CHAPTER 6

ENCAPSULATION & TRANSPLANTATION OF ISLET INTO DIABETIC MOUSE MODEL FOR EFFECTIVE THERAPY

6.1. Introduction

Several modern treatments are available for managing diabetes mellitus, including insulin, metformin, sulfonylurea, DPP inhibitor, etc. However, there are certain limitations of current treatment such as hypoglycemic shock, various side effects of drugs. Hence, there is a need to cure diabetes mellitus with exogenous insulin independence. The use of regenerative medicine could be a possible cure for diabetes.

6.1.1. Regenerative Medicine:

Regenerative medicine is a modern inter-disciplinary area targeting at the replacement, repair or restoration of normal function to disease organs/tissues by the delivery of risk-free, efficient and reliable treatment composed of cells or tissues, executed either alone or in combination with the extraordinary configuration of bio-materials (Langer and Vacanti 1993). This field of medicine comprises of various disciplines as depicted in figure no:6.1.

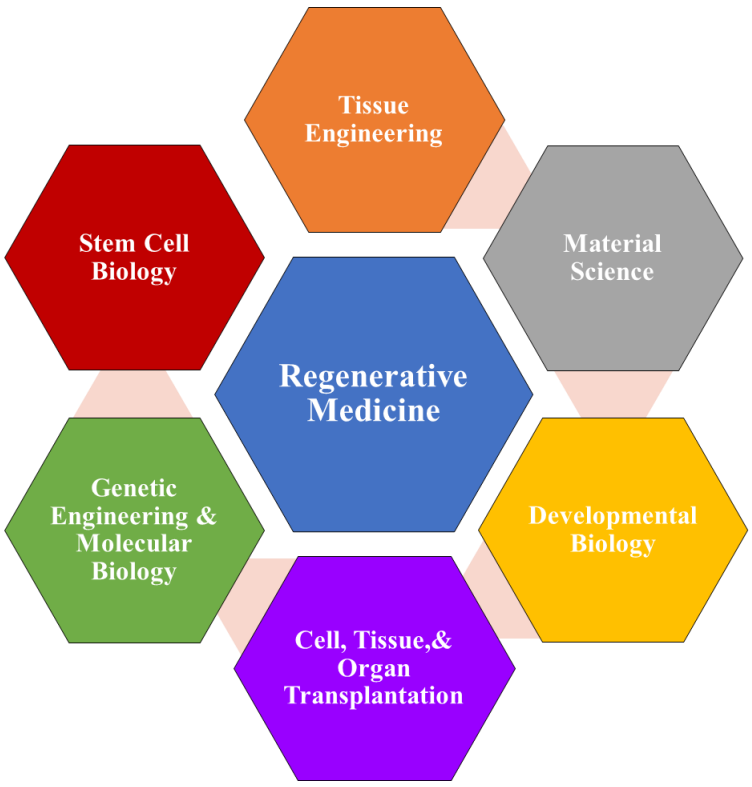

Figure 6. 1: Regenerative medicine is a multi-disciplinary field which comprises of various distinct disciplines. This fugure is adopted and modified from (Polak 2010).

Regenerative medicine mainly focused on cell, tissue & organ transplantation. Thus, pancreas & islets transplantation can be used as a therapeutic procedure to cure diabetes mellitus.

6.1.2. Islet transplantation:

Islet transplantation can be dated back to 19th century when the pancreatic fragment was first successfully transplanted subcutaneously into a dog. The major breakthrough in human islets transplantation came in the year 2000 when Shapiro & his colleagues established the Edmonton Protocol, where allogeneic transplant with 100% insulin independence was achieved for approximately 11 months with subsequent islet transplantation along with immunosuppressive drug (Shapiro *et al.* 2000).

A. The strategy of islets transplantation

Human islets transplantation protocol involves, explanation of the pancreas from human cadavers. Collagenase is perfused into the pancreas & chopped into tiny pieces. The chopped pancreas is then placed in ricordi chamber for digestion and purified by the density gradient centrifugation process. After islets purity is confirmed by DTZ staining, islets are transplanted into the intra-hepatic portal vein. Diagrammatic representation of human islets transplantation protocol is given below (Figure no: 6.2).

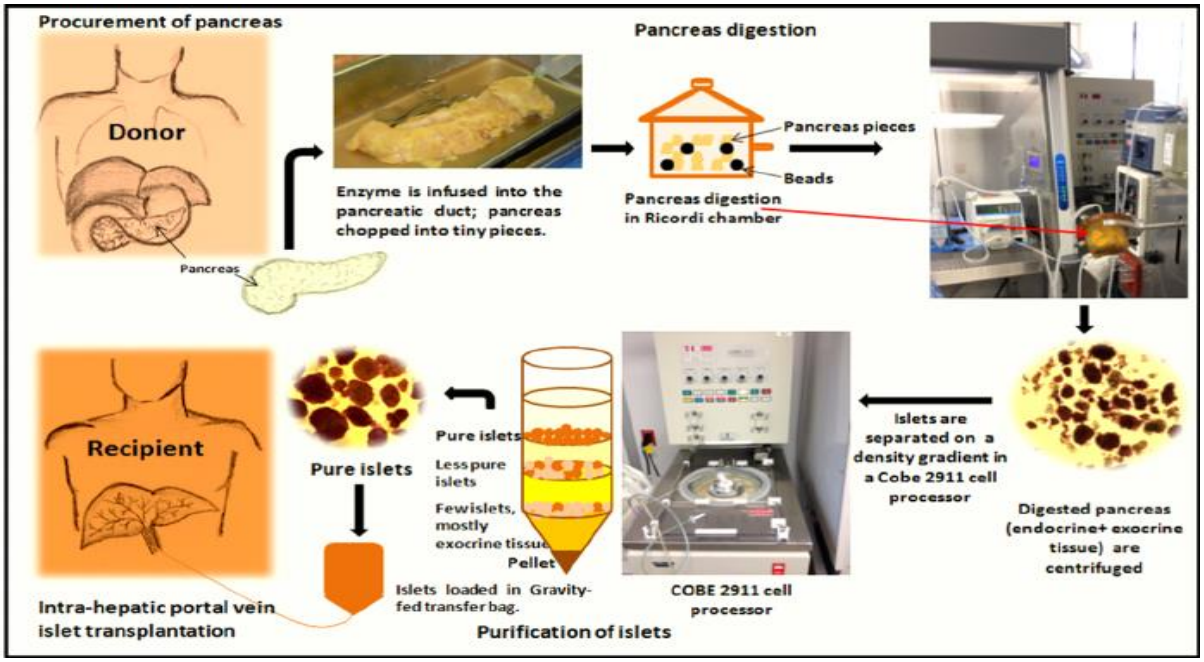

Figure 6. 2: Schematic representation of various stages involved in human islets transplantation.This figure is adopted from (Chhabra *et al.* 2014).

B. Limitations of islets transplantation

In spite of marked clinical success in islet transplantation, there are certain shortcomings to islets transplantation protocol mentioned below: (1) Donor availability, (2) Site of

transplantation, (3) Graft rejection, (4) Longevity of transplanted Islets, (5) Immunosuppressant Therapy (Haider and Ashraf 2008).

To overcome such problems combined therapy i.e. a combination of two or more approaches can be used where:

- **Approach 1**- Islets encapsulation can be used to prevent graft rejection & intake of immunosuppressants.
- **Approach 2-** Stem cells can be differentiated into islets like cell clusters to increase islets mass to combat the problem of islets availability.

6.1.3. Islets Encapsulation:

Cell encapsulation techniques include immobilization of the pancreatic islets/cells inside a polymer-based semi-permeable membrane that allows the bilateral diffusion of molecular components like an exchange of O_2/CO_2 gases, supplements (like amino acid, glucose), various growth factors/hormones along with metabolic waste products for cell metabolism. Additionally, the semi-permeable characteristic of the membrane obstructs immune cells as well as antibodies from destroying the encapsulated pancreatic islets/cells, considering them as potential external intruders (Robles *et al.* 2014). Thus, the most important function of the encapsulation device is to create an environment that allows for normal insulin secretion in response to fluctuating blood glucose levels while maintaining pancreatic islets/cell viability through sequestration from the immune system including antibodies (Desai and Tang 2018) (Figure no: 6.3).

The major advantage of cell encapsulation devices is to provide a physical hindrance among transplanted pancreatic islets and their beneficiaries developed as a promising methodology to overcome some of these challenges by wiping out the requirement for immunosuppression (Soon-Shiong *et al.* 1994, de Vos *et al.* 2010, Vaithilingam and Tuch 2011).

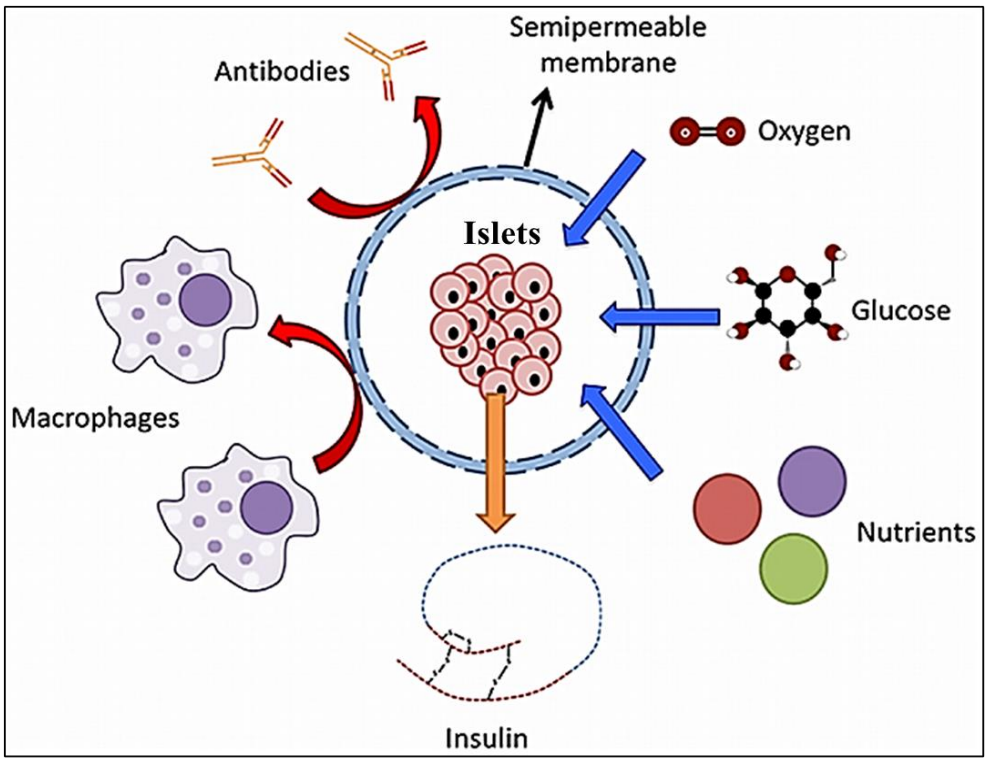

Figure 6. 3: Principle of Encapsulation. The semi-permeable membrane permits the dispersion of molecules like glucose, nutrients (amino acid), oxygen, insulin (in response to glucose), metabolic waste also while protecting the pancreatic islets from the immune cells/antibody of the host. This figure is adopted and modified from (Vaithilingam and Tuch 2011).

