Histol Histopathol (2011) 26: 13-21

http://www.hh.um.es

Histology and Histopathology

Cellular and Molecular Biology

Effects of *Momordica charantia* on pancreatic histopathological changes associated with streptozotocin-induced diabetes in neonatal rats

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Summary. The aim of this research was to determine the effects of *Momordica charantia* (MC) fruit aqueous extract on pancreatic histopathological changes in neonatal STZ-induced type-II diabetic rats. Diabetes mellitus was induced in one day Sprague-Dawley neonatal rats using a single intrapretoneal injection of streptozotocin (STZ) (85 mg/kg body weight) and monitored for 12 weeks thereafter. The diabetic rats were separated into three groups, as follows: the diabetic control group (i.e. nSTZ), the diabetic group (i.e. nSTZ/M) - which was orally given 20 mg/kg of MC fruit extract, and the diabetic group (i.e. nSTZ/G) - that was treated with glibenclamide, 0.1 mg/kg for a period of four weeks. At the end of treatment, the animals were sacrificed and blood samples were collected from the saphenous vein to measure the blood glucose and serum insulin level. The pancreatic specimens were removed and processed for light microscopy, electron microscopy examination and immunohistochemical study. The results of this study showed that MC fruit aqueous extract reduced the blood glucose level as well as glibenclamide and increased the serum insulin level in the treated diabetic rats ($P<0.05$). The fruit extract of MC alleviated pancreatic damage and increased the number of ß-cells in the diabetic treated rats ($P<0.05$). Our results suggest that oral feeding of MC fruit extract may have a significant role in the renewal of pancreatic ß-cells in the nSTZ rats.

Keywords: Diabetes, Neonatal rats, Streptozotocin, Pancreas, ß-cells, *Momordica charantia*

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Introduction

Diabetes mellitus is a systemic metabolic disorder characterized by elevated blood glucose due to absolute or relative deficiency of insulin secretion from pancreatic cells (Leonardi et al., 2003). Non insulin dependent diabetes mellitus (NIDDM) or type II diabetes is the most common form of the disease, usually accompanied by insulin resistance and defective ß-cell function (Lupi and Del Prato, 2008). Insulin resistance is a major factor in pathogenesis of NIDDM and occurs when the cellular mechanisms fail to respond to the effects of insulin (Shulman, 2000). Pancreatic ß-cell mass is markedly reduced in insulin-dependent diabetes mellitus (IDDM) or type I diabetes and moderately reduced in NIDDM (Rahier et al., 2008). The neonatal rats treated with STZ at the first day of birth showed hyperglycemia and a reduction in pancreatic insulin amount during the neonatal period and this could be maintained up to adulthood. Therefore, these animal models can be taken to reproduce human type II diabetes (Portha et al., 2007). These rats also become ß-cell deficient and exhibit hypoinsulinemia with relatively mild diabetes (Pari and Ashokkumar, 2005).

Although medications such as sulfonylureas are widely used to treat type II diabetes, however, they are associated with side effects. Recently, some researchers had manifested an increasing interest in traditional medicinal plants. Many traditional plants have been used for diabetes therapy (Kim et al., 2006). One of these plants is *Momordica charantia*, or bitter melon, which belongs to the *cucurbitacea* family and is consumed in South Asia, South America and oriental countries as a food item and medicinal plant for treating various diseases such as diabetes mellitus (Grover and Yadav, 2004). Hypoglycemic activity of MC fruit (Miura et al.,

2001; Virdi et al., 2003), seeds (Sathishsekar and Subramaniam, 2005) and whole plant (Krawinkel and Keding, 2006) has been previously confirmed in experimental animals. MC is capable of reducing fasting serum glucose in patients with type II diabetes (Ahmad et al., 1999). Some studies had shown the effects of MC on improving insulin sensitivity in high-fat rats (Sridhar et al., 2008). Welihinda et al. (1986) demonstrated that MC improves the glucose tolerance in diabetic patients. Previous studies had also reported that MC enhances insulin secretion (Fernandes et al., 2007; Sathishsekar and Subramaniam, 2005) and increases the number of pancreatic ß-cells in islets of Langerhans (Ahmed et al., 1998). There are several possible mechanisms of hypoglycemic activity of MC. Previous studies had revealed that MC increases the glucose uptake in liver via promoting glucose-6-phosphate dehydrogenase and declining glucose-6-phostatase (McCarty, 2004). It could also increase the mRNA expression of glucose transporter 4 (GLUT4) proteins in skeletal muscles (Shih et al., 2009). Mahomoodally et al. (2007) suggested that MC fruit extract can reduce the glucose transport via the brush border of small intestine in treated diabetic animals.

