Abstract
The purpose of this study was to determine whether the treatment with recombinant plasmid consisted of human GLP1 promoter and insulin gene can treat diabetic rats. Rats were induced type-1 diabetes mellitus (T1DM) by a single dose of intraperitoneal injection of streptozotocin (STZ) at dose of 55mg/kg. The induction of diabetes was confirmed in rats by checking the blood glucose level for seven days. The recombinant plasmid, GLP1/Ins/pBud plasmid, was wrapped with chitosan and then transferred to diabetic rats by force feeding. The blood glucose level was checked from the tips of the tails by needle puncture using a glucometer and test strips. The blood levels of human and rat insulin were assessed by enzyme-linked immunosorbent assay (ELISA). The results showed no significant effects of orally treatment with recombinant plasmid DNA at both doses of 100 and 600 µg/mL on the human insulin level in diabetic rats (p>0.05). The human insulin level was significantly increased by orally treatment at dose of 300 µg/mL (p=0.04). The findings indicated that the intraperitoneal injection of 300 µg/mL of this nanoparticle complex prominently increased the human insulin level in diabetic rats in contrast to both doses of 100 and 600 µg/mL. Despite above results, both methods was not effective enough to decrease the blood glucose levels in diabetic rats. It was concluded that the treatment of diabetic rats with recombinant plasmid consisted of human GLP1 promoter and insulin gene was not effective to reduce the blood glucose levels in diabetic rats.

Keywords: Diabetes Mellitus; T1DM; Plasmid; Chitosan; L cells

Introduction
Type-1 diabetes (T1DM) is a chronic autoimmune disease caused by damaging the beta-cells in the islets of Langerhans. This destruction results in insulin deficiency and hyperglycemia (1). Poor glycemic control in diabetes can cause severe secondary complications. Exogenous insulin cannot completely prevent complications of diabetes, thereby morbidity and mortality of diabetes are increased significantly (2). In 2007, 437,500 children were affected by type 1 diabetes worldwide. Approximately, 70,000 children less than 14 years old develop T1DM per year with an annual increasing rate of 3%. The primary clinical signs of T1DM are ketoacidosis and chronic hyperglycaemia. Diabetic ketoacidosis can lead to coma and death. In addition, chronic hyperglycaemia can cause macrovascular and microvascular complications such as retinopathy, peripheral neuropathy, cardiovascular disease, and renal disease (3). It is a plan to treat type-1 diabetes by gene therapy through in vivo or in vitro transfer of insulin gene into germ line or somatic cells (4). One of target cells in gene therapy is gasterointestinal L-cells, which produce Glucagon-like peptide 1 (GLP-1)
hormone (5). This hormone is an incretin hormone that is able to promote insulin secretion, inhibit the glucagon secretion in pancreas, and increase the synthesis of proinsulin. Furthermore, GLP-1 promotes the proliferation of $\beta$-cells, and inhibits $\beta$-cell apoptosis. Meanwhile, it enhances the hepatic glucose uptake following increased glycogen synthesis activity (3).

A specific part of proglucagon promoter can activate L cells to produce GLP-1 and insulin (6). In addition, the engineered L-cells can be used to synthesize, process and secrete mature insulin (7). Thus, the combination of GLP-1 promoter with insulin gene transferred by nanoparticles in the gastrointestinal cells can potentially regulate the blood glucose level and treat T1DM (5). The human insulin gene can be transferred by chitosan-DNA nanoparticles in the gastrointestinal tract (8,12). Chitosan is a biodegradable polysaccharide composed of D-glucosamine and N-acetyl-glucosamine. It is a safe, nontoxic and cationic carrier (9) that can be used as a carrier to deliver GLP-1 gene and transfer insulin gene into the gastrointestinal epithelial cells of L cells (10) in order to treat diabetes. However, the exact cause of T1DM is unknown. Apparently, a genetic predisposition, environmental factors and distinctive metabolic changes contribute to initiation, development and progression of diabetes (3). There are many attempts to cure diabetes. Thus, this study aimed to investigate the effects of gene therapy via transferring plasmid-insulin gene-GLP1 promoter into the gastrointestinal L cells on the regulation of glucose and insulin levels in the blood of diabetic rats.