A. Types of encapsulation

Intending to create immune-protected β-cells, there are two encapsulation approaches which have been developed over several decades these are (1) **micro-encapsulation-** this approach uses micro-scale capsules with each one containing a single cell or islets. (2) **macro-encapsulation-** In this approach, devices house a large number of cells or islets (Scharp and Marchetti 2014). The fundamental distinction between microdevices and macrodevices is a matter of scale, with both having their pros & cons. Table 6.1 shows the advantages & limitations of each approach:

Sr. No.	Micro-encapsulation	Macro-encapsulation
Advantages		
1	Maximizes surface area to volume ratio	Contains many islets
2	Promotes nutrient & oxygen exchange	Have greater control over membrane parameters like pore size, thickness, etc.
Limitations		

1	Irregular membrane thickness & pore size	Limited nutrient & oxygen supply
2	Large no. of such microcapsules required	Inner membrane permeable to antibodies & inflammatory factors
3	Live imaging & tracking a big challenge	Central necrosis & islets aggregation

Table 6. 1: Advantages & limitations of macro & microencapsulation devices (Desai *et al.* 2006)

B. Challenges in islets encapsulation

Despite advancements made in the field of islets encapsulation, still, there are certain challenges which must be dealt with (1) Islets availability (2) Islets survival & viability (3) Nutrient & O_2 Supply (4) Vascularization (5) Immune Response (O'Sullivan *et al.* 2011).

6.1.4. Encapsulation materials and its characteristics

An encapsulation material or a device should possess certain characteristics to be ideal for encapsulation such as (1) Biocompatible i.e. no host response against encapsulating material. (2) Serve as an immune barrier i.e. prevents direct contact/ interaction between immune cells & encapsulated cells. (3) Provide ample blood supply- encapsulating material should promote vascularization to facilitate the exchange of nutrients, glucose, insulin & waste products. (4) Appropriate glucose and insulin kinetics should be maintained such that insulin is released as soon as glucose enters encapsulated β cells (Desai and Shea 2017).

Thus, the choice and design of encapsulation materials can enhance engraftment and promote islets survival post-transplantation. A challenge to engraftment is the host response to the material, which can lead to a fibrotic response that can exacerbate mass transport limitations, and the material choice or chemistry can modulate the extent of fibrosis. In the search for optimal encapsulation materials, many types of natural and synthetic polymers are being explored. Alginate has been the predominant microencapsulation material of choice owing to availability, cost, and ease of production (Dusseault *et al.* 2006). A wider range of materials has been investigated for macro-encapsulation. PTFE (Polyterafluoro-ethylene) and PCL (Polycaprolactone) have been shown to induce limited fibrosis and to exhibit good vascularization, allowing for better cell viability (Nyitray *et al.* 2015). There are various materials & devices available which can be used for both micro- & macro- encapsulation & some of them are shown in Figure: 6.4.

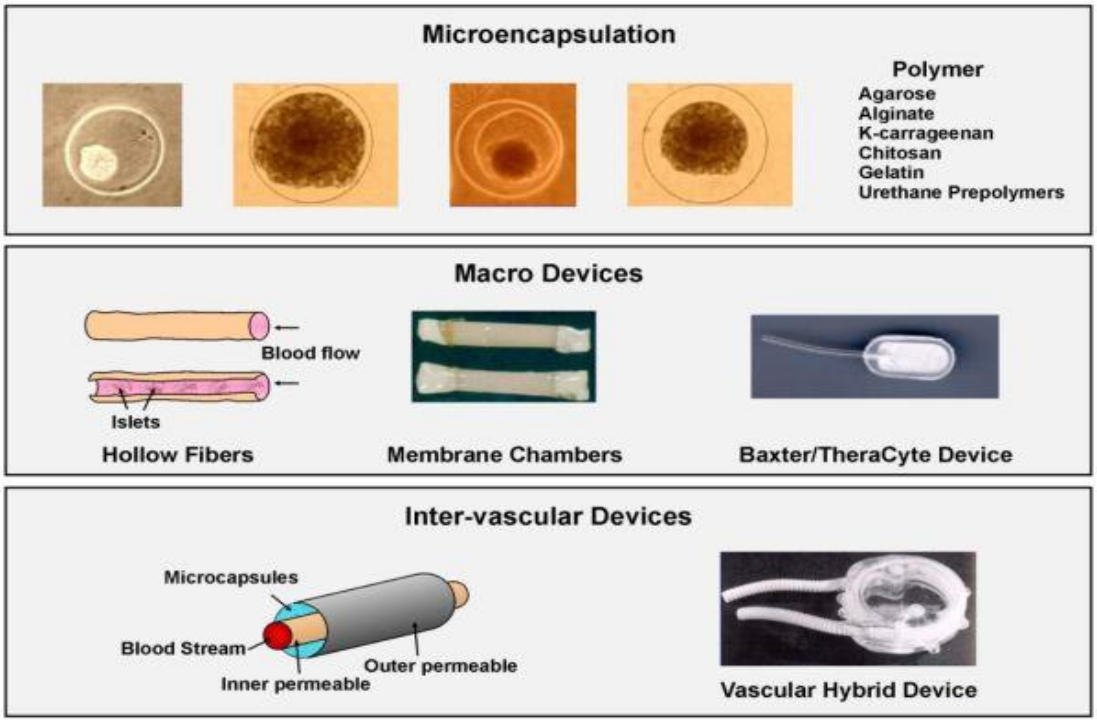

Figure 6. 4: An illustrative depiction of various micro & macro encapsulating materials and devices, which have been exploited for islets encapsulation and act as an immuno-isolation membrane. This figure is adopted from (Desai and Shea 2017).

There are various encapsulation materials/devices which are under clinical trials, properties of these materials/devices are described in Table 6.2.

No	Devices	Experimental intervention	Properties
1	Seranova Cell Pouch	Implantation of allogenic islets into Cell Pouch following Pre- vascularisation	Subcutaneous transplantation, 2-12 weeks of pre vascularisation
2	Diabecell	Laparoscopic delivery of alginate encapsulated porcine islets	Transplantation in Peritoneal cavity, No Immuno-suppression required
3	Alginate encapsulation	Implantation of alginate encapsulated allogeneic islets	Subcutaneous transplantation, Immuno-suppression required
4	Viacyte	Encaptra containing hESCs derived islets	Subcutaneous transplantation, No Immuno-suppression required
5	β-Air artificial pancreas	Microencapsulation of allogeneic islets in ß Air that provides oxygen to cells	Transplantation in the peritoneal cavity, No Immuno-suppression required

Table 6. 2: Various islets/β cell encapsulation systems undergoing clinical trials (Desai and Shea 2017).

6.1.5. Hollow Fiber Membrane (HFM):

After a thorough review of various encapsulation materials & devices literature, we screened out two potential encapsulation devices i.e. Theracyte (commercially available) & Hollow Fiber membrane (HFM) made of polysulfone- TPGS (Psf-TPGS). Both have their pros & cons but we decided to go with HFM because of following advantages:

- HFM is a double-layered membrane with an inner membrane having a pore size in the nanometre range while the outer diameter is in the micrometer range (Figure 6.5).
- HFM is made of polysulfones which helps encapsulated islets to release VEGF as shown in *in vitro* studies, which is an important factor for angiogenesis to occur & also supports vascular growth on the outer surface due to its rough outer surface (Silva *et al.* 2006).
- The inner diameter of HFM is ≈900µm which is suitable to prevent islets aggregation inside HFM & at the same time facilitates easy loading of islets into HFM with help of needle (Teotia *et al.* 2017).
- Molecular cut-off of HFM is 20KDa i.e. any molecule with molecular weight >20 KDa is inhibited (Approximately:100 % rejected). Thus, allows easy passage to small molecules like insulin and glucose while inhibits diffusion of IgG & other antibodies (Figure: 6.6).
- FTIR spectroscopy of HFM made of Psf-TPGS shows minimal insulin & protein adsorption on its surface for encouraging insulin exchange through the HFM membrane (Figure:6.6).

