EFFECTS OF INSULIN AND EMBRYONIC STEM CELLS LOADED PLGA NANOPARTICLES ON PANCREATIC BETA TC CELLS

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Abstract: The present study, aims to investigate and compare the effect of insulin and embryonic stem cells (ESC) loaded nanoparticle formulations (NPs) on pancreatic-beta-TC-cell regeneration. Characterization studies of NPs were performed. Permeability of insulin and the effect of ESC on pancreatic beta cells were investigated. Insulin or glucose levels were determined and histologic investigations were also performed. ESC encapsulation efficiency was calculated by western blot analysis. The particle sizes of insulin and ESC-loaded-NPs were determined as $0.665 \pm 0.202 \mu\text{m}$ and $0.650 \pm 0.310 \mu\text{m}$. The mean zeta potentials of insulin and ESC-loaded-NPs nanoparticles were found as $6.88 \pm 0.729 \text{ mV}$, $5.13 \pm 0.631 \text{ mV}$. The polydispersity index of insulin and ESC nanoparticles were 0.660 ± 0.175 , 0.620 ± 0.205 respectively. Encapsulation efficiency of insulin and ESC-loaded-NPs were found to be $50 \pm 1.53\%$ and 51% . Insulin release from nanoparticles was found to be 72.8% over 48h. Glucose concentrations were decreased to 201 and 202.7 mg/dL from 250 mg/dL in streptozocin (STZ) induced diabetic mice group after insulin and ESC-loaded-NPs administration. Insulin and ESC-loaded-NPs improved the blood insulin levels in all experimental groups. These NPs may be used for repairing of pancreatic cells. Healing or some degree of regeneration was observed when insulin and ESC-loaded-NPs were administered to mice. ESC-loaded-NPs can be a potential source for cell replacement therapy in the treatment of diabetes.

Keywords: embryonic stem cell, insulin, PLGA nanoparticles, diabetes, pancreatic beta TC cell

Diabetes is one of the most common diseases that affect people all over the world. When insulin cannot be secreted from the pancreas or if the body cannot effectively use insulin, diabetes occurs. In diabetes, abnormal metabolisms of carbohydrates, fats, and proteins are involved (1-3). Diabetes mellitus is divided into two groups as insulin dependent type I and insulin independent type II. In both types, any defect of beta cell in Langerhans islets causes insulin insufficiency or absence (2, 4). Insulin is a major anabolic hormone, which is the most effective molecule for the control of blood glucose levels in diabetes (1, 2). Glucose uptake by liver, muscle, and fat tissues can be achieved by the action of insulin

(2). Insulin is the standard treatment for Type 1 and Type 2 diabetic patients. Insulin and other protein or peptide drugs are administered by intramuscular or subcutaneous injections, which unfortunately cause a great discomfort for patients (5-7). Insulin injections are the only available option for diabetic patients to control their blood glucose level. Parenteral administration of insulin has many disadvantages such as common infections, pain caused by subcutaneous injections, stress due to long-term use, and low patient compliance (1, 6, 8).

The number of diabetic stem cell related studies are increasing in popularity (4). ESCs have been proposed to be a powerful tool to overcome pancre-

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atic disease, as well as a potential source for cell replacement therapy in the treatment alternatives of diabetes. ESCs can generate many different cell types and they are potential sources of pancreatic beta cells because of their self-renewing properties (9). ESCs are multipotent cells derived from the inner cell mass (ICM) of 3-5 days old embryos (10-12).

Many previous studies indicate that polymeric nanocarriers have some advantages for peptide and protein delivery. Polymeric carriers, nanoparticles

are solid and spherical structures (13) that have been used in diagnostic kits, therapeutics, and biomedical tools; they can protect encapsulated drugs from severe external conditions and provide a sustained or controlled drug release (1, 14, 15). These NPs can be prepared with proteins (albumin, gelatin, etc.). NPs can degrade in biological mediums, representing a significant improvement over traditional delivery systems in terms of efficiency and effectiveness (16, 17). Preparations of these systems were reported to be simple and rather easy to scale-up, they

Figure 1. (A) Polarize microscope image of nanoparticles (x100). (B) TEM of insulin nanoparticles, (C) ESC nanoparticles (x2000), (D) Immunoblots of cell control, ESC NP and positive control for encapsulation efficiency

Table 1. Design of experimental animal groups (n = 3).

