Study Protocol

Effect of encapsulated human islet cells implantation on insulin dependency in diabetes mellitus: An innovative approach

Abstract:

Background: Globally, the number of diabetic patients has quadrupled in last 30 years and diabetes mellitus (DM), is among the major causes of death with rank 9 worldwide. Approximately,. there is 1 patient with~~of~~ DM in 11 people worldwide. Asia is one of the major areas of rapidly emerging T2DM global epidemic, with China and India as top two epicenters. Studies have shown that without immunosuppression in diabetic animal model, normoglycemia is maintained after implantation of encapsulation technique (macro/micro-encapsulation) containing islets at different sites in body. If this study could work well, it will be possible to implant a capsule in diabetic patient reducing dependency on insulin from outside using other methods like multi-layer-by-layer encapsulation. Purpose of this study will be to determine whether encapsulation of pancreatic β-cell in subcutaneous region could protect human islets from rejection and autoimmune destruction in diabetic patients, by overall decreasing insulin dependency in T2DM patients without immunosuppression.

Methods and analysis: Protocol will be based on multi-layer-by-layer encapsulation procedure.

Discussion: To our knowledge, there are no such studies which are performed in phases making this study to be a best example of evidence based protocol. Appropriate subgroup analysis has been planned while performing animal experimentation. Routine postoperative monitoring have been planned to ensure Ethics and dissemination. Analysis of encapsulation~~e~~ in different conditions i.e. in-vitro and in-vivo will provide diverse data on effects of encapsulated islet cells on different environmental conditions. In-vitro characteristics will be correlated with the in-vivo data to find out specific successful outcomes. According to this protocol, if we will be able to develop encapsulated human islet cells that will produce insulin and decrease insulin dependency then this study will be a novel interventional research to decrease burden of insulin therapy in low income population such as of India.

Keywords: type 2 diabetes mellitus, multi-layer-by-layer encapsulation, insulin dependency

Introduction: β-cells of pancreas are essential for normal blood glucose homeostasis via release of insulin in response to elevated glucose levels. 8.5% of global population is affected by type 1 diabetes, and type 2 diabetes mellitus (T2DM). β-cells failure is one of the major factors for the development of diabetes, which unable enough insulin to be released in order to decrease blood sugar levels. A high blood sugar itself accelerates failure of β-cell in a malicious way (1).

Islet cell transplantation appears to be an attractive option over exogenous insulin therapy in treatment of diabetes (type 1 and secondary diabetes) because transplanted islets respond to physiologic needs of body (1,2). However, inherent problems such as intense immunosuppression, lack of renewable sources of β-cell, availability of biocompatible device, and easily accessible sites that facilitate minimally invasive clinical islet transplantation are to be resolved in this regard.

It is a potential option for type 1 diabetes and significant advancements have been achieved, particularly in past few years (3,4). However, clinical success of this approach in humans is still hampered in type 2 diabetes. Lifelong need of immunosuppressive drugs to prevent islet cell destruction by immune system of recipient are some reasons for lack of research in this field (5,6). Unfortunately, immunotherapies required to avoid immune rejection of transplanted islets can be toxic, impair islet function and survival, and increase susceptibility of recipients to serious infections and tumors (7). A promising strategy to overcome chronic immunosuppression is "immune isolation", i.e. encapsulation of islets with biocompatible materials which are able to shield cells from immune attacks, while allowing nutrition supply to and insulin release from beta cells (8,9).

Encapsulation protects islets from host immune responses and thus has potential to eliminate need for immunosuppression (10-12). In addition, this approach might permit use of allogenic and xenogenic islets that might obviate lack of renewable sources of beta cells (10). Despite encouraging results obtained in animal models, loss of graft function in microcapsules has limited their use in islet transplantation (13).

More recently, nano-medical technologies have been employed in this field, including conformal coating of isolated islets based on layer-by-layer nano-encapsulation (14). Major advantages of this approach are minimization of capsule size and graft volume (with possible reduction of islet core hypoxia) and better preservation of dynamics of insulin secretion (14).

Concept of self-assembly of nano-particles around cells was first introduced in late 60's,(15) and some years later this approach was used to model multi-layer nano-films to be applied in biological systems (16). Since then, layer-by-layer nano-encapsulation methodologies have been implemented in various biomedical fields, including islet transplantation (17).