This study investigated the effects of MC fruit aqueous extract on pancreatic histopathology of diabetic neonatal rats induced with STZ, a validated animal model of NIDDM. Its efficacy was compared with glibenclamide, as a standard and common hypoglycemic medication.

Material and methods

Animals

The protocol for animal experiment for this study was approved by Animal Care and Use Committee of Faculty of Veterinary Medicine, University Putra Malaysia. Normal females Sprague-Dawley rats (200-250g) were caged overnight with normal males. Natural birth occurred 22 days after mating. One-day-old neonatal rats received a single intraperitoneal injection of streptozotocin (85 mg/kg) (Sigma, S0130-USA) freshly dissolved in 0.9% saline solution (Li et al., 2004). Meanwhile, the normal control neonatal rats received an equivalent volume of 0.9% saline solution only. The neonatal rats were kept with their own mothers for one month and kept in suitable temperature (22±2°C), humidity and 12 hours of day-night cycle in plastic cages. The animals were considered as diabetic only if their blood glucose concentration was more than 11 mmol/l on the second post injection day (Li et al., 2004). Twelve weeks after STZ injection, the diabetic animals were divided into three groups with seven animals in each group. The treated groups were as follows: the nSTZ control group (STZ-injected neonatal rats), the nSTZ/M group (STZ-injected neonatal rats treated with MC fruit aqueous extract) and the nSTZ/G group (STZ-injected neonatal rats treated with glibenclamide). The non-diabetic rats were considered as a normal control group.

Preparation of momordica charantia fruit aqueous extract

Fresh green whole fruits of MC were purchased from the local shops within 5 kilometer radius from the preparation venue. Small pieces of fruit were soaked in water at a ratio of 10:25 w/v for 1 hour, and stored at room temperature. It was then filtered and evaporated by rotary evaporator to dry under reduced pressure to produce yield (Virdi et al., 2003).

Mode of feeding

Treatments were given twice daily for a period of four weeks. The extract powders were orally fed in nSTZ/M at a dosage of 20 mg/kg body weight, and glibenclamide was orally administered in nSTZ/G at a dosage of 0.1 mg/kg body weight (Virdi et al., 2003).

Measurement of blood glucose concentration

For detection of blood glucose level, the blood samples were collected from the saphenous vein. The blood glucose was then measured once a week during four weeks treatment using the Accu-Chek Instant Plus blood glucose monitor (Roche Diagnostics Corp.).

Tissue preparation for light microscopy

The male rats were sacrificed by being given ketamine (80 mg/kg) and xylazine (8 mg/kg) anesthesia at the end of treatment, and the pancreatic islets were examined under the light microscope. The specimens were obtained from the splenic lobes of pancreas, fixed in 4% paraformaldehyde/ PBS overnight at temperature 4°C (Li et al., 2004). The fixed specimens were embedded in paraffin and sectioned at 3 μm. From each block, 50 slides of two sections each were prepared. The prepared slides were stained using hematoxylin and eosin (H&E) and then examined under the light microscope (Olympus Bx51, Japan) in a blinded manner. The histopathological findings were scored as (–) if the extent of the injury was less than 5% or there was no injury, as (+) if a mild injury was observed (5-25%), as (++) if a moderate injury was apparent (25-50%), and as (+++) if it was a severe injury (>50%) (Soltani et al., 2005).

Tissue preparation for electron microscopy

The small pieces of splenic lobes of pancreas were fixed with 4% glutaraldehyde (Agar, R1010) for 24 hours at temperature 4°C, washed with 0.1 M sodium cacodylate buffer for 30 min and post-fixed in 1% Osmium tetroxide (Agar, R1017) for two hours at temperature 4°C. After washing with 0.1 M sodium cacodylate buffer (30 min), the specimens were

dehydrated in an acetone series and infiltrated overnight with acetone and resin mixture and embedded in epoxy resin (Agar, R1043). The prepared ultra-thin sections were examined using the H-7100 scanning electron microscope (Hitachi, Japan). The number of insulin secretory granules and non- degenerated mitochondria were calculated using the image analyzer software.