Group numbers	Group names	Treatment
Group 1	Control	No formulation
Group 2	Insulin solution (10 IU)	Intraperitoneal
Group 3	Insulin nanoparticles (10 IU)	Intraperitoneal
Group 4	ESC (suspond stem cell) (1.2×10^6 /mL cell)	Intraperitoneal
Group 5	ESC nanoparticles (1.2×10^6 /mL cell)	Intraperitoneal
Group 6	ESC (suspond stem cell) (1.2×10^6 /mL cell)	Intrapancreatic
Group 7	ESC nanoparticles (1.2×10^6 /mL cell)	Intrapancreatic

have improved biodistribution, and their therapeutic efficacy allows sustained drug release at the targeted site over a period of days or even weeks after the administration (18).

Poly lactic-co-glycolic acid (PLGA), is one of the most successfully developed biodegradable polymers that has been extensively used as a drug delivery system for protein and peptides (6,13,15). PLGA is approved by the US Food and Drug Administration (FDA) and European Medicine Agency (EMA) for various drug delivery systems. PLGA based nanoparticles can protect drugs from degradation and enhance their stabilities (13, 15).

Western blot analysis is one of the most widely used methods to analyze specific proteins. To determine protein loading, the most commonly used control material is β -actin. β -actin is a 43-kDa protein that is found nearly in all eukaryotic cells at various concentrations. One advantage of using β -actin is that it is very common material and it can be used for many different species (18, 19).

The encapsulation efficiencies were determined by western blot analysis. This study compares the effect of insulin and ESC-loaded NPs formulations on pancreatic beta TC cell regeneration. Bovine Serum Albumin (BSA) was used as a reference protein for ESC using the Lowry method. BSA has been widely used in drug delivery systems because of easiness of purification, low cost, and representing no unusual ligand-binding properties (12).

In this study, insulin and ESC-loaded NPs were prepared and their effects on pancreatic beta TC cells were determined. Characterization studies of NPs were performed. The effect of insulin and ESC loaded NPs were investigated by examining blood insulin and glucose levels. Lower blood glucose levels were obtained after ip administration of insulin or ESC loaded NPs. Some degree of healing or pancreatic cell regeneration was also observed with these formulations.

MATERIALS AND METHODS

Materials

Insulin was purchased from Lilly France (Humulin® R 100 IU/mL), Bovine Serum Albumin

(BSA), Poly lactic-co-glycolic acid (PLGA), streptozocin (STZ) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) were obtained from Sigma, USA. Polyvinyl alcohol (PVA) was provided by Wecker- Germany. Dulbecco's modified Eagle's medium (DMEM) was purchased from Biochrom, Germany. Insulin ELISA kit was from RayBio, Germany. Glucose Liquicolor kit was purchased from Stanbio Lab, USA. Pancreatic Beta TC (β TC) and embryonic stem cells were obtained from American Type Culture Collection (ATCC), Manassas, USA.

Methods

In vitro studies

NPs preparation

NPs were prepared using emulsification and solvent evaporation technique (13, 15). PLGA was used as a polymer. PLGA (400 mg) was dissolved in dichloromethane:methanol (9 : 1) at room temperature then injected slowly (0.5 mL/min) into aqueous phase containing insulin and 0.4% PVA. The mixture was stirred at 13500 rpm by Ultraturrax® for 10 min and 100 mL of distilled water added after evaporation, NPs suspensions were centrifuged at 10000 rpm for 30 min at 4°C and insulin-loaded NPs were obtained.

ESC 1.2×10^6 /mL cell and suspension of blank NPs incubated for 1 h for cell labeling. The medium was then changed and cells were incubated for further 48 h (20). NPs suspensions were centrifuged at 10000 rpm for 30 min at 4°C and ESC loaded NPs were obtained.

Characterization of NPs

Particle size, polydispersity index (PDI) and zeta potentials of NPs were measured by Malvern Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Physical appearances of the NPs were determined using a microscope (Leica DMEP polarized microscope). Amount of the drug in the supernatant was determined and then encapsulation efficiency (EE) of insulin was calculated (21-23). The protein content was determined by Lowry method, and used for the quantification of insulin. Spectrophotometric determination was carried out at 754 nm.

Table 2. AUC values of insulin-loaded formulations and insulin solution after intraperitoneally administration.