Diabetic burden in World

Global epidemic of diabetes has been rapidly spreading to every Asian country. Presently, population with diabetes in Asia accounts for more than 230 million individuals. This includes approximately 55% of the world's diabetic population, and by 2020 it is expected to surpass 355 million. In the world, India and China are two Asian countries, home to have diabetes patients with largest number (69.2 million and 110 million, respectively). Three other Asian countries, Japan, Indonesia, and Bangladesh, also have highest number of diabetic patients worldwide. Rapid adaptation to westernized lifestyle as well as increasing obesity prevalence in many Asian countries impose burden on health system in population (18). Diabetes prevalence among adults in Bangladesh was 11.0% (19) and Sri Lanka it was 10.3% (20) having similarity to Indian diabetic patients.

Diabetic burden in India

Diabetic population in India has increased from 26.0 million in 1990 to 65.0 million in 2016 (19). India is considered to be diabetes capital of the world having 74 million diabetic patients accounting for approx. 8.7% among the adult population (20). It ranges from as low as 1.1% in urban Lucknow in 1973 to as high as 25.2% in New Delhi in 2015 (21). In 2011 prevalence of diabetes was 8.4%, 10.4%, 5.3% and 13.6% in Maharashtra, Tamil Nadu, Jharkhand and Chandigarh, respectively. In urban areas of Tamil Nadu, it rises from 7.8% in rural to 13.7%. In Jharkhand, rates when compared with rural areas were 13.5% in urban areas which were four times higher than that in rural areas (3%). Chandigarh and Maharashtra also followed similar trends (urban: 14.2 vs rural: 8.3%) (Urban: 10.9 vs rural: 6.5%) respectively (22). According to a report published in The Times of India on July 2017, it was given that prevalence of diabetes in South Indian states such as Tamil Nadu, Karnataka it was 10.4% and 7.5% respectively (23).

Studies have not been conducted where nano-encapsulated islets are implanted in subcutaneous region without immunosuppression (humans). The viability and functionality of subcutaneously implanted nano-encapsulated islets also have not been established over a long-term period in absence of immunosuppression.

Thus, present study will be done in phases to evaluate viability and assess functions of nano-encapsulated islet cell implantation on insulin dependency when implanted at subcutaneous site maintained without immunosuppression first in in-vitro condition, then in-vivo i.e. healthy animal model followed by animal model with diabetes mellitus and finally trials in diabetic patients.

Aim: To study effect of encapsulated human islet cells implantation on insulin dependency in diabetes mellitus.

Objectives:

- To culture human pancreatic beta cells in suitable culture medium by isolating them from cadaver.
- To encapsulate β-cell of pancreas in capsule and check β-cell viability and insulin release: an in-vitro approach.
- To implant encapsulated pancreatic β-cell in subcutaneous region of Wistar rats.
- To replicate encapsulation process and implantation in subcutaneous region of diabetic patients.

Materials and Methods:

Study Design:

This thesis protocol will be done in phases. In phase 1, isolation of pancreas will be done from cadaver donor which will be followed by isolation of islet cells from pancreas. After this, a process i.e. encapsulation of islet cells will be done. This step will be confirmed by Histo-pathological examination of encapsulated islet cells, Cell viability of encapsulated islet cells, Gene expression analysis of insulin produced by encapsule. This will be further followed by Phase 2 of this protocol which is Implantation of encapsulated islet cells in abdomen/thigh of Wistar Rats subcutaneously. After successful implantation, post implantation monitoring will be done. This will include Insulin viability, histopathology, Gene expression analysis etc. Statistical analysis will be done.

Explantation of encapsule will be done in extreme condition which will be followed by analysis of islet cells function.
*Patient and Public Involvement: No patient/public involved.

Methodology:

PHASE1: Preparation of encapsule

1. **Harvesting of pancreas:** In a fresh dead body pancreas dissected and removed in situ and preserved in required temperature.

2. **Isolation of pancreatic beta cells**

1. Collection of Pancreatic tissue from cadaver

a) After collection, whole pancreas specimen will be placed in icebox which will be instantly sent to the laboratory.

2. Selection of tissue for isolation of islets

a. We will select tissue which seems to be soft and in good physical shape. Damaged tissue (Fibrotic tissue) and offering < 2 g of tissue that can be used for islet isolation will be excluded.
b. Pancreatic tissue will then be dipped in Euro Collins Solution for preservation.