Immunohistochemical localization

The slides were deparaffinised with xylene and hydrated with ethanol. After 20 min of boiling in the target retrieval solution (Tris EDTA), the slides were brought to Tris-buffered saline (TBS) with Tween 20. The slides were incubated with peroxidase block solution for five minutes and then incubated with mouse monoclonal insulin primary antibody (dilution 1:1000) (abcam, ab6995 England) for 30 min in room temperature. The slides were then washed three times in TBS with Tween 20 and incubated with Dextran coupled with peroxidise molecules and goat secondary antibody molecules against rabbit and mouse immunoglobulins (Real Envision Polymer) for 30 min, washed in TBS with Tween 20 (3 times) and then incubated with DAB substrate (dilution 1:50) for 10 min (Dako REAL EnVision Detection System, Peroxidase/DAB+, Rabbit/Mouse, U.S.A). The slides were counterstained with haematoxylin and mounted with coverslips and examined under the light microscope. The number of insulin-positive cells was calculated using the image analyzer software.

Statistical analyses

One-way analysis of variance, ANOVA (SPSS 15.0) and the corresponding post hoc test were used for analysis of data. Non-parametric data were evaluated using Kruskal-Wallis H test. All data expressed as mean ± SD (Standard Deviation) and $P<0.05$ were identified as significantly different.

Results

Effects of MC fruit extract on blood glucose and serum insulin level

The results obtained from the effects of MC fruit extract on blood glucose and insulin level are shown in Figures 1 and 2 respectively. In the nSTZ rats, the blood glucose concentration was significantly higher than that of the normal rats ($P<0.05$). The treated diabetic rats in nSTZ/M and nSTZ/G groups showed a blood glucose lowering activity at the 2nd, 3rd and 4th weeks post-treatment compared to the nSTZ group ($P<0.05$).

In the nSTZ group, the serum insulin level after four weeks treatment was lower than that of the normal group ($P<0.05$). Serum insulin level in the nSTZ/M and nSTZ/G groups was higher than that of the nSTZ group ($P<0.05$)

Light microscopy findings

The sections of islets of Langerhans are shown in Figure 3. In the normal control group, the histological appearance of pancreatic islet cells was normal. In the nSTZ rats, the most significant histopathological findings were necrotic and degenerated cells in the islets of Langerhans. Interestingly, in the nSTZ/M and nSTZ/G groups, the severity extent of abnormal histological signs of pancreatic islets was less than those of the nSTZ group (Table 1).

Electron microscopy findings

Under the electron microscope examination of the pancreatic ß-cells, was observed normal structural of mitochondria in the normal group. Also, numerous

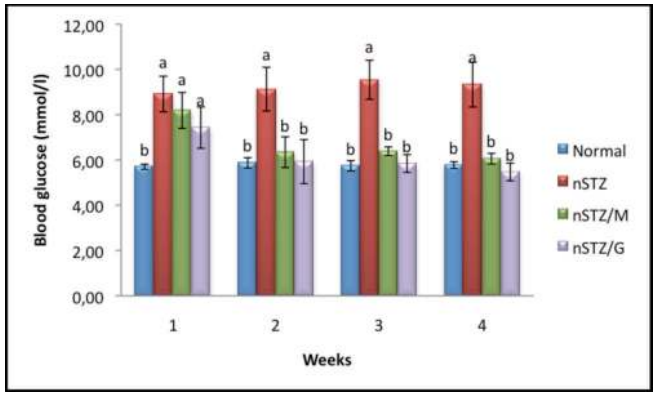

Fig. 1. Non-fasting blood glucose concentration changes during four week treatment. Error bar: ± SD (n=7). [a,b]: Bars with different alphabet notation differ significantly at $P<0.05$.

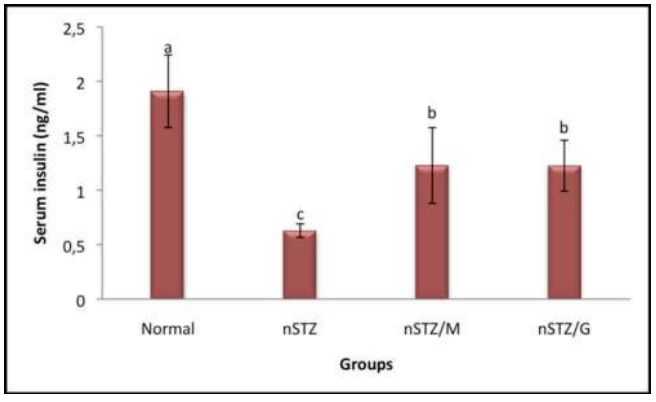

Fig. 2. The serum insulin level in non-fasting experimental animals after four week treatment. Error bar: ± SD (n=7). [a,b,c]: Bars with different alphabet notation differ significantly at $P<0.05$.