Formulations	Solution	Nanoparticle
AUC (μ g/mL)	30127 ^a	21026.25 ^b
AUC (%)	120630 ^a	84105 ^b

a-b = p < 0.001

The method was found to be linear ($r^2 = 0.999$) and reproducible.

$$EE(\%) = \frac{\text{Theoretical amount of insulin-free insulin in supernatant}}{\text{Theoretical amount of insulin}} \times 100$$

Insulin release from NPs

Release studies were performed using Franz diffusion cells with attached 12000 Dalton pore size dialysis membranes. 2.0 mL insulin NPs suspensions were placed into the donor compartment of the diffusion cells. The receiver compartments were filled with 2.0 mL of phosphate buffer (pH 7.4). Insulin release studies were performed for 48 h at 37°C. 2.0 mL of samples were withdrawn at predetermined time points and fresh buffer was immediately replenished with the same volume. The samples were then analyzed by Lowry method as described above.

Cytotoxicity assay

Cytotoxicity of all formulations was examined. Cell viability on pancreatic beta TC cells – after insulin loading, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide) tests were carried out. The effects of formulation components on cell viabilities were also investigated for 48-h. The color densities were measured at 570 nm using multi-well ELISA reader.

Transport experiments from cell monolayers

Beta TC cells were seeded with the concentration of 2.5×10^5 cells/cm² on polycarbonate membranes with a pore size of 0.4 mm. The beta TC monolayers containing membranes were placed between the donor and receptor compartments of vertical diffusion cells. 95% O₂ and 5% CO₂ were delivered to the system at 37°C to maintain cell viability. Transport experiments were performed from the apical to the basolateral compartment. Samples were collected from the basolateral compartments at 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 720, 1440, 2160 and 2880 minutes. Monolayer integrities were assessed by transepithelial electrical resistance (TEER) measurements across the cell monolayers. TEER was measured before and after the experiments. Insulin contents of the samples were then analyzed by the Lowry method and Papp values were calculated using following equation (24-26):

$$P_{app} = \frac{dQ}{dt} \frac{1}{AC_0}$$

dQ/dt refers to the permeability rate, A (cm²) is the area of the membrane (diffusional area), and C_0 (mg/mL) refers to the initial concentration of doxycycline in donor compartment.

Incubation of living cells with NPs

Beta TC cells (2.5×10^5 cells/mL) were seeded to the wells and three different groups were studied. No treatment was applied to the first group. Initial amount of insulin and glucose levels were determined. Glucose and insulin concentrations in the samples were measured using Glucose Liquicolor and Mouse Insulin ELISA kit.

In the second group, beta TC cells incubated with Krebs-Ringer bicarbonate buffer (KRB) (119 mM NaCl, 4.7 mM KCl, 2.54 mM CaCl₂, 1.19 mM MgSO₄, 1.19 mM KH₂PO₄, 25 mM NaHCO₃) for 1 h. The KRB was removed and all cells were incubated with KRB containing glucose (25 mM) for 2 h (27). Prepared cells were incubated with insulin and ESC-loaded NPs for 48 h. After the incubation, treated cells were centrifuged at 800 rpm, for 5 min and insulin and glucose levels were measured from the supernatants.

In the third group, beta TC cells incubated with STZ. Glucose level equal or over than 250 mg/dL were considered diabetic in the study (28). Insulin and ESC-loaded NPs incubated with this group for 48 h. After the incubation, treated cells were centrifuged at 800 rpm, for 5 min and insulin and glucose levels were measured from supernatants as described above.

After the experiments, immunoblot analyses were performed. Protein levels were measured using a Bio-Rad assay kit. Membranes were probed with antibodies against to β -actin. The immunoreactive proteins were detected by chemiluminescence (West Pico chemiluminescent substrate/Thermo-Pierce). Nitrocellulose membranes were stripped out and re-probed with antibodies against the β -actin. Protein bands were digitally imaged for densitometric quantitations.

In vivo studies

Balb-C type, 12–13 weeks old male mice were used. Animal studies were conducted under the protocol approved by the Animal Care and Use Ethical Committee of Gazi University (G.U.N.ET-12.075).

To induce experimental diabetes, STZ solutions in sodium citrate buffer (pH: 4.5) were injected ip (150 mg/kg) (29) to the animals. Blood glucose levels were measured by glucose kit 2 days after STZ injections. Glucose levels > 250 mg/dL were accepted to be diabetic (30).