3. Human islet cell isolation

1. Tissue that is above preserved will be weighed and placed into a dish of 10cm.
2. Using a syringe and 300mmHg pressure, liberase will be injected into pancreatic duct which will distend the pancreatic tissue.
3. The distended pancreatic tissue will then be placed in a sterilized container, which will be warmed to 37°C with constant and gentle agitation in order to assist digestion.
4. Repeated samples of digested pancreatic tissue will be taken out to monitor process of digestion preferably by observing presence of free islets.
5. During digestion process, staining will be done using Dithizone allowing islets to distinguish them clearly from exocrine tissue of pancreas.
6. After finishing digestion process, enzyme activity will be stopped by cooling and bovine serum albumin (BSA) addition.
7. To increase difference in densities between endocrine and exocrine fractions, the digest will then be added to cold UW solution.
8. More efficient islet isolation will be obtained using centrifugation on a discontinuous density gradient prepared with Hanks' balanced salt solution and Histopaque (polysucrose and sodium diatrizoate).
9. After centrifugation process, interface containing islets will then be removed; it will be prepared for culture in suitable media in either a rotational cell culture system or conventional static culture.

10. These isolated islets will then be cultured in M199 medium containing 5.5 mM glucose, 10% bovine serum, 100UI/L penicillin, 100 mg/L of streptomycin, 750 µg/L amphotericin and 50 mg/L gentamycin.
11. Culture of islets will be maintained in a humidified incubator at 30°C with 5% CO2.

4. Multi-layer-by-layer nano-encapsulation

a. This type of encapsulation will be performed by electrostatic binding of oppositely charged polymers.

b. In this two polymers i.e. Chitosan [(Poly(D-glucosamine) deacetylated chitin)] which is a weak polycation positively charged at a pH below 7, and PSS [(Poly(styrene- 100 sulfonic acid, sodium salt)], a strong polyanion permanently negatively charged will be dissolved (1 mg/ml) in medium M199 and stored at 37 °C for about 48 hours before initiation of encapsulation procedure.

c. This step is obligatory to form a random coil structure for weak polycation.

d. After this islets will be suspended in chitosan containing medium (~100 islets/ml) for about 15 min.

e. This step will be followed by washing.

f. Again, Islets will be suspended in medium containing PSS for about 15 min.

g. Steps d-f will be repeated so as to obtain 9-10 layers by alternate binding of oppositely charged polymers.

h. Finally, collection of nano-coated islets will be done and cultured in medium M199.

5. Viability of Islet cells

a) Viability of islet cells will be monitored qualitatively in all of the encapsule using dithiazone staining as well as microscopic examination.

b) Quantitative analysis of islet cells that have undergone apoptosis will be assessed on flow cytometer. Then, approx. 200 IEQ will be recovered from encapsule using 0.25% trypsin-EDTA and single-cell suspension.

c) islet cells will then be sorted based on their auto-fluorescence using flow cytometer.

6. Gene Expression Analysis

a) Total RNA will be isolated from 50 IEQ.

b) First-strand cDNA will be synthesized using random hexamer primer.

c) Genes coding for insulin and glucagon will be amplified using specific primers (insulin: CTTTGTGAACCAGCACCTGTG as forward primer and GCGGGTCTTGGGTGTGTAG as reverse primer; glucagon: CATTGCTTGGCTGGTGAAAGG as forward primer and CAGAGAAAGAACCATCAGCATGTC as reverse primer).

d) Reaction mixtures without reverse transcriptase will serve as negative controls.

e) Insulin and glucagon expressions will also be confirmed by immune-staining of paraffin-embedded sections of islet encapsulated devices.

7. Statistical Analysis

Analysis will be performed using statistical package of social service (SPSS, version 23.0) and G Power software. Measurements such as body weight will be expressed as mean ± standard deviation. Data will be analyzed using appropriate test.

Staining score will be based on intensity of reactive cells. p-values will be considered significant at < 0.05 level.

PHASE 2: Implantation of encapsule in Wistar rats

a. Experimental animals:

21 Male Wistar rats, 4-8 months old weighing (100–250g), will be used for experimental study. Animals will be maintained at 21 ± 2^0C temperature with a relative humidity (30% to 70%) throughout the year, in a 12h/12h light/dark cycle, with adequate veterinary care. Animals will have free access to standard pellet chow diet and water ad libitum. Animals will be acclimatized to laboratory conditions for at least 2 weeks prior to study and will be provided with social enrichment.

b. Grouping of animals:

21 Male Wistar Rats will be divided into 2 groups;
Group 1: Control group (7 rats)
Group 2: Experimental group (14 rats) in which diabetes is induced by alloxan