insulin secretory granules composed of an electron-dense core and a translucent halo were observed. The electron microscopic evaluation of nSTZ rats revealed that the pancreatic ß-cells manifested swelling and vacuolization in the mitochondria. The mitochondria looked round-shaped and the mitochondrial internal membrane and cisterns were not identifiable in this group. MC fruit extract and glibenclamide alleviated severities of abnormal changes induced by diabetes in the nSTZ/M and nSTZ/G rats (Fig. 4). A significant decrease was also observed in the number of insulin secretory granules and non-degenerated mitochondria in the nSTZ group compared to the normal group ($p<0.05$). Interestingly, MC fruit extract increased the number of insulin secretory granules and non-degenerated mitochondria in the nSTZ/M rats ($P<0.05$). Similarly, the nSTZ/G rats exhibited increment of insulin secretory granules and non-degenerated mitochondria in pancreatic ß-cells ($P<0.05$) (Table 2).

Table 1. Comparison of histopathological characteristics found in pancreatic islets of the experimental animals after four weeks treatment.

	Necrotic cells	Degenerated cells
Normal	–	–
nSTZ	++	++
nSTZ/M	+	+
nSTZ/G	+	+

–: no injury; +: mild injury; ++: moderate injury; +++: severe injury.

Immunohistochemical study