The animals were divided into 7 groups, 3 animals were used in each group. Formulations were administrated to the animals ip, ESC suspensions and ESC loaded NPs were administrated to the animals both intrapancreatic and ip. The design of the animal groups was shown in Table 1.

Blood samples were collected at predetermined time points (0.25, 0.5, 1, 2, 4, 6, 8, 24 h). The plasma glucose and insulin concentrations were measured using a glucose kit and mouse insulin ELISA kit respectively and the areas under curves (AUC) were calculated.

Histopathological studies

After the treatment, pancreases of mice were taken out and kept in formaldehyde solutions (10%). Tissue sections (110 μ m) were stained with Hematoxylin & Eosin dye and evaluated under a light microscope (Olympus BX51). The severity and prevalence of degenerations of the pancreas, surface areas were classified semiquantitatively (0: normal; 1: minimal; 2: slight; 3: middle; 4: distinctive; 5: severe). Also, semidiameters and covered areas of Langerhans islets were measured.

Statistical analysis

All data in this study were considered as means $\pm$ SD, and one-way ANOVA tests were used for statistical analyses. GraphPad InStat computer program was used.

RESULTS AND DISCUSSION

In this present study, it was observed that insulin and ESC-loaded NPs are effective on pancreatic beta cells and insulin and ESC-loaded formulations found to induce pancreatic beta TC regeneration.

Characterization Studies

The physical appearance of nanoparticles was determined using a polarized light microscope and TEM (Fig. 1). Mean particle sizes of insulin and

Figure 2. Insulin with different concentration on β TC cell viability (A) Free nanoparticle effects on β TC cell viability (B)

Figure 3. (A) In vitro release profiles of insulin from nanoparticles at pH 7.4 phosphate buffer from dialysis membrane, (B) Cumulative amount of insulin from nanoparticles transported through β TC cell monolayers (error bars represent standard deviations, $n = 3$)

ESC NPs were determined to be $0.665 \pm 0.202 \mu\text{m}$ and $0.650 \pm 0.310 \mu\text{m}$. The mean zeta potentials of insulin and ESC NPs were $6.88 \pm 0.729 \text{ mV}$, $5.13 \pm 0.631 \text{ mV}$. The polydispersity indexes of insulin and ESC NPs were found to be 0.660 ± 0.175 , 0.620 ± 0.205 respectively. Encapsulation efficiency of insulin NPs was found to be $50 \pm 1.53\%$. For ESC NPs encapsulation efficiencies were measured with three different methods (Lowry method 51%, cell counting 50.4% and western blot analysis 51.2%). Encapsulation efficiencies were also obtained from the literature. Insulin-loaded chitosan nanoparticles were prepared and encapsulation efficiency of these nanoparticles was reported to be 52.76% (6). Another study was carried out to evaluate the encapsulation efficiency of BSA-loaded PLGA microspheres. Total protein assay was performed using the Lowry method and the total protein loading and surface-associated proteins were found to be 8.61% and 16.6%, respectively (31). Our encapsulation efficiency of insulin NPs appeared to be comparable and good enough.

Western blot analysis of the densitometric quantitation of the β -actin signals is shown in Figure

1D. Control was accepted as 100%. Western blot percentages found by cell counting and Lowry; both determinations were found to be compatible with each other.

Citotoxicity

The effect of insulin-loaded and unloaded NP formulations on beta TC cell viabilities were investigated for 48 h using the MTT method. The effect of Dulbecco's modified Eagle medium (DMEM) was also tested. The viability of cells as percentages is given in Figure 2A and B. According to MTT test results, the insulin concentration was selected with the value of 10 IU.

MTT tests were carried out to determine the toxic levels of insulin and NPs containing PLGA and PVA. Insulin was not found to cause any cellular toxicity with used dose (10 IU). Our dose was 10 IU/30g (67 IU/kg) for IP administration. The dose in the literature was found to be varied for IP administrations for rats (33 – 80 IU/kg) (32), 667 IU/kg dose was used for intraduodenal administration for rats in the literature (33) but, the ip dose used in this study was found to be applicable.