Group 2 is further divided into two;
Group 2a: Control Diabetic rats group (7 rats)
Group 2b: Diabetic rats in which beta cell encapsule will be implanted (7 rats)

c. Alloxan induced type 2 diabetes mellitus:

A single dose 120 mg/kg of alloxan Monohydrate (Sigma) dissolved in sterile Phosphate Buffer Saline (PBS) will used for induction of diabetes mellitus in overnight fasted Wistar rats through intraperitoneal injection of freshly prepared. The basal glucose level of animals will be measured before they injection. Diabetes will be confirmed after 3 days of alloxan injection by determining fasting blood glucose concentration; only animals with fasting blood glucose >300mg/dl will be considered diabetic used for experiment. Diabetic animals will be allowed free access to tap water and pellet diet and will be maintained at room temperature in clean iron cages.

d. Number of days

Group 1: Will recieve normal saline for 180 days, Left untreated for 180 days with regular Parameter examination.
Group 2: Will recieve 1 dose of Alloxan (120MG/KG BW i.p.) dissolved in sterile PBS, Left untreated for 180 days with regular Parameter examination.
Group 2a: Will recieve normal saline for 180 days, Left untreated for 180 days with regular Parameter examination.
Group 2b: Will recieve implant beta cell encapule, Left untreated for 180 days with regular Parameter examination.

e. Parameters to be studied:

1. **Behavioral**: unexpected signs of illness or infection, body weight, food intake, body temperature will be examined.

2. **Biological**:

- Blood glucose levels will be monitored daily for 2 weeks followed by every week for 1 month and subsequently on monthly basis.
- Antioxidant enzyme assay: superoxide dismutase (SOD), catalase, glutathione peroxidase will be assessed to measure activity of antioxidant enzyme.
- Lipid profile:
  - Low-density lipoprotein (LDL)
  - High-density lipoprotein (HDL)
  - Triglycerides
  - Total cholesterol

  Serum lipid profile will be tested on the day of sample collection. Levels of all these parameters will be checked.

- Pancreatic exocrine (amylase, lipase), hepatic (Liver function test), and renal functions (Kidney function test) will be monitored.
- Islet cell viability will be assessed qualitatively by dithiazone staining and microscopic examination.

3. **Molecular:** Genes coding for insulin will be determined by RT-PCR and insulin expression will be confirmed by immune staining.
4. **Histopathological**: H&E staining will be used and explanted beta cell encapsule will be scored.

f. Duration of experiment: 8 months.

g. Samples to be collected: Blood, beta cell encapsule (6 months after implantation).

h. Statistical Analysis: Analysis will be performed using statistical package of social service (SPSS, version 23.0) and G Power software. Measurements (e.g. body weight) will be expressed as mean ± standard deviation. Data will be analyzed by paired t test. Staining score will be based on intensity of reactive cells. p-values will be considered significant at < 0.05 level.

Ethics and dissemination: This is to certify that the project proposal no' IAEC/AIIMS/Rish/Med/14/21 entitled "Encapsulated human islet cells implantation in subcutaneous region: Effect of insulin dependency in Wistar rats" submitted by Dr. Ravi Kant, Additional Professor & Head, Division of Diabetes and Metabolism, Department of General Medicine has been approved by the IAEC of All lndia Institute of Medical Sciences (AIIMS), Rishikesh, Uttarakhand in its 3rd meeting held on 15-06-2021. Monitoring is performed in accordance with currently valid rules and regulations, Good Clinical Practice.

Discussion: Patients often avoid treatments because of high cost of treatment facilities. Poorly

controlled diabetic status leads to chronic complications of diabetes mellitus. India being a developing country with some peculiar problems to it warrants unique solutions. Taking care of diabetes in these conditions poses problems in providing quality healthcare to patients. A diabetic patient takes insulin approximately 3 times (30 Units) per day. This cost around 8-10 lakh in 10 years. Beta cell encapsule implantation will be a good solution to this problem. After successful results in Wistar rats, we will replicate this study in diabetic patients. Once implanted subcutaneously it will work for long time. This will not only decrease financial burden on diabetic patients but also diabetic patient need not to puncture them again and again.

Clinical Message: In this study, if we will be able to decrease insulin dependency in patients with type 2 diabetes mellitus then this study will be a novel interventional research to decrease burden of insulin therapy in low income population such as of India.

Ethical Approval: All authors hereby declare that "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) will be followed, as well as specific national laws where applicable. All experiments have been examined and approved by the appropriate ethics committee.

COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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