Immunohistochemical staining of ß-cells showed the

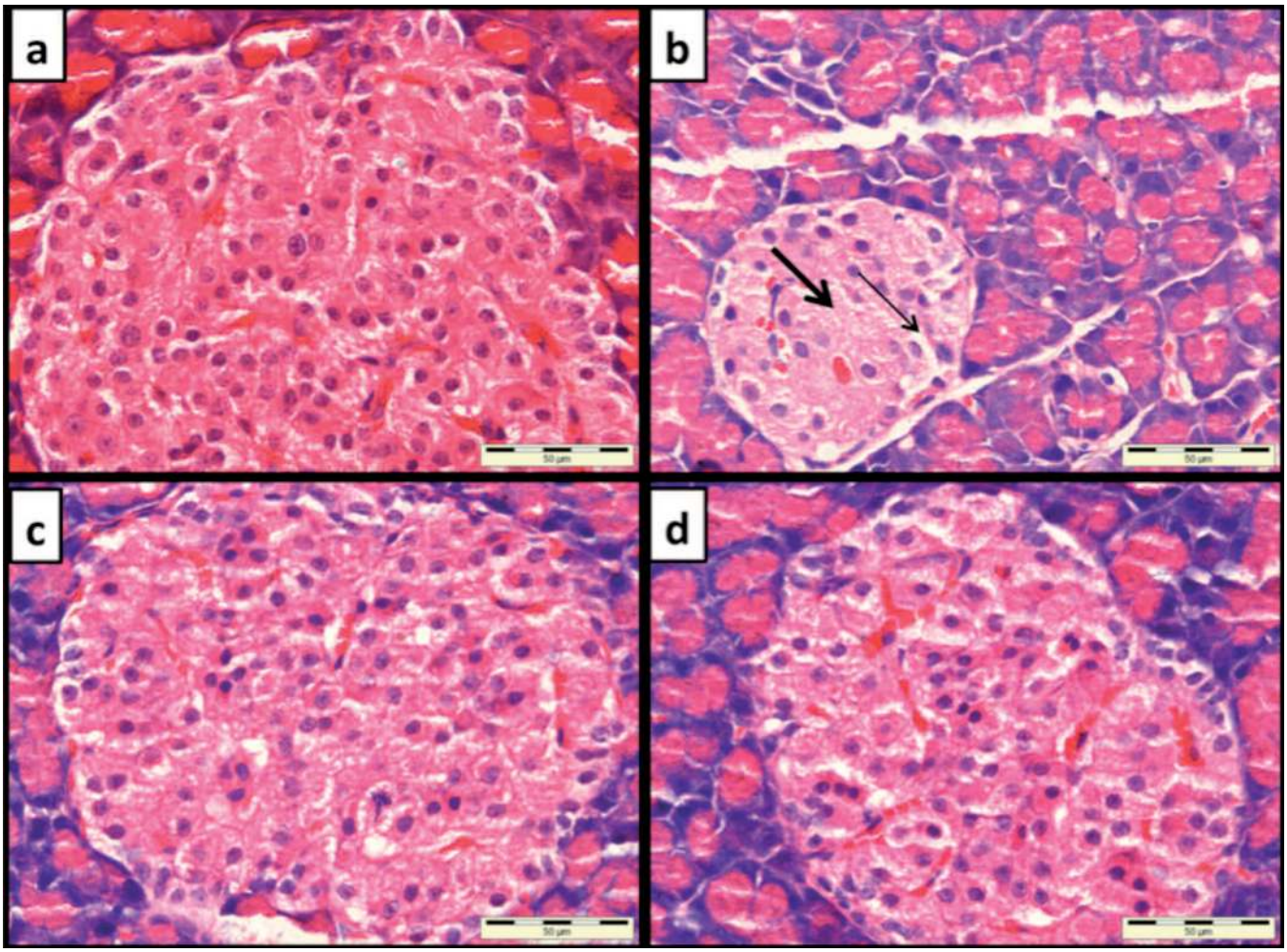

Fig. 3. a. The histology of islets of Langerhans exhibiting a normal structure in the normal group. **b.** Degeneration (thin arrow) and necrotic cells (thick arrow) clearly existed in the nSTZ group. In nSTZ/M **(c)** and nSTZ/G **(d)**, the severity of these changes was less than those in the nSTZ group. Seven animals were used in each group. H&E. Scale bar: 50 μm.

existence of insulin-positive-cells in all groups (see Fig. 5). The number of insulin-positive-cells had significantly reduced in the nSTZ rats compared to the normal control ($P<0.05$). The most striking result achieved from the immunohistochemical study was the increase in the number of insulin-positive-cells in the nSTZ/M group

Table 2. The number of insulin secretory granules and non-degenerated mitochondria in the pancreatic B-cells of experimental animals after four weeks treatment.

	Normal	nSTZ	nSTZ/M	nSTZ/G
The number of insulin secretory granules	625.20±118.98[a]	131.6±39.60[b]	556.40± 83.32[a]	490.20±122.89[a]
The number of non-degenerated mitochondria	40±11.34[a]	11.20±3.56[b]	35.40±10.14[a]	32.80±9.68[a]

*Values are means ± SD (n=7). [a,b]: Superscripts with different alphabet notation within row differ significantly at $P<0.05$.