Figure 4. (A) Control groups, Glucose % after incubation with KRB buffer, glucose and streptozocin solution using pancreatic β TC cells, (B) Glucose levels after incubation with insulin and ESC loaded NPs using glucose-induced pancreatic β TC cells and (C) Glucose levels after incubation with insulin and ESC loaded NPs using STZ-inhibited pancreatic β TC cells (error bars represent standard deviations, $n = 3$)

Figure 5. (A) insulin % after incubation with glucose and streptozocin solution against to control group using pancreatic β TC cells, (B) insulin levels % after incubation with insulin and ESC loaded NPs using glucose-induced pancreatic β TC cells and (C) STZ-inhibited pancreatic β TC cells (error bars represent standard deviations, $n = 3$)

Additionally, empty nanoparticles were also not found to be toxic to cells at any concentrations. In other studies in the literature, MTT assay was performed using Caco-2 cell with blank PLGA-NP. It was reported that cell viability did not alter after 3 h of the incubation with blank PLGA-NP at 1-25.5 mg/mL concentrations (6).

In vitro releases and transport studies of insulin from NPs

In vitro insulin release experiment from NPs formulation was performed using Franz-type diffusion cells. Insulin release from NPs was found to be 72.8%. Release profiles of insulin from NPs are given in Figure 3A.

Transport experiments of insulin from NPs from the apical side to the basolateral compartments were evaluated. Results are given in Figure 3B. Cumulative amounts of insulin at the end of the 48-hour time period were calculated. P_{app} values were

calculated for NP formulation as 1.06 cm/h. The values of TEER were measured before and after the experiment for NPs. TEER values were found to be 79 and 72 ohms for the control group and insulin NPs, respectively.

In vitro insulin release experiment from NP formulation was performed through dialysis membrane using Franz-type diffusion cells at pH 7.4 and 37°C. Insulin release from NPs was found to be 72.8%. In the literature, insulin solid lipid nanoparticles formulation (Witepsol E85/PVA at 1%) was prepared and *in vitro* release studies were performed at pH 7.4. Insulin was reported to be released from the formulation 39% after 48 h (34).

Incubation of cells with NPs

Glucose level determinations of cells after incubated with NPs following exposure of KRB buffer having glucose and STZ are given in Figure 4A. Insulin and ESC loaded NPs were administered

to beta TC cells and glucose level analyses were performed. Induction or inhibition of glucose levels against to control groups were given (glucose-induced groups (Fig. 4B), STZ-inhibited groups (Fig. 4C)).

Concentrations of glucose and insulin in diabetic cells were determined using assay kits after 48 h. Glucose concentrations were found significantly increased from 193.5 mg/dL to 247.1 mg/dL and 257.7 mg/dL ($p < 0.0002$) for glucose-induced and STZ-inhibited cells respectively. Glucose concentrations were found significantly reduced back after incubated with insulin and ESC loaded NPs for both glucose-induced and STZ inhibited cells (glucose-induced cells and insulin-loaded NPs applied group: 199.9 mg/dL; glucose-induced cells, and ESC loaded NPs applied group: 201 mg/dL, STZ-inhibited cells, and insulin-loaded NPs applied group: 200 mg/dL, STZ-inhibited cells, and ESC loaded NPs applied group: 202.7 mg/dL ($p < 0.001$)).

After the treatment, decreased glucose levels were found to be comparable with the control group. Glucose levels were found to be 103.31% and 103.88% for insulin and ESC loaded NPs for 48 h, respectively (control group:193.5 mg/dL (100%)) in

the glucose-induced group. Similarly, in STZ-inhibited group, glucose levels were also decreased and they were found to be 103.36% and 104.75% for insulin and ESC loaded NPs respectively.

Insulin levels, after exposure to KRB buffer having glucose or STZ, are given in Figure 5A, insulin and ESC-loaded NPs on beta TC cells were performed after induction or inhibition of glucose (Fig 5B) of STZ groups against control group are given Fig 5C. In glucose and STZ-induced cells insulin concentration was measured as 59.8% and 52.1%. After the treatment with insulin and ESC loaded NPs for 48 h, these levels increased to 81.4% and 79.3% in a glucose-induced group. In STZ-induced group, these levels increased to 80.9% and 80.3% respectively.

In vivo studies

Insulin solution and NPs were applied to animals ip or through intrapancreatic route (Fig. 6). The plasma glucose % (Fig. 7a) and insulin concentrations (Fig. 7b) were measured using a glucose assay kit and mouse insulin ELISA kit respectively. After IP administration, the areas under curves (AUC) were calculated and given in Table 2.

Figure 6. (A) Mouse pancreas, (B) intrapancreatic administration

Table 3. The total semidiameter, footprint and degenerations of a cross-sections taken in Langerhans islets (48 h).