Methods

**DNA extraction:** Bacteria (E.coli strain TOP-10F) in frozen glycerol stocks were grown on LB agar (Lysogeny broth) (1st Base, Malaysia) plates without antibiotics overnight. A single isolated colony from the plate was cultured into 5 ml of LB broth medium (1st Base, Malaysia) (without antibiotics) at 37°C with shaking overnight. After that, the harvested cells were centrifuged at 9000 rpm for 30s to collect bacterial pellet. The extraction procedure was continued by following the protocol of High-purity Plasmid DNA Mini-Preparation Kit (BioTeke Corporation; China). The extracted DNA concentration was measured by nanometer drop (Eppendorf AG, Hamburg, Germany).

**Preparation of Nanoparticle:** The preparation of DNA-chitosan nanoparticles was based on a protocol as previously described (11). Briefly, chitosan was dissolved in sodium acetate followed by mild stirring and heating at about 60°C overnight to form chitosan solution. Equal volumes of filtered 0.02% chitosan in 5mM sodium acetate buffer and 100 $\mu$g/mL plasmid-DNA in 50mM sodium sulfate were preheated to 55°C, then mixed together quickly and vortexed for 30s. This mixture was kept at room temperature for 30 min that was for stabilization. The chitosan powder with the medium molecular weight and 75-85% deacetylated (Sigma, USA) was used to prepare chitosan-DNA nanoparticles. Meanwhile, particle size and charge characterizations were carried out using a Zetasizer (Nano ZS-Malvern) at 25°C. This optimization was through various concentrations of chitosan and different pH levels of sodium acetate solution. In addition, the transmission electron microscopy (TEM) (Hitachi H-7100 TEM) was used to evaluate the distribution and morphology of nanoparticles. A DNase degradation test was applied to assess the efficiency of encapsulation and DNA stability against nuclease degradation.

**Procedure:** The effect of human insulin gene transferring was studied in diabetic rats using GLP1/Ins/pBud plasmid. Plasmid consisted of the human insulin gene and the specific promoter of L-cells. This recombinant plasmid delivered and transferred into animal model using chitosan nanoparticles as gene carrier. Rats were divided into three sub-groups, where each subgroup contained 12 rats. The first diabetic group received nanoparticles containing GLP1/Ins/pBud plasmid. The second group was diabetic control and only treated with chitosan. The last group was healthy rats that did not receive STZ.
injection or treatments. Nanoparticles were administered intraperitoneally and orally to diabetic rats. The treatment doses were 100, 300 and 600 µg/mL of DNA solution in a 300 µL volume for each rat. The repetition for oral and intraperitoneal treatment was 7 and 4 times respectively that was repeated every two days.

**Determination of insulin efficiency through blood glucose testing:** The effects of recombinant plasmid consisting human insulin gene on the blood glucose level in diabetic rats were studied. Following the nanoparticle treatment, the blood glucose concentration was measured every other day for 35 days in all groups. The blood glucose level was checked from the tips of the tails by needle puncture using a glucometer and test strips (Expeed VIVO, HuBDIC, Korea). The blood glucose levels in the treated groups were compared with the untreated diabetic rats and healthy rats.

**Detection of insulin protein by ELISA:** The human insulin protein secretion in blood was detected using the ultrasensitive human insulin ELIZA kit (ALPCO, USA). The serum was separated from blood and used for the ELIZA test. Blood was collected from rats’ heart, incubated at 37°C for 1 hr and allowed to clot. Then, blood samples were centrifuged at 3000 rpm for 10 min. The yellowish serum in the supernatant was transferred to a new tube and stored at -80°C.

**ELIZA Test for Human and Rat Insulin:** The amount of insulin present in the serum collected from blood samples was determined using the ultrasensitive human and rat insulin ELIZA kits (ALPCO, USA). The human ELIZA kit had 100% cross reactivity with mature human insulin, whereas no reaction was reported with other forms, such as pro-insulin, C-peptide. ELIZA test was carried on as below. An about 25 µl of each standard (reconstituted control) and sample were loaded into their respective wells. Then 100 µl of detection antibody was added to each well. The microplate was covered and incubated for 1 hr at room temperature on a shaker (700-900 rpm).