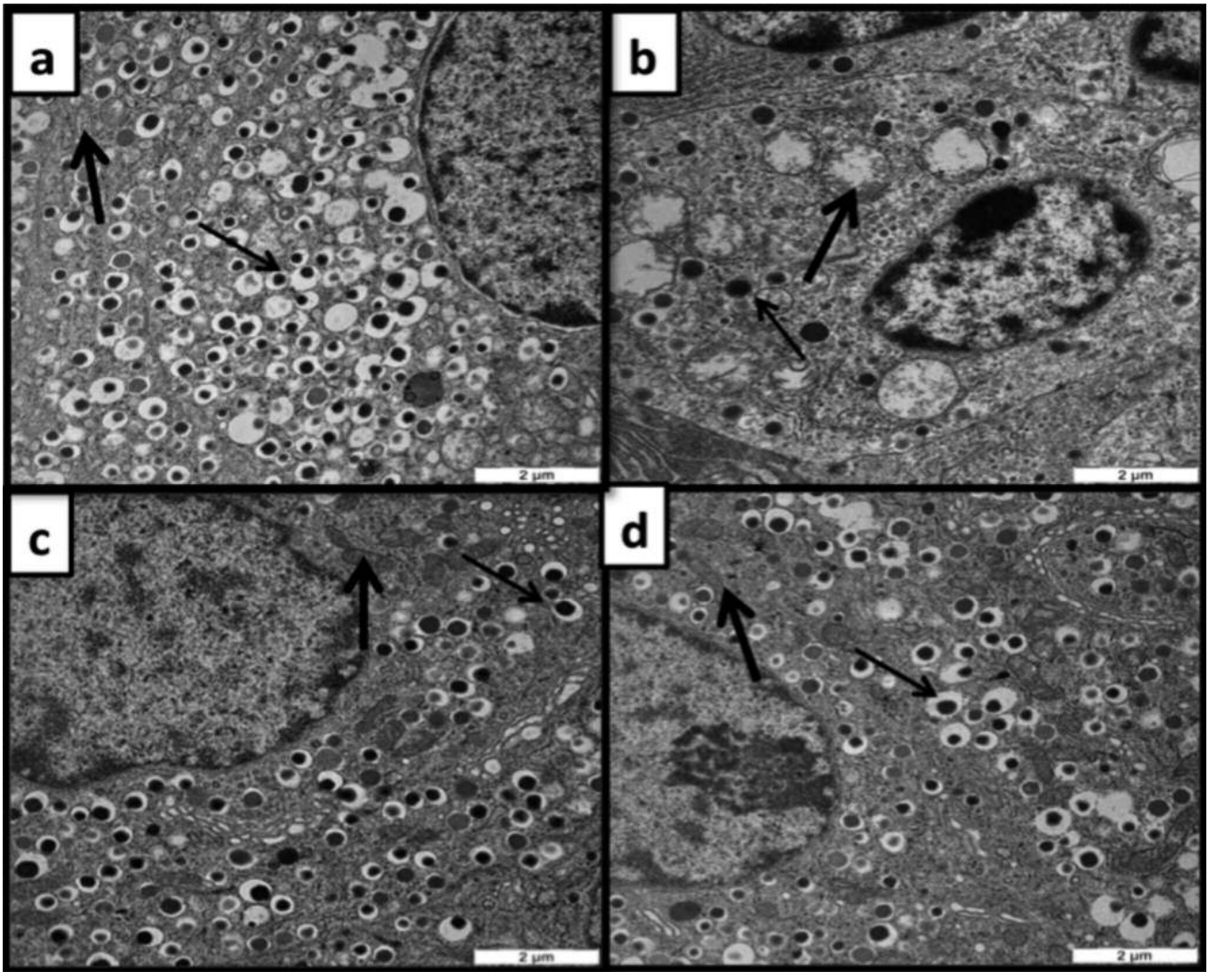

Fig. 4. a. Electron micrographs showing B-cells contained numerous insulin secretory granules (thin arrow), a normal structure of mitochondria (thick arrow) in the normal group. **b.** Degenerated mitochondria (thick arrow) and a few insulin secretory granules (thin arrow) in the nSTZ group. **c.** B-cells containing numerous insulin secretory granules (thin arrow) and improved mitochondrial structure (thick arrow) in the nSTZ/M and nSTZ/G **(d)** groups. Scale bar: 2 μm.

Table 3. The number of insulin-positive-cells after four weeks treatment in the experimental animals.

	Normal	nSTZ	nSTZ/M	nSTZ/G
The number of insulin- positive-cells	87.57±19.19[a]	22.86±7.09[b]	55.29±22.04[c]	49±16.94[b]

Values are means ±SD (n=7). [a,b,c]: Superscripts with different alphabet notation differ significantly at P<0.05.