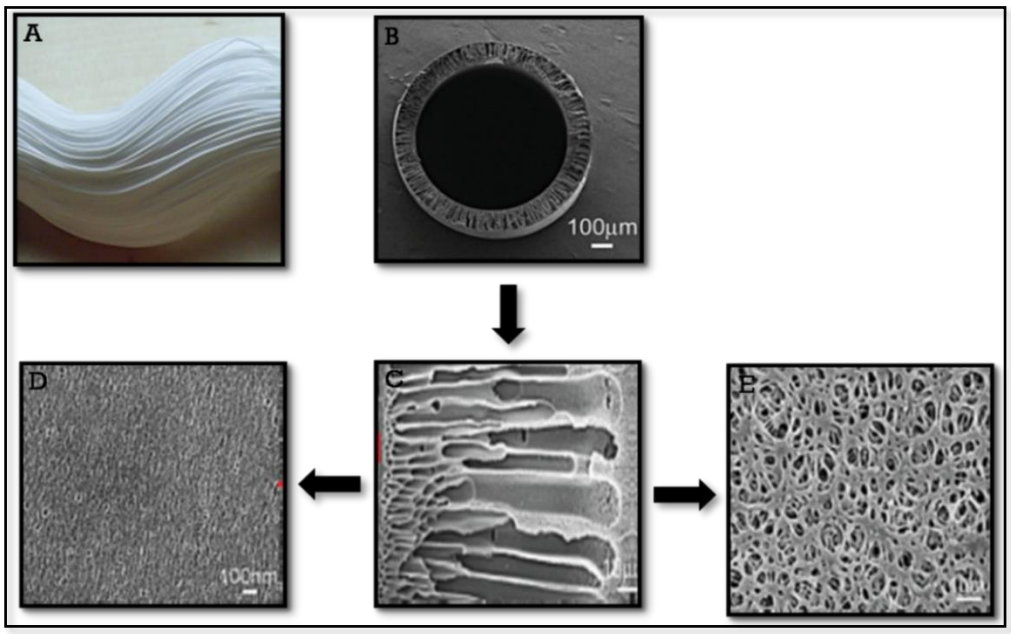

Figure 6. 5 Representative images regarding microstructure details of HFM (A) Psf-TPGS HFM. (B) SEM microstructure of HFM. (C) SEM micrographs of HFM showing asymmetric cross-

section. (D) Inner membrane pores are in the nanometre range. (E) The outer membrane is a macro porous. This figure is adopted from (Teotia *et al.* 2017).

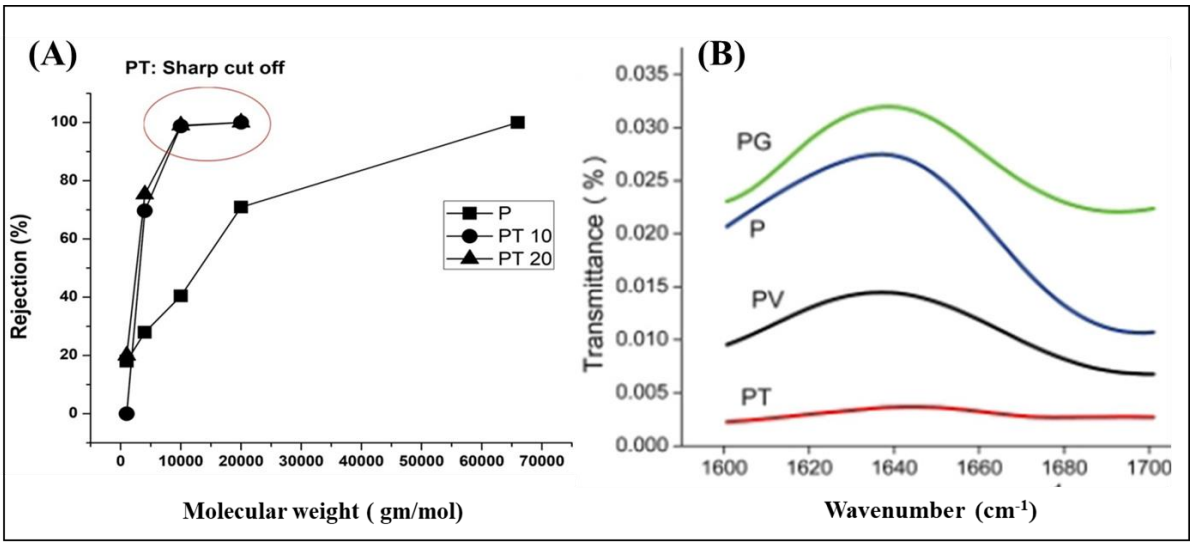

Figure 6. 6: Representative graphical analysis of HFM properties. (A) The graph shows the molecular cut-off of HFM. (B) Insulin adsorption of different membranes on FTIR spectra (PT-Psf+ TPGS). This figure is adopted from (Teotia *et al.* 2017).

As discussed previously use of combined therapy i.e. combination of two or more approaches can be applied to overcome limitations of islets transplantation where (1) The first approach is to use islets encapsulation which can be employed to avoid graft rejection & intake of immunosuppressant which we have discussed above. (2) The second approach is to use mesenchymal stem cells (MSC) which can be differentiated into islets like cell clusters (ILCCs) to increase islets mass & release insulin in case of hyperglycemia to combat the problem of islets availability.Several growth factors and synthetic factors are used for supporting the islet encapsulation process for islet transplantation study but the use of bioactive molecules will promise an attractive option.

6.1.6. Bio-active Molecules

Our lab findings demonstrated that swertisin having islet neogenesis and anti-apoptotic as well as pancreatic islet protective effect (Dadheech *et al.* 2013, Srivastava *et al.* 2016, Srivastava *et al.* 2019). Curcumin is a known anti-oxidant & has shown to increase islets viability in hypoxic conditions (Zhang *et al.* 2013, Pathak *et al.* 2016). Genistein (Liu *et al.* 2006), isolated from soybean, directly acts on pancreatic islets and enhanced insulin secretion. Thus, these bioactive molecules play (direct or indirect) crucial role in increased pancreatic islet viability (by

preventing central necrosis and apoptosis in islets) and islet functionality, as previously described in chapter 3.

Considering all the above knowledge and scientific reports, few key questions are raised (I) Does bioactive molecules enhance the viability of encapsulated differentiated ILCCs and rat islets? (II) Whether HFM encapsulation protocol, increase islet longevity in "Rat to Mouse" xenotransplantation model? To address these questions, we conducted *in vitro* study, in which, we have taken a combination of three bioactive molecules such as swertisin, curcumin, genistein in two types of cell systems (1) ILCCs (Derived from hBMSCs) (2) Rat islets. Another important aspect of this study is to prove islet longevity using HFM (Follow Fiber Membrane) containing encapsulated rat islets into a diabetic mouse model. To achieve our objective, we design the following sub-objectives and utilized HFM an immune-isolating device being prepared by IIT Mumbai,India.

(a) ILCCs encapsulation study (*In vitro* study)

In this sub-objective, we attempted islet viability study during the encapsulation process using encapsulated differentiated ILCCs derived from hBMSCs (human subject) in the presence of bioactive molecules (*In vitro*).

(b) Rat islet encapsulation study (*In vitro* study)

We also attempted an islet viability study during the encapsulation process using encapsulated rat islet in the presence of bioactive molecules (*In vitro*).

(c) "Rat to mouse" for xeno-islet transplantation study using HFM (*In vivo*)

To understand xeno-islet transplantation study, we explored (encapsulated rats islets packed into HFM) HFM as biocompatible/islet longevity in STZ induced diabetic mouse model. The experimental design to undertake this work has been described in the figure below: 6.7,6.8,6.9,6.10.

6.2. Experimental design for chapter-6

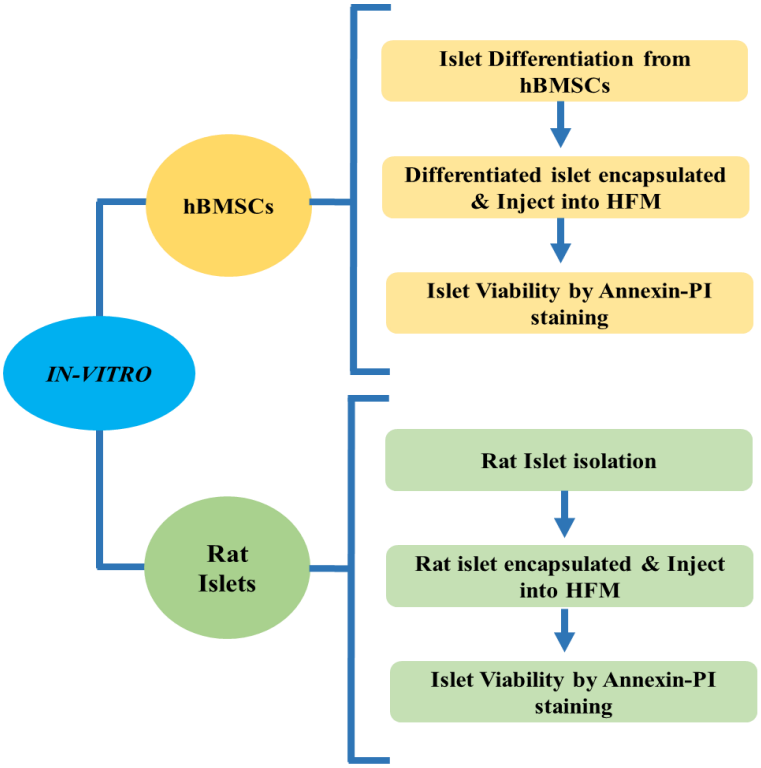

Figure 6. 7: plan of work for Chapter-6 (*In vitro*)

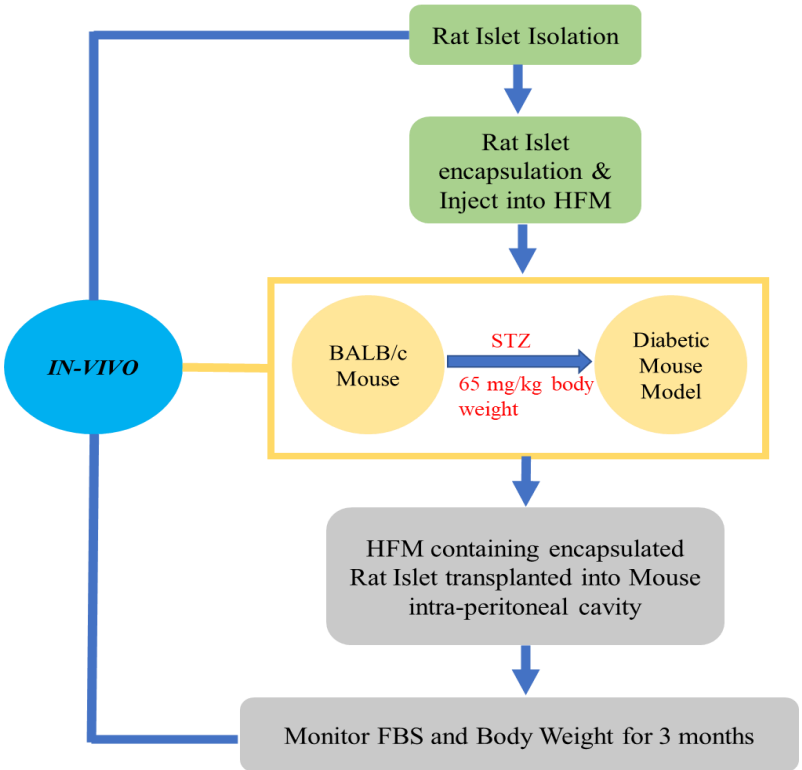

Figure 6. 8: plans of work for Chapter-6 (*In vivo*).