After that, the microplate was washed six times with working strength wash buffer. After the final wash step, any residual wash buffer and bubbles was removed from the wells by inverting and firmly tapping the microplate on absorbent paper towels. After that, 100 µl of TMB substrate was added to each well of microplate and then was covered and incubated for 30 min at room temperature on a shaker (700-900 rpm). The stop of reaction was achieved by adding 100 µl of stop solution to each well and then shaking gently.

Finally, the microplate was analysed using a Bio-Tek EL800 Microplate Reader (LABEQUIP, Canada) with 450 nm filter. In the rat insulin ELIZA kit, the 96-well microplate was coated with a monoclonal antibody that was specific for insulin. About 10 µl of standards, controls, and samples were added into the microplate wells and then mixed with 75 µl of Working Strength Conjugate. The microplate was sealed and incubated at room temperature on a microplate shaker at 700-900 rpm. After that, wells were washed 6 times with wash buffer and then well dried. About 100 µl of TMB substrate was added into each well and then the microplate was covered and incubated for 15 min at room temperature on a shaker (700-900 rpm). Once incubation finished, 100 µl of stop solution was added, and then the optical density (OD) was measured by a Bio-Tek EL800 Microplate Reader (LABEQUIP, Canada) at 450 nm. The calculation of insulin level for both human and rat insulin kits was based on manufacture recommends.

**Induction of T1DM in Rats:** Animal study was on albino rats according to the guidelines for the care and use of experimental animals and the ethics committee that was approved by the Animal Care and Use Committee (ACUC), Faculty of Medicine and Health Sciences, University Putra Malaysia (UPM). Type I diabetes mellitus was induced in albino rat model, 7-8 weeks old using streptozotocin (STZ; N-nitroso derivative of glucosamine) (Sigma, USA). Rats were starved overnight and then treated with a single intraperitoneal injection of 55mg/kg (mg per body weight) STZ. STZ was dissolved in cold.
Na-Citrate solution (0.01 M citrate buffer, pH 4.5) and injected within 5 min to avoid degradation of STZ. After that, rats were supplied with 10% sucrose water overnight to avoid sudden hypoglycemia post-injection. Animals had access to food and water. When the blood glucose level was increased greater than 27.75 mmol/L for 7 consecutive days (12), the injected rats were considered as T1DM cases (13) for subsequent experiments. Animals had access to food and water. Readings were usually taken in morning when rats were under fasting and no fasting conditions. Glucometer read "HI" if the blood glucose exceeded 33.3 mmol/L. Those readings were set on 33.3 mmol/L for averaging.

Results and Discussion

The results showed that the orally treatment of albino rats with GLP1/Ins/pBud plasmid was not significantly effective to decrease the blood glucose level (p>0.05). Giving orally different doses of recombinant plasmid including 100, 300 and 600 µg/mL to diabetic rats did not significantly decrease the fasting and no fasting blood glucose levels in diabetic rats (p>0.05). After the orally treatment of diabetic rats with 300 µg/mL of DNA solution, the release of human insulin and rat insulin was significant (p= 0.04) and non-significant (p> 0.05), respectively. Tables 1 and 2 show the mean values of blood levels of glucose and insulin after the treatment of diabetic rats with GLP1/Ins/pBud plasmid. The injection of 100, 300 and 600 µg/mL doses of DNA solution in few diabetic rats showed that a dose concentration of 300 µg/mL prominently increased the level of human insulin secretion compared to the concentrations of 100 and 600 µg/mL (Table 1). Such effects are more likely due to the multiple mechanisms involving in the regulation of insulin gene expression attributed to nutrients, hormones and pharmacological factors (14, 15).

