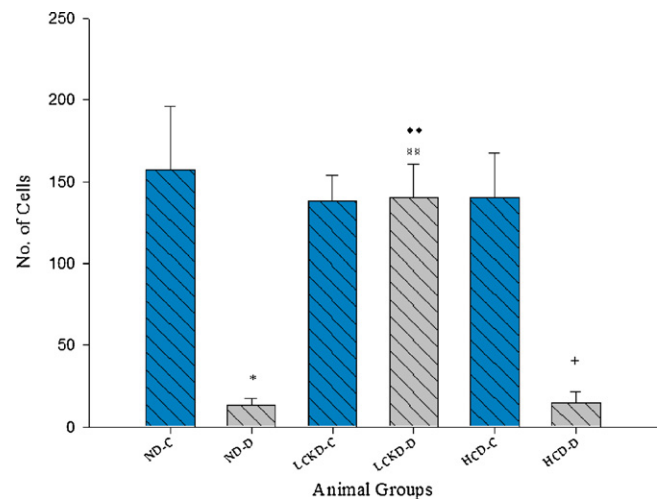**Fig. 9.** The effect of different diets: normal diet (ND), high carbohydrate diet (HCD), and low carbohydrate ketogenic diet (LCKD) on number of β cells in control (C) and diabetic rats (D). The values are mean ± SEM ($n = 42$). $^* = p < 0.05$, ND-C compared to ND-D. $+ = p < 0.05$, HCD-C compared to HCD-D. $\bowtie = p < 0.01$, ND-D compared to LCKD-D. $\blacklozenge\blacklozenge = p < 0.01$, HCD-D compared to LCKD-D.

an increase in lipogenesis, rather than oxidation, leading to fat deposition and obesity.

Several mechanisms are involved in the reduction of body weight using LCKD. There is a greater loss of glycogen and water in this diet compared to the other diets that leads to an initial rapid weight loss. This occurs during the depletion of glycogen storage as each gram of glycogen is stored in 3 g of water (Kreitzman et al., 1992). Also, due to ketosis, there is energy loss in the form of ketones in urine, sweat or feces. Ketones have a diuretic effect and therefore lead to an even greater water loss, which has been measured from 4.5 to 15 lbs (Phinney et al., 1983). Further reduction in weight results from thermogenesis and increased fat loss, with preserved lean body mass (Willi et al., 1998). In contrast to our results other investigators (Kettelhut et al., 1980; Siegel et al., 1980) showed an increase in the body weight of LCKD fed rats. However, the LCKD composition used in these studies were different from what we used in our studies.

Consumption of high glycemic index diet (ND and HCD) was associated with a higher risk of diabetes (Hodge et al., 2004). However, the results presented here shows that STZ injected LCKD group showed no substantial change in the blood glucose level and remained within the normal range of 100 mg/dL. Previous studies from other investigators have also shown the protective effect of high-protein, CHO-free diet in the induction of diabetes using STZ (Eizirik and Migliorini, 1984; Eizirik et al., 1985). They found that rats adapted to a high-protein, CHO-free diet for period of 15–21 days prior STZ injection, showed decrease in the severity of diabetes and reduced the diabetogenic effect of STZ.

Although the LCKD group showed the least food intake and body weight gain comparing to other groups, all the diets used were approximately isocaloric. This is believed to be due to regulation of food intake through the action of a number of peptides. One of them is cholecystokinin (CCK), which is a satiation signal that is released predominantly from I-cells in the small intestine in response to the presence of fat or protein, which have a high satiety value (Kerstens et al., 1985). Plasma CCK concentrations are elevated in response to fat digestion, not by carbohydrates digestion (Torregrossa and Smith, 2003).

Other peptides that affect food intake are peptide YY (PYY), neuropetide Y (NPY) and leptin. PYY is shown to reduce food intake in high-fat fed rodents and also elevate fat oxidation (Adams et al., 2006). On the other hand, leptin can modulate energy expenditure, appetite and sympathetic nervous activity (Haynes, 2005). It can directly inhibit NPY and enhance melanocortin action which is responsible for suppression of feeding, which means that leptin can promote weight loss (Haynes, 2005).

Beside these peptides, ketone bodies in LCKD also play a role in the regulation food intake. In a study involving 3-hydroxybutyrate (3-OHB) infusions in Sprague–Dawley and Osborne–Mendel rats, which were fed low-fat or high-fat diets, it has been found that both groups exhibited reduced food intake and body weight, indicating that increased circulating levels of 3-OHB act as a satiety signal (Arase et al., 1988).

After STZ injection, the diabetic groups of ND and HCD showed polyphagia as expected while the food intake remained constant in LCKD group, which was in agreement with the studies of other investigators (Yancy et al., 2004). Also, polydipsia was observed in diabetic rats that were fed with normal and high carbohydrate diet, but the water intake of LCKD diabetic rats remained constant. Similarly, rats fed on ND and HCD showed a significant increase in urine output and the STZ injected rats fed on LCKD did not show a significant increase in urine output. In support to the data presented in this study, other studies using CHO restricted diet (Schmidt et al., 1980) or CHO-free diet (Eizirik and Migliorini, 1984; Eizirik et al., 1985) also showed similar results in water intake, urine excretion and food consumption.

Urine glucose analysis showed the presence of glucose in ND-D and HCD-D groups while glucose was absent in the LCKD-D group. The presence of glucose in the urine also correlated with the polyurea condition in these groups since it has a diuretic effect (Shihabi et al., 2001).

In our studies, STZ injection showed a decrease in the number of β cells in rats belonging to the ND and HCD groups as demonstrated by other investigators (Lazarys and Shapiro, 1972; Mythili et al., 2004). However, there was no difference between LCKD control and diabetic groups as well as between LCKD control and control of ND and HCD. Based on these observations, we suggest that LCKD, specifically ketone bodies functions as antioxidants that prevent the diabetogenic effect of STZ. In this regard, it has been shown that inducing ketosis either by the administration ketone bodies or LCKD, elevates the antioxidative capacity in the central nervous system, and improves the conditions of patients with neurologic disorders (Peterson et al., 2005; Maalouf et al., 2007). Veech et al. (2001) also suggested that ketones might reduce oxidative stress in cardiac tissue. Nazarewicz and his colleagues (2007) found that ketone bodies, particularly beta-hydroxybutyrate, significantly increased the redox status of healthy human blood.

In conclusion, this study suggests that LCKD prevents the development of streptozotocin in duced diabetes and its complication in rats. However, further studies are necessary to understand the underlying cellular mechanisms. Further studies on the therapeutic role of LCKD in diabetes are in progress in our laboratory.

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