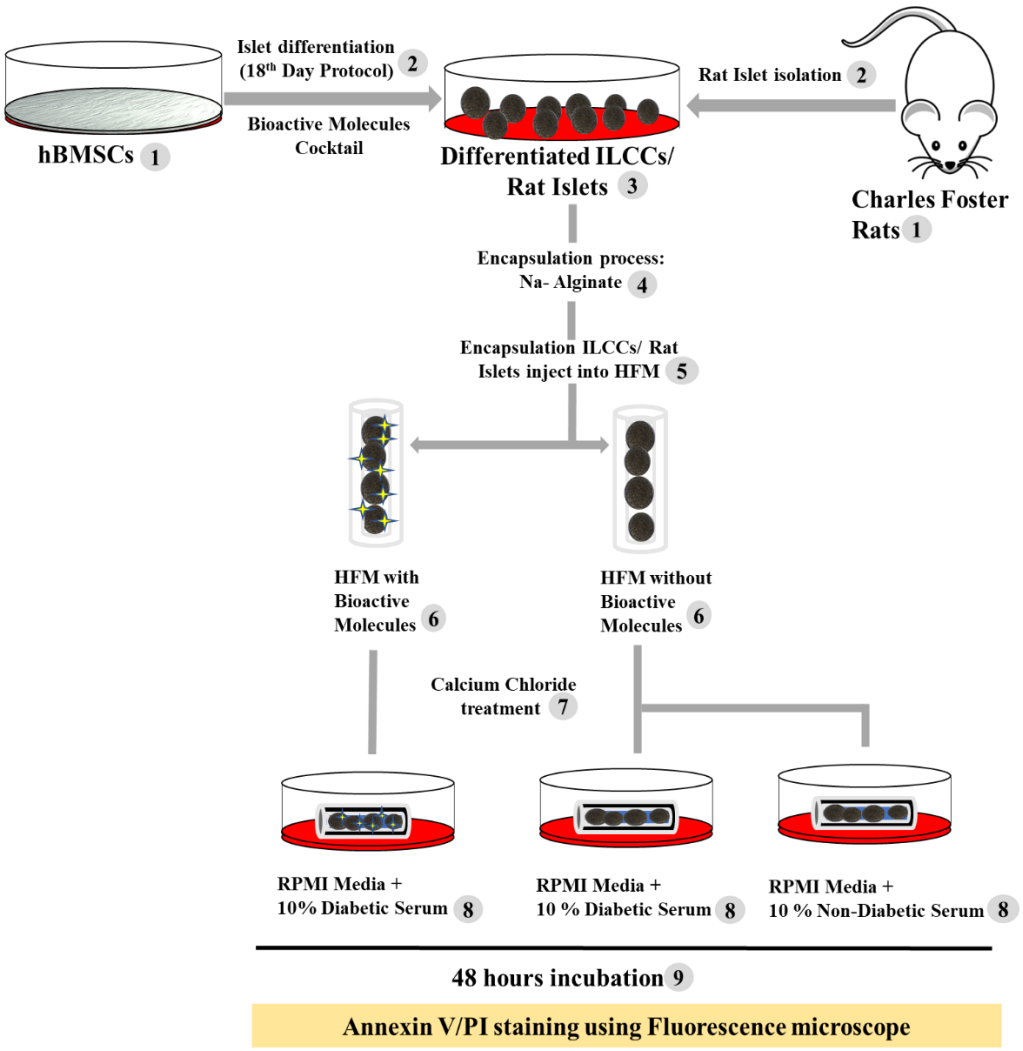

Figure 6. 9: Encapsulated rat islet viability study (*In vitro*).

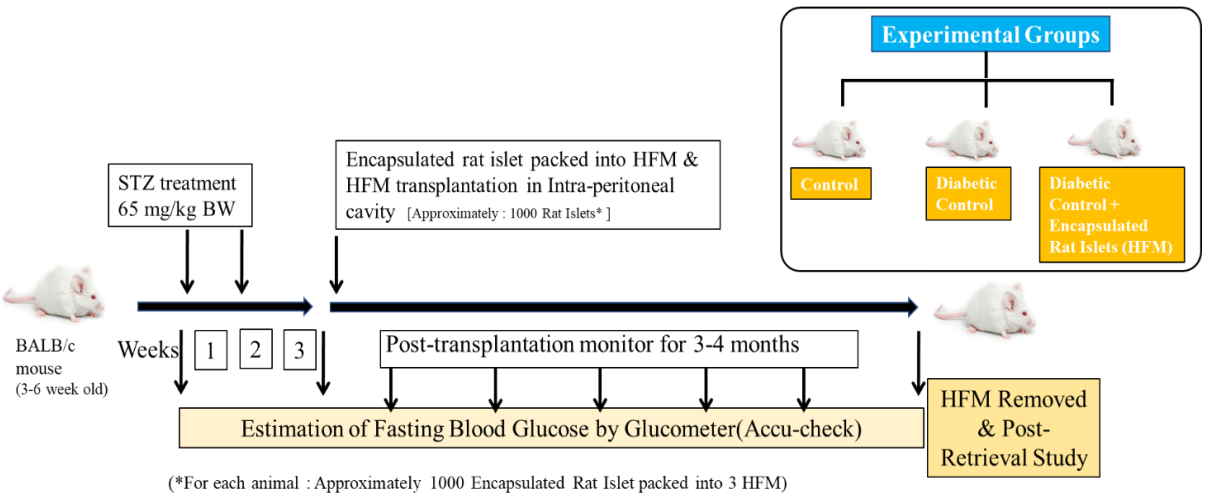

Figure 6. 10: Experimental time-line for xeno-transplantation study (*In vivo*).

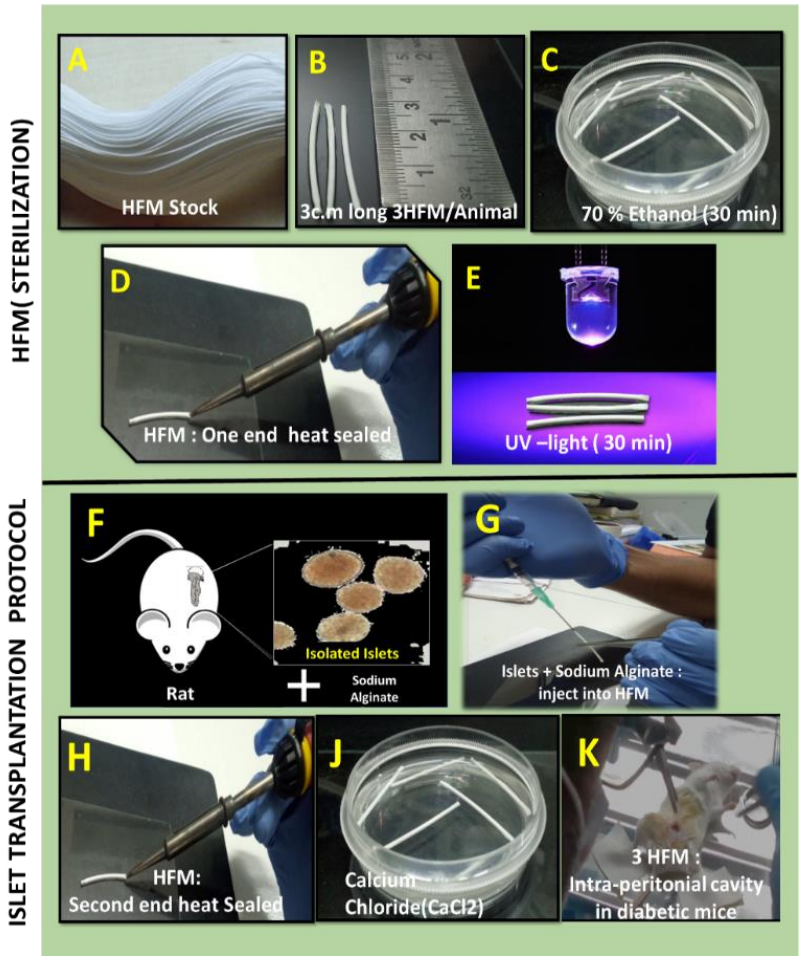

Figure 6. 11: Experimental process (steps) for xeno- transplantation study (*In vivo*).

6.3. Material and Method

6.3.1. Material

A. Chemicals, cell culture media, and HFM

All chemicals, media, and plastic-wares used in the present study were purchased from Thermo Scientific, USA. Annexin V-PI kit was purchased from BD scientific, USA. HFM was obtained from Prof.Jayesh Bellare lab, IIT- Mumbai (MOU was signed between MSU and IITB).