The human insulin level after treating with the concentration of DNA solution at 600 µg/mL was less than 300 µg/mL that was probably due to hyperinsulinemic-insulin resistance following the impaired L cells and GLP-1 release in response to insulin and heterologous GLP-1 secretagogues (16). Contrary to the report of Bowman and co-workers (17), our study showed that the increased dose of DNA solution to 300 µg/mL elevated the efficiency of gene delivery regarding the enhancement of insulin level. It was probably due to the increased plasma copy numbers and transferred insulin gene to L cells. However, the results showed that T1DM was not reversal to normal state after this treatment. Our project showed a relative impact of transferred human insulin gene and GLP1 promoter into the gastrointestinal L cells carried by nanoparticle chitosan for releasing insulin in the diabetic cases. Such effect has been previously reported by Jean and colleagues (10) indicating such function of gastrointestinal epithelial cells and GLP1 on the increased level of insulin. Until now, exogenous insulin or gene transfer for insulin or glucokinase alone has failed to treat diabetes. Thus, further investigations are required to clarify the synergistic action of insulin and glucagon promoter to treat diabetes. Accordingly, this study aimed to investigate the effects of the transfer of the insulin gene and GLP1 promoter on the regulation of blood insulin and glucose levels in animal model for treating diabetes.

This study confirmed previous reports those introduced plasmid as a non-viral carrier of DNA in gene therapy conducted by chitosan (8, 10, 18, 19). Contrary to some earlier research (8, 19), our results showed non effectiveness of the transfer of the human insulin gene in rats to decrease blood glucose level. Such effect can be related to genetic polymorphism in rats (1), and the type of insulin in each species. Human insulin is similar to bovine and porcine insulins. Porcine and bovine insulins structurally differ from human insulin in one amino acid (alanine replaces threonine at B30) and three amino acids (A8 alanine, A10 valine and B30 alanine), respectively. Bovine insulin is more immunogenic than both porcine and human insulins (20).

In addition, rapid turnover of intestinal epithelial cells can decrease the efficiency of transfection of recombinant plasmid DNA in the
gastrointestinal L cells (21). These cells are in
the distal jejunum, ileum, colon, and rectum (22).
The maximum density of L-cells is in the ileum
in most species (23). While intestinal epithelial
cells renewal in mammals is 3-5 days (21),
average ileum turnover in rats is 1.4 days (24).

The results can also be associated with the
type of chitosan, which can affect gene delivery
and DNA chitosan nanoparticles. Such effect can
be related to the variety of chitosan in molecular
weight and degree of deacetylation. Since, lower
degree of deacetylation of chitosan can reduce
the efficiency of DNA binding and stability of
nanoparticle complex in a dose dependent manner (17); therefore, further optimization is
needed to find a synthetic degradable cationic
polymer for protecting plasmid effectively and
release. However, the results were against what
we expected. Such insufficiency may reflect slow
intracellular DNA release from chitosan
nanoparticles (17). The findings of our in vivo
study did not confirm the results of gene delivery
found in an in vitro research by Rasouli and co-
workers (5). It suggests that the results achieved
by in vivo studies may not always be similar to in
vitro results. This difference is a confirmation to
the presence of disparity amongst different
studies (17), which can be due to diversity in
 genetic polymorphism and immune responses
(1).

All research showed no therapeutic effects
of treatment with gene transfer on diabetes,
which can be attributed to low levels of insulin
and high variability in gene delivery (17).
However, the technique of gene delivery using
nanoparticles tries to produce detectable amount
of insulin in order to treat diabetes. Accordingly,
this study aimed to use plasmid as a non-viral
gene carrier to deliver human insulin gene and
GLP1 promoter to diabetic rats following in vitro
study by Rasouli and colleagues for treating
T1DM (5).