Groups	The total semidiameter of a cross section taken in the islets of Langerhans (µm)	The footprint of a cross section taken in the islets of Langerhans (mm²)	Degeneration of Langerhans islets*
Control	430	0.54	0
Solution	289	0.36	0.10
Insulin nanoparticle	238	0.18	0

*0: normal; 1: minimal; 2: slight; 3: middle; 4: distinctive; 5: severe

Figure 7. (A) insulin concentration % (B) glucose concentration % and time profile for insulin solution and nanoparticle (error bars represent standard deviations, n = 3)

Figure 8. Pathological examination results of pancreatic sections of control group (A), ip administration of insulin-loaded NP (B), intra-pancreatic administration from ESC-NP (C), ip administration of NP (D) (Light microscope × 20)

Table 4. Glucose concentrations after ESC NP administrations to diabetic mice (after 48 and 120 h).

Groups	Glucose concentration (mg/dL) (ip) (48 h)	Glucose concentration (mg/dL) (intrapancreas) (48 h)	Glucose concentration (mg/dL) (ip) (120 h)
Control	104.5 ± 0.325*	105.3 ± 1.25 ^a	104.5 ± 0.320 ^c
ESC Nanoparticle	106.4 ± 0.437*	109.8 ± 1.30 ^b	103.2 ± 1.47 ^d
ESC (suspended)	105.1 ± 0.528*	----	110.9 ± 3.01 ^e

Values are expressed as mean ± standard deviation (n = 6), * Not significant from control group p > 0.05, a-b = p < 0.002, b-c = p > 0.005, d-e = p < 0.05

Histopathological studies

After IP administration of insulin solutions and insulin-loaded NPs to diabetic mice, the severity and prevalence of degenerations of pancreas cells, surface areas, semidiameters, and covered areas of Langerhans islets are given in Table 3.

In the islet of Langerhans cells, tissues were found quite limited, vacuolar degenerations and

degenerative changes were observed. A significant difference was not observed between the groups.

After both ip and intrapancreatic administration of ESC NPs and suspended cells to diabetic mice, blood samples were taken after 48 h and glucose concentration measurements are given in (Table 4).

Figure 9. *In vitro-in vivo* correlation results between cumulative amount of insulin (%) passed through pancreatic beta TC cells and insulin concentration in blood (µg/mL) for insulin solution (A) and nanoparticle (B)

Table 5. The total semidiameter, footprint of a cross-section taken in Langerhans islets and degeneration after intraperitoneal ESC formulation administration. (48 h and 120 h).

Groups	Control	ESC (suspended)	ESC Nanoparticle	Control	ESC (suspended)	ESC Nanoparticle
	48h			120h		
The total semidiameter of a cross-section taken in the islets of Langerhans (µm)	654	214	335	756	692	1092
The footprint of a cross-section taken in the islets of Langerhans (mm²)	1.34	0.438	0.686	0.450	0.530	2.24
Degeneration of Langerhans islets*	0	0	1.0	0	0	0.1

*0: normal; 1: minimal; 2: slight; 3: middle; 4: distinctive; 5: severe

Table 6. The Number of a cross-section taken in the islets of Langerhans and degeneration after ESCs and nanoparticle given intrapancreatic. (48 h).

Groups	Control	ESC (suspended)	ESC Nanoparticle
Number of a cross section taken in the islets of Langerhans	5.6	5.0	2.7
Degeneration of Langerhans islets*	0	1.5	1.6

*0: normal; 1: minimal; 2: slight; 3: middle; 4: distinctive; 5: severe

After administration of ESC loaded NPs to diabetic mice, the severity and prevalence of degeneration of pancreas surface areas and semidiameters and covered area of Langerhans islets after formulations are given in Table 5 and 6.

The intrapancreatic administration led to severe and widespread degenerations and immune response and infected cells were observed in all formulations especially with NPs. Due to the route of administration, the trauma, inflammatory necrotic reactions, and degenerations have been observed (Fig. 8C).

In vitro-in vivo correlation

In vitro-in vivo correlation studies were also performed considering the cumulative amount of insulin (%) passed through apical to the basolateral side of the pancreatic beta TC cell monolayers and blood insulin concentrations values from animal studies. The correlations were shown in Figure 9A and 9B.