B. Animal (Mice & Rats)

Young male Balb/c mice, 4-5 weeks old, weighing around 25-35 grams, were placed at an animal house with 12 hours light and dark cycle with regular pellet diet ad libitum and water. Male gender was selected to rule out female menstrual cycle changes in experiments. Diabetes was induced with STZ injection (STZ 65 mg per kg body weight) intraperitoneally for 6 days with 10-12 hours overnight fasting. Diabetes status of Balb/c mice was confirmed by

monitoring (tail vein) fasting blood sugar (FBS) using Accu check Performa glucometer (Accu-check, Roche, USA) at regular intervals as shown in the figure: 6.10 till the length of the experiment. Male Charles Foster rats (age: 6-8 weeks; weight:200±20 gram) were used for the isolation of pancreatic islets. All animal experiments were executed after approval from CPCSEA committee meeting (MSU/Biochemistry/IAEC/2018/02), at the Department of Biochemistry, The M.S. University of Baroda, Vadodara, Gujarat, India.

6.3.2. hBMSCs differentiation into ILCCs using Bioactive molecule cocktails

As described earlier in the chapter: 3 (Section 3.3.2-H)

6.3.3. Rat islets isolation

Rat isolation protocol was performed as our group previously described in (Srivastava *et al.* 2016). Briefly, rat pancreas was digested by collagenase solution (collagenase V& collagenase I solution). Briefly, the rat pancreas was isolated and chopped into very fine small pieces, these pieces were digested with the collagenase solution (collagenase V& collagenase I solution) for 10-15 mins on a magnetic stirrer at 37 C. Digested pancreati tissue was filtered through a cell strainer and recovered rat islets were spun down at 1200 rpm for 5 min. Further, the centrifugation was done 3–4 times and the rat islets were kept (non-adherent plates) in serum-containing RPMI medium for a day. The purity of isolated rat islets was performed by DTZ staining and then later encapsulated.

6.3.4. DTZ Staining

Isolated rat islets subjected to DTZ staining after overnight incubation in RMPI media with 10 % FBS. DTZ staining protocol was performed exactly as described earlier in the chapter: 3 (section number: 3.3.2-I).

6.3.5. Islets Encapsulation:

We performed *in vitro* islet encapsulation process as illustrated in (Teotia *et al.* 2017). Briefly, HFM of length 3 cm was cut from stock HFM & subjected to sterilization process i.e. 30 min in ethanol & 30 min in UV exposure in the chamber. After the sterilization process, one end of HFM was heat-sealed with the support of a soldering machine (25-watt soldering iron, Soldron, India)(Figure:6.11). In the mean time isolated rat islets or differentiated ILCCs (from hBMSCs) were spun down at 1200 rpm for 2 minutes & 300 µl of 1% sodium alginate solution was added to the pellet (contain rat islet / ILCCs). This suspension was equally distributed into three 0.5

ml eppendorf tubes, & in one of the tubes, the respective concentration of bioactive molecules (i.e. swertisin, curcumin & genistein) were added (Bioactive molecules concentration was used as described in chapter number: 3). With help of hamilton syringe (Hamilton, USA) 15 µl of suspension was added to HFM & the other end was heat-sealed using soldering machine. Rat islets / ILCCs containing HFM were dipped in $CaCl_2$ solution for 5 min. & washed twice with PBS (figure:6.11). And further, incubated for 48 hrs. in a culture dish containing RPMI medium with Non-diabetic (control) & diabetic mice serum & viability assay (Annexin V/PI staining) was performed.

6.3.6. Annexin V/PI Staining:

Islets/ILCCs were explanted from HFM after incubation with diabetic and nondiabetic serum, collected in 0.5ml Eppendorf tube & centrifuged at 800 rpm for 1 min. The supernatant was removed, while the pellet was given two PBS wash & centrifuged an 800 rpm for 2 min. The supernatant was removed & 90 µl of 1X binding buffer was added to the pellet. Further, Annexin V /PI staining protocol was performed as per kit's manufacturing instruction (FITC Annexin V Apoptosis detection kit, BD Bioscience, USA). Briefly, 3µl Annexin V antibody was added to each vial, along with 10 µl of propidium iodide (working concentration 20 µg/ml) & incubated for 15 minutes in dark at 25 ^{o}C. After incubation, rat islets/ILCCs were observed under the inverted fluorescent microscope (Eclipse Ti2, Nikon, Japan) for annexin V- FITC (green fluorescence- green filter) & for propidium iodide (Red fluorescence- red filter). A graph of Annexin V/PI level was plotted against fluorescence intensity against the sample using Image J software (NIH, Bethesda, USA).

6.3.7. Xeno-islets transplantation study :

We performed a xeno islet transplantation protocol as described in (Teotia *et al.* 2017). Briefly, bodyweight & fasting blood glucose of STZ treated diabetic mice were taken before transplantation. 40 mg/kg body weight of thiopental sodium (Thiotone powder, Flagship Biotech International PVT LTD, India) was injected into the intraperitoneal cavity to anesthetize the diabetic animal. Hairs from abdomen were removed with help of scissors & hair removal cream & wiped with a povidone-iodine solution (Betadine, India). A 2 mm incision was made into the abdomen with the help of pointed scissors. 3 HFM (each of length 3 cm) with approximately 1000 encapsulated rat islets were transplanted into the intraperitoneal cavity of the STZ induced diabetic mouse. The incision was sealed with the help of surgical sutures (Lotus surgical Pvt Ltd, Mumbai). The transplanted animal received an i.p. injection of

an antibiotic such as ampicillin & cloxacillin combination (20 mg/kg body weight), gentamycin (3 mg/kg body weight), and painkiller i.e. diclofenac sodium (0.5 mg/kg body weight) for every three consecutive days of transplantation. Additionally, topical ointment such as soframycin (Sanofi, India) and neosporin powder (Glaxo SmithKline, India) was applied on the transplantation site. Fasting blood sugar (FBS) and body weights were monitored for all groups till three months (Figure:6.10).

6.3.8. H & E staining

After completion of study, all animals from three groups were sacrificed. HFM containing encapsulated rat islets were explanted animals. HFM were fixed in 4 % PFA in PBS solution. Fixed HFM vertical sections were stained with H & E staining kit (Rapid H & E kits, Bio Lab Diagnostics, India), and images were captured under different magnification in light microscope (Eclipse Ti2, Nikon, Japan).

6.3.9. Statistical analysis:

The experimental data are presented as mean ± SEM. The signification of difference was evaluated by the paired student's t-test using graph pad analysis software. When more than one experimental group was compared with one control group, significant was assessed according to one -way analysis of variance (ANOVA).

6.4. Results

6.4.1. Encapsulated ILCCs viability: *In vitro* study

Firstly, hBMSCs were isolated from bone marrow by previously described methods (chapter :3) of hBMSCs isolation. hBMSCs were analyzed for specific mesenchymal stem cell surface markers growing in the exponential phase of culture. To drive pancreatic islet differentiation of hBMSCs towards ILCCs, we developed high reproducible and feasible a culture protocol comprised of four stages using bioactive molecule cocktail (18^{th} Day protocol) as described earlier in chapter: 3.

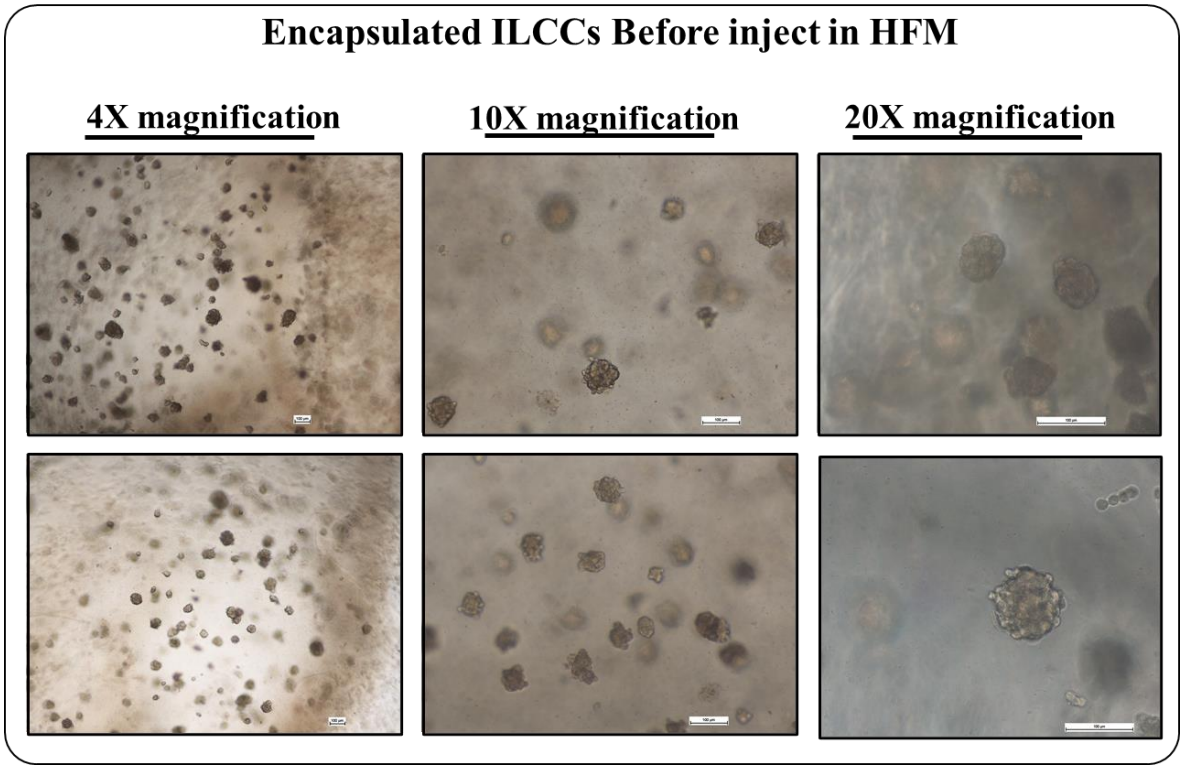

Figure 6. 12: Encapsulated ILCCs (hBMSCs differentiated into ILCCs using 18-day protocol) were observed by phase-contrast microscopy. Different sizes of ILCCs fixed using calcium-alginate encapsulation method before injecting into HFM. Eventually, encapsulated ILCCs covered by calcium-alginate mess. Scale bar: 100 µM; Magnification 4X,10X,20X (N=3).