In the current study, survival rate in sample
groups was significantly more than diabetic
control groups (p<0.001). Mortality rate in diabetic
rats after treatment with recombinant plasmid
was less than those without treatment. The period
time of survival in diabetic rats was more than
60 days without having any objective problems
such as wound, which was more than expectation
in severe diabetes with a blood glucose level
more than 33.3 mmol/L. Such effect was possibly
related to the reduction of complications after
treatment. For example, positive impact of this
treatment on GLP-1 receptors may subsequently
prevent complications such as cardiovascular
diseases and neuronal degeneration (9). The
subsequent stimulation of PI-3K signaling
pathway (25) and MAP Kinase signaling pathway
(26) can aslo result in cell growth and lifespan
extension. However, some problems including
DNA-plasma degradation in GI tract, partial
degradation of plasmid DNA during the transport
of chitosan nanoparticles complex across cells,
incomplete DNA protection (27) and the poor
intracellular release of DNA from chitosan
nanoparticle complexes (28) can limit utility of
oral gene therapy as a method to treat diabetes
completely. Such limits can decrease the
expression level of insulin gene in the cells (17).
Furthermore, the expression of insulin gene
following gene therapy and using nanoparticles
is valid for a limited period and needs to be
repeated periodically after shedding epithelial
cells. Meanwhile, lack of typical prohormone
carrier for processing of proinsulin to insulin
hormone in gastrointestinal cells such as L cells
can lead to partially production of insulin, which
limits use of these cells as target cells in gene
therapy for diabetes (8). Our study was the first
research, which used plasmid with both insulin
gene and GLP1-promoter wrapped with chitosan
for treating T1DM in animal model. Further
investigations are needed on stem cells, gene
polymorphism, immune reactions and system
controls for the proliferation and regeneration of
gastrointestinal cells such as L cells and K cells
for finding a better approach to treat diabetes.
Meanwhile, the increase of therapeutic effects
of gene delivery by nanoparticles requires further
studies regarding gene transfer barriers, exact
cells for transfection, proper stage for releasing
DNA from chitosan and optimal optimization for polymer carrier.

**Limitations**: Despite challenges, the strategy of gene delivery into the gastrointestinal cells using the complex of plasmid containing insulin gene and nanoparticle regarding the treatment of diabetes needs further examination. The success of treatment is associated with different parameters such as the efficiency of gene transfer via oral administration. Our project have been faced some limitations, which could affect the interpretation of findings. Biological differences between human and rat was a limit that might cause different reactions to insulin and glucose levels. In addition, our study was limited by the modest levels of produced insulin in the intestinal cells and high variability in gene transfer through gene therapy by nanoparticles. A large numbers of L cells transfected with insulin gene are needed to produce enough insulin for the treatment of diabetes. Meanwhile, the long term action of insulin may have a limitation. However, the shedding of gastrointestinal epithelial cells such as L cells can decrease their efficiency as target cells in gene therapy. The release of transgenic DNA from chitosan-DNA nanoparticles in the bloodstream and transport to systemic tissue by crossing the gastrointestinal tract can affect the efficient transport of DNA to the gastrointestinal cells such as L cells as well.

**Conclusions**

Our results suggest that the transport of transfected insulin gene into L cells could secrete human insulin hormone and execute relative effects on diabetic rats in a dose dependent manner. It was concluded that the concentration of 300 µg/mL of DNA solution was the efficient dose compared to doses including 100 and 600 µg/mL to increase the level of human insulin protein in diabetic rats. The blood glucose level

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<tr>
<th>Table 1. Insulin levels after treatment with recombinant plasmid DNA (100, 300,600 µg/mL) in diabetic rats</th>
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<tr>
<td><strong>Human Insulin Level</strong></td>
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<td>Conc. (µg/mL)</td>
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<tr>
<td>Orally</td>
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<td>Injection</td>
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Conc.= Concentration dose

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<th>Table 2. FBS and NFBS Glucose levels after the treatment with recombinant plasmid DNA (100, 300,600 µg/mL) in diabetic rats</th>
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<tr>
<td><strong>FBS Glucose level (mmol/L) p value</strong></td>
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<tr>
<td>Conc. (µg/mL)</td>
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<td>Orally</td>
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<td>Injection</td>
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*p less the 0.05 is significant using one way-ANOVA analysis test
FBS=Fasting blood glucose; NFBS=No fasting blood glucose
Conc.= Concentration dose

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was not significantly decreased after the treatment with all concentrations. The findings indicated that the transport of GLP1/Ins/pBud plasmid into L cells wrapped with chitosan stimulated the release of insulin hormone into the bloodstream in a dose dependent manner. This study supports the view that the transport of transfected insulin gene into L cells can be a potential approach to regulate insulin level, make free patients from repeated insulin injections and reduce complications. However, further investigations are needed to prove the efficiency of gene therapy and genetic engineering using L cells in the treatment of diabetes mellitus.

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Conflict of interest: Authors declare that there is no conflict of interests

References