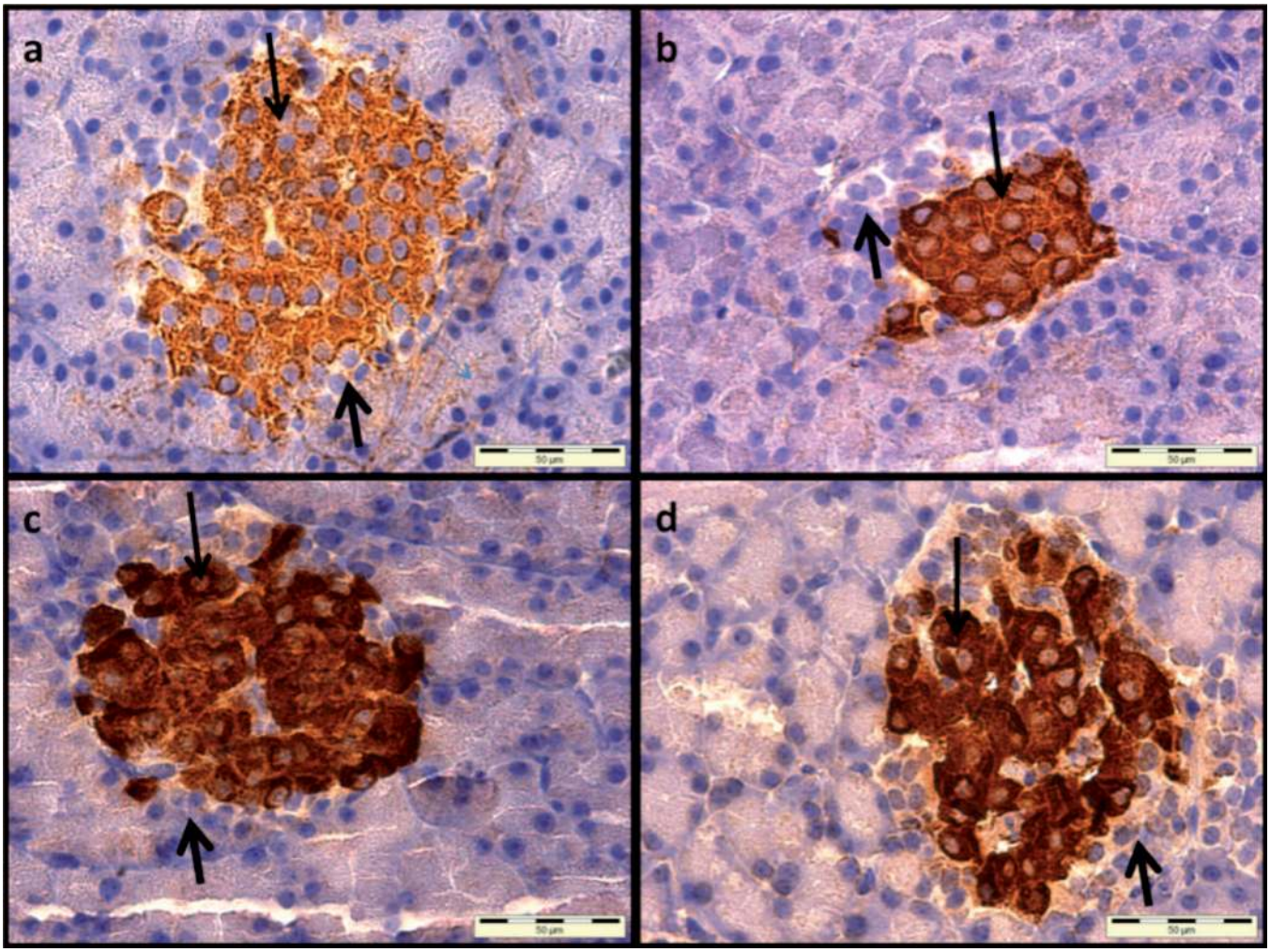

Fig. 5. a. Light micrographs of the normal group showing the insulin-positive-cells of islets of Langerhans were stained with anti-insulin antibody. **b.** A few insulin-positive-cells found in the diabetic group. **c.** Note the numerous insulin-positive-cells in the treated diabetic group with MC fruit extract (nSTZ/M). **d.** The treated diabetic group with glibenclamide (nSTZ/G). Insulin-positive-cells (thin arrow). Insulin-negative-cells (thick arrow). Seven animals were used in each group. Immunohistochemical staining, hematoxylin counterstained. Scale bar: 50 μm.

compared to the nSTZ group (P<0.05). Although the number of insulin-positive-cells in nSTZ/G rats were more than those in nSTZ rats, there was no significant difference between them (P>0.05) (Table 3).

Discussion

The neonatal rats treated with streptozotocin on the first day of birth exhibited mild hyperglycemia, impaired glucose tolerance and insulin resistance, which resemble the development of NIDDM in humans (Portha et al., 2007). The present study was designed to evaluate the effects of MC fruit aqueous extract on the improvement of damage in pancreatic ß-cells of the nSTZ rats.

The results of this study showed a significant effect in reducing the blood glucose level in the treated

diabetic rats with MC fruit extract as well as glibenclamide. Similar findings had been made previously in IDDM experimental animals (Virdi et al., 2003; Sathishsekar and Subramanian, 2005). Glibenclamide is one of the most common widely used medications against hyperglycemia, which stimulates insulin secretion from ß-cells through inactivation of ATP-sensitive potassium channel (Sakamoto et al., 2006). It also increases the number of insulin receptors (Hribal et al., 2001). Previous studies had reported that the hypoglycemic components of MC consist of a mixture of saponins, such as charantin, insulin-like peptides and alkaloids that are concentrated in its fruit (Krawinkel and Keding, 2006).