After 18 days of the differentiation process, differentiated ILCCs were encapsulated with sodium alginate and calcium chloride method. We observed different sizes of ILCCs immobilized with complex calcium-alginate mess before injected into the Hollow fiber membrane (HFM). Phase-contrast microscopy demonstrated encapsulated ILCCs with different magnification views such as 4X, 10X, 20X. We observed each islets cluster, perfectly trapped with calcium-alginate mash in this encapsulation method (Figure: 6.12). Further, we monitored that the encapsulation process leads no changing ILCCs morphology, integrity and no sign of cell proliferation in ILCCs or cluster formation within calcium- alginate matrix (Figure :6.12).

Next, our study aimed to increase islet longevity by protecting encapsulated pancreatic islet from oxidative stress and apoptosis. Hence, we used bioactive molecules cocktail in alginate encapsulation process to protect differentiated islets from oxidative stress and central necrosis along with HFM as an immune-isolation device. Several recent studies have shown a reduction of islets viability & function on exposure to diabetic serum and high glucose concentration due

to oxidative stress. Hence, we chose diabetic mice serum for mimicking diabetic condition for *in vitro* study. HFM containing encapsulated ILCCs were incubated with non-diabetic and diabetic serum for 48 hours, in the presence and absence of bioactive molecules cocktail. After 48 hours islets were explanted from HFM & islets viability was assessed by Annexin-V/PI staining protocol using fluorescence microscopy. We found that control mice's serum group contains comparatively low level of cell death as compared to diabetic's mice serum group, with high level of cytokines and ROS present in diabetic serum due to high grade of inflammation. Further, we analyzed the apoptosis parameter using Annexin V staining in both groups and we found that a notable high level of Annexin V/PI staining in diabetic mice's serum group due to the accumulation of inflammatory cytokines. Interestingly, when we encapsulated ILCCs along with bioactive molecules cocktail (before incorporating into HFM), remarkably reduced PI staining in diabetic mice's serum group, indicating that bioactive molecules cocktail combat against inflammatory cytokines. Additionally, densitometric analysis of representative fluorescence images demonstrated significantly reduced PI intensity in bioactive molecule cocktail group as compared to diabetic serum group (Figure: 6.13).

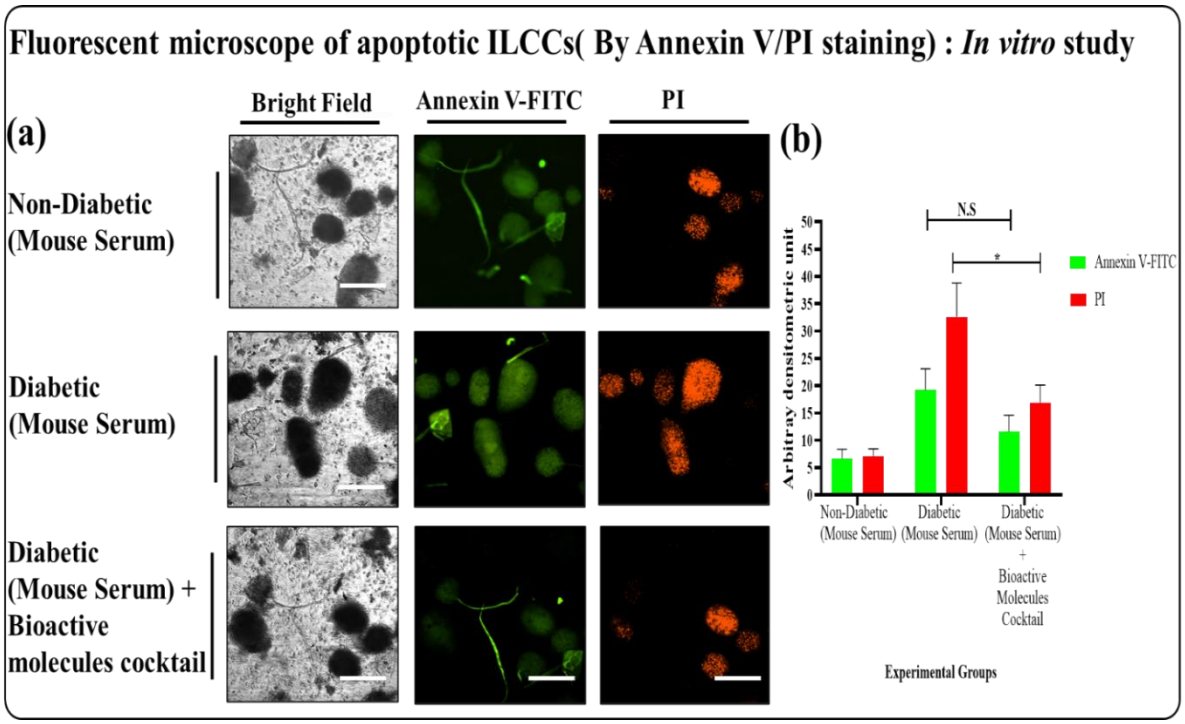

Figure 6. 13: Fluorescence microscopies of Annexin V-FITC (green) & propidium iodide (red) staining of retrieved encapsulated ILCCs (differentiated from hBMSCs) from HFM, after 48-hrs. incubation in RPMI media containing non-diabetic mouse serum & diabetic mouse serum. In control, rat islets encapsulated in HFM were placed in non-diabetic (normal) serum while in the

other two groups (Diabetic & Bioactive molecule cocktail), islets encapsulated in HFM were placed in diabetic serum. Scale bar- 100µm (magnification 20 X); (b) Densitometric analysis of Annexin V /PI of fluorescence microscopic images. Annexin V-FITC staining of ILCCs incubated with diabetic serum vs ILCCs incubated with diabetic serum along with BMC shows the non-significant change. * P≤ 0.05 PI staining of rat islets incubated with diabetic serum vs rat islets incubated with diabetic serum along with BMC show significant change. The graphs are plotted with mean ± SEM (N=3).

However, there was no significant difference in Annexin V staining intensity between the BMC group and diabetic serum group. To our knowledge, this was the first study in which hBMSCs derived ILCCs and bioactive molecules cocktail has been used in islet encapsulation along with HFM scaffold for effective islet transplantation.

6.4.2. Encapsulated rat islet viability: *In vitro* study

Next, this study aimed to assess the long-term biocompatibility of alginate encapsulated ILCCs (hBMSCs derived ILCCs) after transplantation in the STZ diabetic mouse model. Hence, we need to culture a massive number of hBMSCs for islet differentiation and to generate a bulk amount of ILCCs to conduct xenotransplantation study. But, due to scarcity of differentiated ILCCs, we modified our further experimental strategy from shifting hBMSCs derived ILCCs to rat islets as proof of concept in xenotransplantation.

Rat insulin has considerable sequence homology with human insulin, thus can be a source of xenotransplantation study. Rat islets from young rat pancreas were successfully isolated & cultured overnight before encapsulating them in HFM. Isolated rat islets purity was assessed by DTZ staining. Rat islets had taken up DTZ stain indicating insulin-positive islets. The rat islets demonstrated different sizes ranging from 50 to 200 µM (Figure: 6.14).

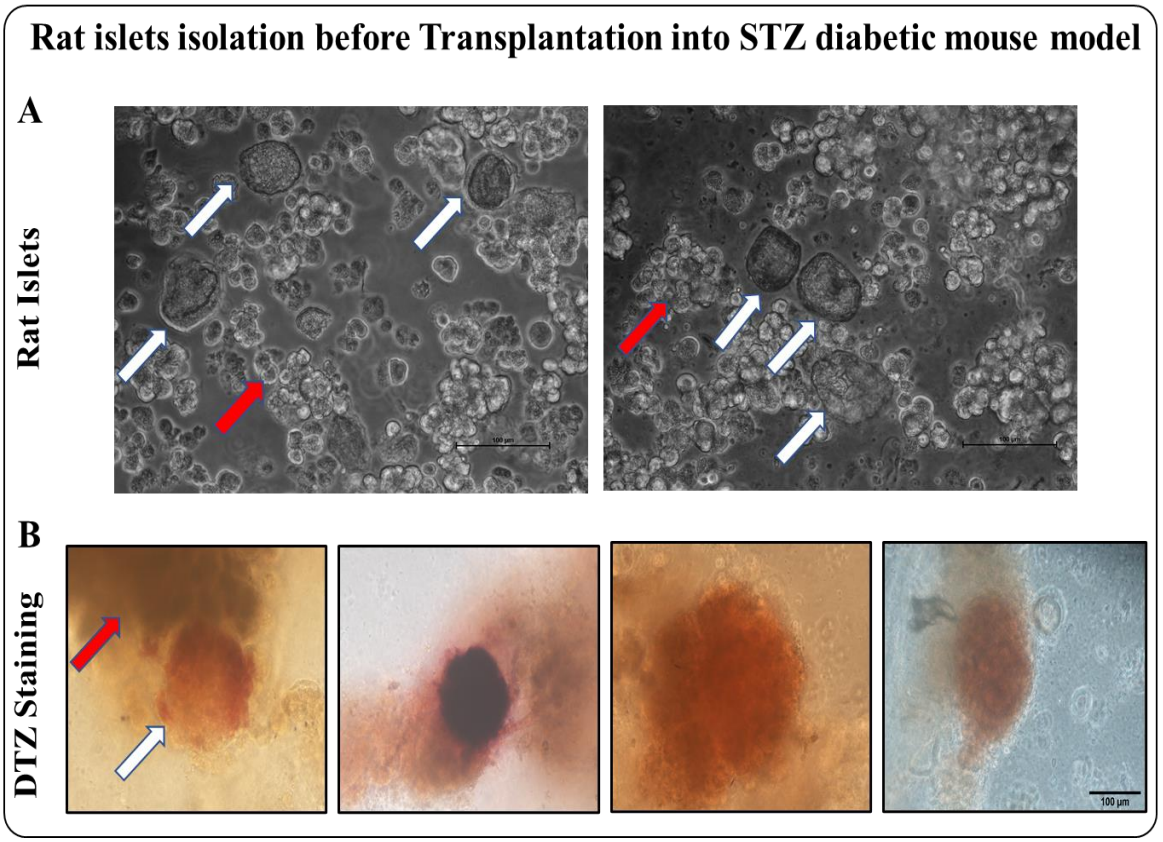

Figure 6. 14: Freshly isolated and overnight culture rat pancreatic islets (A) Phase-contrast microscopy shows free-floating rat pancreatic islets along with acinar cells. Scale bar: 100 µM; Magnification 10 X (B) Phase contrast microscopy shows positive DTZ staining in pancreatic islets and negative DTZ staining in pancreatic acinar cells and ductal tissue. "White" arrow marks indicating islets and "Red" arrow marks indicating acinar cells. Scale bar-100µM; Magnification 20X (N=3).