Considering both positive charges and their physical appearances of NPs under TEM, it is the first study in the literature which shows that NPs can actively bind to the surface of ESCs and it is even more interesting that after the ip administration these NPs can move together with ESCs finalizing the trip at the damaged area in the pancreas which is proved by TEM. NPs found to be present on the cell surface and this may provide a sustained action and more opportunity for internalization or penetration (Fig. 1). On the other hand, the possible relationship between glucose and insulin concentrations in diabetic cells induced with glucose and STZ was also investigated. The reduction of insulin concentration was synchronous with the increased glucose level. In the beginning, the level of insulin of the control group was found to be 50 µg/mL (100%) using mouse insulin ELISA kit. Glucose and STZ-induced cells insulin concentrations were also measured and this difference was found to be significant ($p < 0.0001$). After the treatment with insulin and ESC loaded NPs for 48 h, these levels of insulin increased to 180 % in both glucose and STZ-induced groups (Fig. 5).

In the literature, alginate-poly(L)lysine-alginate (APA)-βTC6 and APA-MIN6 cells were encapsulated and evaluated considering their insulin secretions and glucose responses of pancreatic beta cell lines. It was reported that glucose concentrations (400 mg/dL) of hyperglycemic cells decreased to normal levels (100 mg/dL) by the secretion of a high concentration of insulin after the treatment with APA-MIN6 cells (27).

Western blot analyses were performed to determine ESC encapsulation efficiency using β-actin. It is becoming a standard practice to measure a house-keeping gene, typically actin, in Western blots (35). Density of the bands measured against to control group and ESC cell activity as a percentage of the installation has been determined. Some studies reported that beta-actin determined by Western blot analysis at various concentrations of beta-actin bands show the protein density. It has been found that increased protein concentrations gave increased density of the bands (35, 36).

Diabetes is a serious and prevalent metabolic disease in the world. Scientists are interested in several new approaches such as the using of stem cells to treat diabetes efficiently (37). Ngoc et al used derived mesenchymal stem cells (MSCs) from mouse bone marrow and differentiated them into insulin-producing cells. These cells were encapsulated in an alginate membrane and grafted into diabetic mouse with streptozocin (50 mg/kg) by ip injections. The treated mice were found to achieve normal blood glucose levels (38).

ESC has two very important features. One of them is renewing itself to proliferate without differentiation, and the other is the potential to create specialized cell types when induced for differentiation. Therefore it was thought that when ESC reached the damaged areas in pancreas in diabetic mice with the help of NPs, the cells possibly transformed into the pancreatic beta cells to overcome the tissue damage there.

ESC NPs were found to be able to repair the damage in the pancreatic cells; even at the 5th day, they exhibited the same glucose levels with the control group. The reason was attributed to less degeneration potential of ESC loaded NPs than ip administrations (Table 6). Intrapancreatic injections caused a strong immune response in pancreatic acinar cells, consequently, it was interpreted that the main problem was widespread degeneration.

The blood glucose levels of ECS loaded NPs applied group were found to be similar to the control group after 5 days because pancreatic beta TC cells were seem to renewed by stem cells.

Figure 8 shows the pancreatic tissue cells under microscope. Healing or regenerations were observed less than the control group for all formulations and the degeneration of the cells in the pancreatic tissues was found to be higher than the control group and ip administration group. Intrapancreatic administration and the immunologic response to stem cells can cause a degeneration of acinar pancreatic cells. The main reason for obtaining undesirable necrotic degeneration was found to be due to

direct invasive injections to the pancreas. It was thought that although formulations have been applied in nonirritant sterile saline solutions, it might cause some traumatic necroses.

Insulin administration and sustained release can extend the dosing interval times but the treatment with stem cells can actively repair the damaged pancreatic cells and this effect can be long-lasting.

This cell therapy with ESC loaded NPs is considered to be an attractive approach and it can be an alternative to conventional insulin therapy for the treatment of diabetes.

CONCLUSION

It was concluded that insulin and ESC loaded NPs improved insulin levels when glucose and STZ induced diabetic animals were used. These ESC loaded NPs may be used to repair pancreatic cell degenerations and this ESC treatment will be a potential source for cell replacement therapy for the treatment of diabetes.

Conflict of interest

The authors state that there are no conflicts of interest pertaining to this manuscript.

Acknowledgments

This work was carried out at the Faculty of Pharmacy of Gazi University as a PhD thesis and supported by the Scientific and Technological Research Council of Turkey (TÜBİTAK, No. 112S405).

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Received: 29. 12. 2017