The results of this study showed the pancreatic insulin release was decreased in diabetic group. Our results also showed that treatment had significant effect in increasing the serum insulin level in both nSTZ/M and nSTZ/G groups. Previous studies had also reported the increasing serum insulin level in NIDDM diabetic mice (Miura et al., 2001), IDDM diabetic rats (Ahmed et al., 2004) and high fat diet diabetic rats (Chen et al., 2003). The serum insulin level is severely reduced (Suzuki et al., 2003) and impaired in NIDDM individuals in relation to the degree of hyperglycemia present (Takada et al., 2007). Some of the reports indicate that MC may have insulin-like secretagogue effect (Kumar et al., 2008). It was also demonstrated that MC is capable to enhance the number of pancreatic ß-cells in treated diabetic rats (Ahmed et al., 2004). Therefore, the increase in insulin levels suggested that MC would enhance the secretion of insulin by increasing the number of pancreatic ß-cells in the treated diabetic rats.

Histopathological examination of pancreatic islet also showed degenerated and necrotic cells in the pancreatic islets of diabetic rats. While administration of MC fruit extract significantly alleviated these abnormalities in the pancreatic islets of treated diabetic rats. Chronic hyperglycemia causes pancreatic islets destruction, leading to pancreatic dysfunction and development of type II diabetes (Hardt et al., 2002; Poitout and Robertson, 2002; Prentki et al., 2002; Nivitabishekam et al., 2009). Diabetes is also associated with oxidative stress that plays an important role in the development of diabetes complications (Baynes and Thorpe, 1999) and cause a variety of destruction of pancreatic ß-cells (Cemek et al., 2008). In support of this association, ß-cell death and a reduction in number of islets were observed in pancrease of diabetic rats by Sathishsekar and Subramanian (2005). Some studies have reported antioxidant effects of MC (Grover and Yadav, 2004; Wu and Ng, 2008) and improvement of stress oxidative induced by diabetes in pancreas of treated diabetic rats (Sathishsekar and Subramanian, 2005). In general, these results suggested that the MC fruit extract would control the pancreatic ß-cell damage through hypoglycemic and also antioxidant activity. This study investigated more structural changes in the islets of Langerhans using immunohistochemical study and transmission electron microscopy. The ultrastructural examination of the study showed degenerated mitochondrial structure in pancreatic ß-cells in the diabetic rats. Electron microscopy also showed that the number of mitochondria and insulin secretory granules decreased in the diabetic rats. It is evident that the MC fruit extract reduced the severity of injuries in mitochondrial structure and decreased the number of degenerated mitochondria in treated diabetic rats. It also showed increased number of insulin secretory granules in pancreatic ß-cells. Some studies had previously reported mitochondria degeneration in ß-cells of type II diabetic patients (Anello et al., 2005; Bruin et al., 2008), and also the reduction of insulin secretory granules in diabetic rats (Degirmenci et al., 2005). ß-cells are particularly sensitive to oxidative stress (Robertson et al., 2003). Severe oxidative damage induced by diabetes mellitus causes degeneration and disruption of pancreatic ß-cell mitochondria (Anello et al., 2005). Therefore, MC fruit extract may restore degenerated mitochondrial structure via its antioxidant properties, resulting in an inhibition of the increase of mitochondrial degeneration in pancreatic ß-cells. MC also, with the increasing number of ß-cells islets of Langerhans, may promote the number of insulin secretory granules in pancreatic ß-cells of the treated diabetic rats.

The results of immunohistochemical staining demonstrated significant changes in the distribution of insulin-positive-cells in the untreated diabetic rats compared to that of the normal rats. The results of this study indicated that there were weak insulin–positive-cells in the pancreatic islets of the diabetic rats. Meanwhile, the MC fruit extract was significantly capable of increasing the number of insulin-positive-cells as previously reported by Ahmed et al., (2004) in IDDM diabetic rats. MC fruit juice may reduce apoptosis in pancreatic B-cells (Sitasawad, 2000). It also may prevent further pancreatic ß-cell death by decreasing the oxidative stress caused by STZ in diabetic animals (Garau et al., 2003). Therefore, these result suggested that MC fruit extract may plays an important role in increasing the number of insulin-positive cells in the pancreas by preventing the ß-cells death and/or recuperation of partly damaged pancreatic ß-cells. In conclusion, the results of this investigation showed that the aqueous extract of MC fruit can alleviate pancreatic damage and enhance the number of pancreatic ß-cells in NIDDM rats.

Acknowledgements. The study was conducted at Universiti Putra Malaysia and supported by the Research University Grant Scheme (04/01/070088RU).

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Accepted June 11, 2010