Before beginning *in vivo* xenotransplantation experiment, we conducted an *in vitro* viability experiment of HFM containing encapsulated rat islet as performed earlier with differentiated ILCC with Annexin V/PI staining in the presence and absence of diabetic serum, with and without BMC. Our results showed that encapsulated rat islets along with bioactive compounds group provided very less red fluorescence (an indicator of dead cells) compared to a diabetic group, suggesting islets are viable & bioactive cocktail prevented necrosis due to their islets protective & antioxidant properties. Apoptosis (green fluorescence) was observed but it was mainly due to apoptosis of acinar cells, while islets (marked by arrow) showed very slight fluorescence when compared to diabetic control (Figure: 6.15). Additionally, the densitometric analysis showed that significant decreased annexin V and PI staining intensity in the BMC

group as compare to diabetic serum group, suggesting that bioactive molecules play crucial protective role in the encapsulation process (Figure: 6.15).

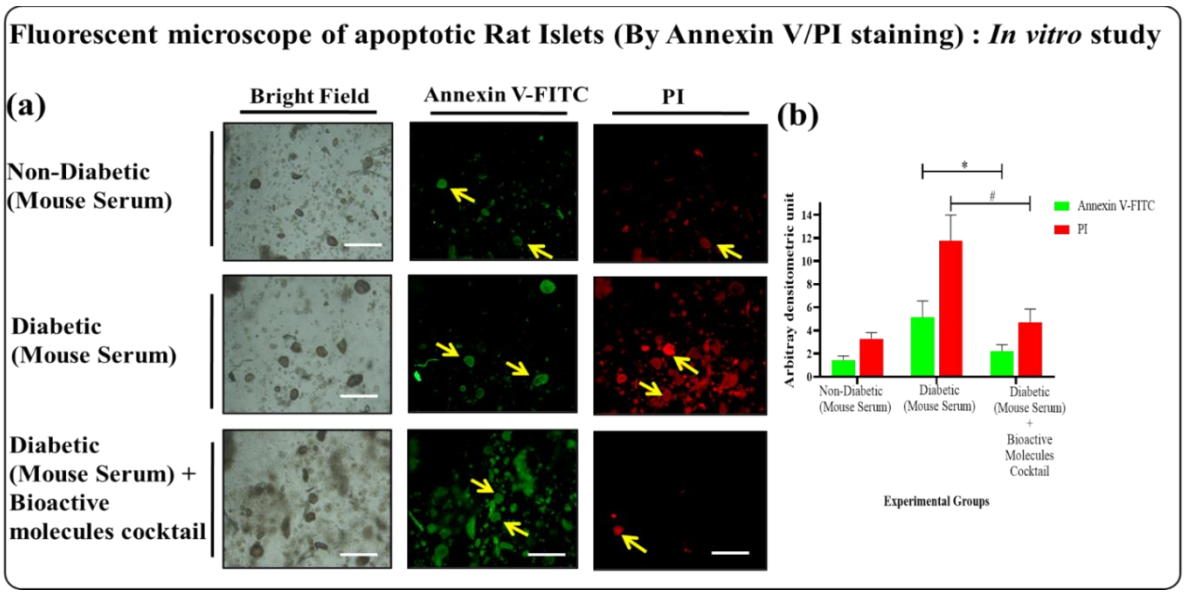

Figure 6. 15: Fluorescence microscopies of FITC-Annexin V (green) & propidium iodide (red) staining of retrieved encapsulated adult rat islets from HFM, after 48-hour incubation in RPMI media containing non-diabetic mouse serum & diabetic mouse serum.(a) In control, rat islets encapsulated in HFM were placed in non-diabetic (normal) serum while in the other two groups (Diabetic & BMC), islets encapsulated in HFM were placed in diabetic serum. Yellow arrow mark denoted rat islets. Scale bar- 500µm (magnification 10 X); (b) Densitometric analysis of Annexin V /PI of fluorescence microscopic images. * P≤ 0.05 Annexin V-FITC staining of rat islets incubated with diabetic serum vs rat islets incubated with diabetic serum along with bioactive molecules cocktail. # P≤ 0.05 PI staining of rat islets incubated with diabetic serum vs rat islets incubated with diabetic serum along with bioactive molecules cocktail. The graphs are plotted with mean ± SEM(N=3).

6.4.3. Encapsulated Rat Islets transplantation (HFM) in STZ treated diabetic mice (Xenotransplantation- *in-vivo* study)

Charles foster Rat islets (young) were chosen as a source of xeno-transplantation material in the STZ induced diabetic Balb/c mice model. Approximately 1000 handpick rat islets encapsulated in calcium-alginate were packed into biocompatible 3 HFMs (Each HFM length:3 cm) & transplanted intra-peritoneally in STZ induced diabetic mice after confirming high FBS.

Bodyweight & fasting blood sugar was initially monitored after every 48 hours (short interval) from 4th day post transplantation and subsequently, with regular large intervals (Post-

transplantation day: 20, 30, 40, 60, 80, 100). The bodyweight of transplanted animals became stable & didn't reduce further as compared with diabetic control (Figure: 6.16). STZ treated diabetic control group showed constant loss in body weight and eventually died by 30 days of post-transplantation. While HFM (encapsulated rat islets) transplanted group demonstrated not much change in body weight throughout the post-transplantation period, suggesting reversal of diabetes in the transplanted group. At the same time, the control group (non-diabetic) showed no change in body weight during pre- & post-transplantation period (Figure: 6.16).

Simultaneously, we monitored an FBS level in all three groups during the pre- and post-transplantation period. HFM transplanted mice group has significantly lower blood glucose levels than the diabetic control mice group over a period of 20 days. In the initial stage of HFM transplanted mice showed a moderate reduction of FBS (Delayed graft function) as compared to a diabetic control group, suggesting slow acclimatization of "HFM graft" in diabetic environmental conditions.

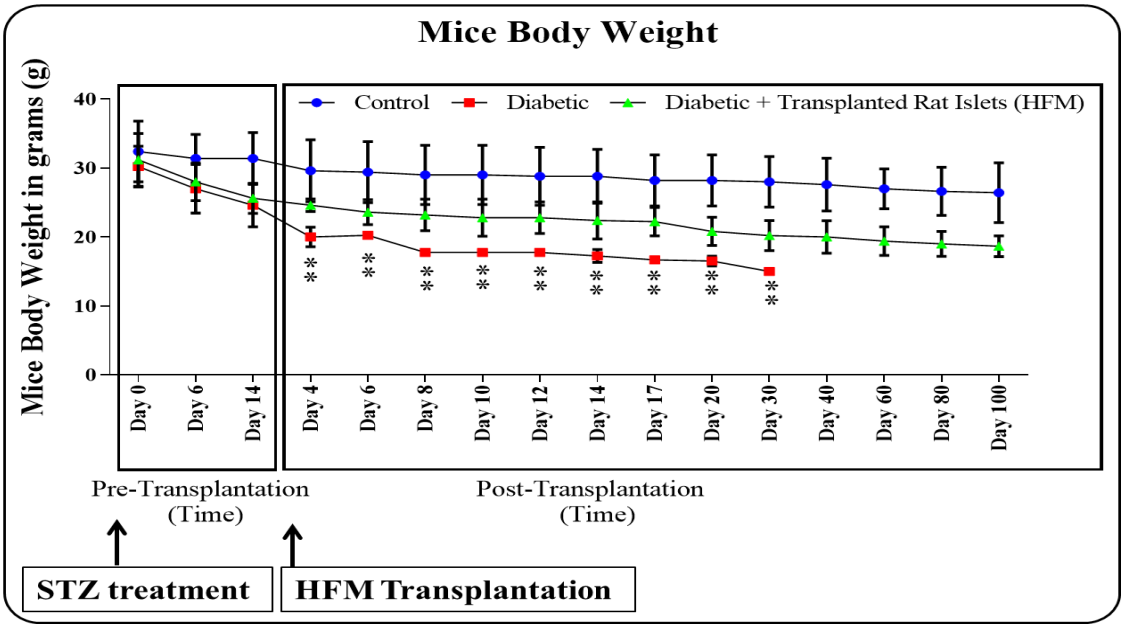

Figure 6. 16: Graph of change body weight (in gram) of pre-and post-transplantation duration in non- diabetic control (blue), Diabetic control (red), diabetic mice transplanted with encapsulated rat islets-HFM (green). Bodyweight decreased in diabetic mice group as compare to transplanted rat islets (HFM) mice group in the post-transplantation period. ** P ≤ 0.01 [Diabetic control group Vs Transplanted rat islets (HFM) group]. Graphs data are shown as mean ± SEM (N=5-6).

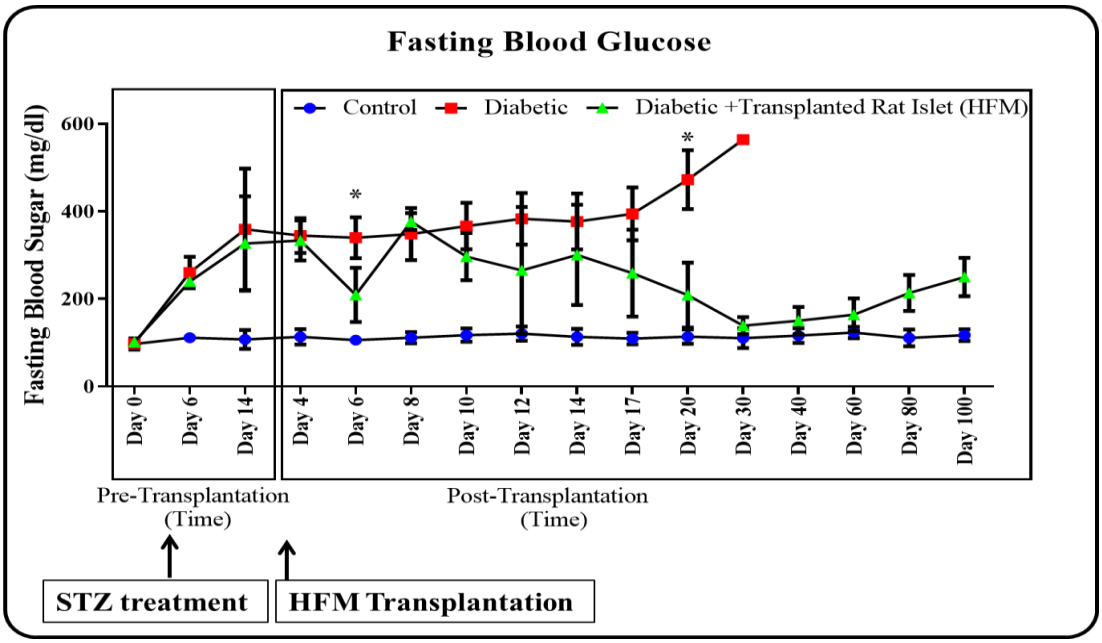

Figure 6. 17: Reversal of experimental diabetes in the STZ-treated Balb/C mice model. Fasting Blood Sugar (FBS) levels (in mg/dl) in non- diabetic control mice (blue), Diabetic control mice (red) and diabetic mice transplanted with encapsulated rat islets into HFM (green) in intraperitoneal cavity area. Hyperglycemia was effectively ameliorated in STZ induced diabetic mice transplanted with encapsulated rat islets post 20 days of transplantation. The collection of the tail vein blood and monitoring of fasting blood glucose levels with a glucometer was performed. *P ≤ 0.05 [diabetic control group Vs transplanted islets (HFM) group]. Graphs data are shown as mean ± SEM(N=5-6).

Interestingly, HFM transplanted mice maintained a blood glucose level of 100-220 mg/dl during post transplanted period (blood sugar above 350 before transplantation), indicating that encapsulated rat islets show long-lasting functionality and HFM having excellent immune-isolation as well as bio-compatibility properties (Figure No: 6.17). Optimal alginate encapsulation in combination with HFM significantly prolonged young rat islet survival into diabetic mice for up to 8-10 weeks, even in peritoneal cavity transplantation site and were able to restore normoglycemia in transplanted mice group without immunosuppression for up to 8-10 weeks *in vivo*.

6.4.4. Retrival of Xenotransplantated Rat islets from HFM

As a pancreas belongs to endocrine organ categories, pancreatic islets contain extensive blood capillary networks. Thus, the present study investigated whether there was adequate blood capillary formation covering HFM. After the termination of experiments, HFM was retrieved

from transplanted mice and the post-retrieved study was conducted. First, we observed that HFM remains intact post -retrieval phase in the peritoneal cavity of transplanted mice and it was covered by an extensive blood vessel, suggesting that HFM outer membrane promotes vascularization [(Figure No:6.18(A;a)]. Additionally,these HFM stayed stable for as long as 3 months in the peritoneal cavity of transplanted mice, with no detectable encapsulated rat islet leaching.To investigate the status of encapsulated rat islets packed in HFM, we took a cross-section of HFM and observed intact encapsulated rat islet inside HFM by phase-contrast microscope. [(Figure: 6.18(B;b)]. And further, we want to study the cellular architecture of the inner and outer sides of the transplanted HFM by H & E staining in the cross-section of HFM, we spotted the number of rat islets with normal morphology along with other cellular debris [Figure no:6.18(C,c,D)].

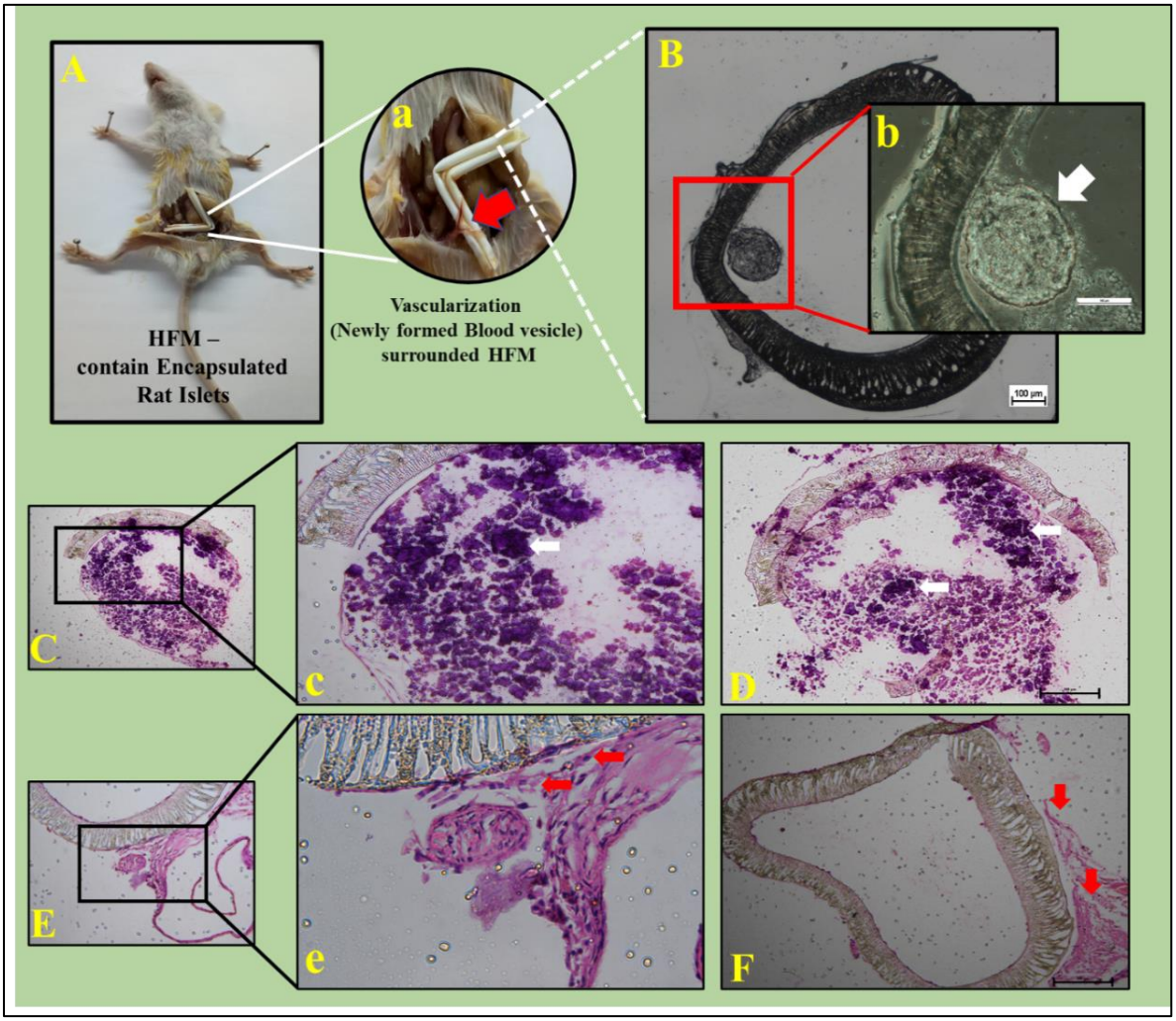

Figure 6. 18: Retrieval of HFM from transplanted diabetic mice model (A) Encapsulated rat islets packed in these HFM remained viable after 3 months. (a) magnified digital photo of the HFM in the i.p. space of a mouse during the retrieval. Newly formed blood vessel (Highly vascularization

indicated by "Red" arrow) surrounding HFM (B) Representative phase-contrast microscopic image of a cross-section of HFM integrated encapsulated rat islets after graft removed from a transplanted diabetic mouse at end of the experiment; scale bar: 100 µM (Magnification 4 X). (b) A magnified image of (B) demonstrates encapsulated rat islets (Indicated with "white arrow"). (C) Representative histological H & E staining images of a cross-section of retrieved encapsulated rat islets from HFM. (c, D) Magnified view of H& E staining displays rat islets ("White" arrow indicates rat islets) and other cellular debris like acinar cells. (E) Representative histological H & E staining images of a cross-section of retrieved encapsulated rat islets from HFM, surrounded by endothelial cells, blood vessels, other supporting cells. (e, F) Magnified view of H & E staining demonstrates vascularization growth outside of HFM (indicated by "Red" arrow) Scale bar: 100 µM; Magnification 10X.

Moreover, histological studies showed no cellular overgrowth inside the HFM, indicating the encapsulation process does not promote cellular growth, even in *in vivo* conditions. Additionally, histological studies reveled increased vascularization around the HFM suggesting, specific plasticity of the endothelial cell population surrounding HFM graft [(Figure no: 6.18 (E,e,F)]. No immune cell infiltration was observed in H & E staining implying, no cellular penetration through HFM. Thus, the present study reveals that xeno-geneic pancreatic islets remain live in HFM with enhanced biocompatibility without immunosuppression in the rodent model (pre-clinical study), bringing us one step closer to applying this therapy in humans.

6.5. Discussion

Over the last two-three decades, many different types of hydrogel and synthetic-biopolymers have been proposed for immune-isolation and encapsulation of pancreatic islets. Among these, alginate is one of the most favourable natural biopolymers, as established by pre-clinical and clinical trials (Orive *et al.* 2003). Numerous capsule/transplanted devices had been screened to test biocompatibility for islet transplantation. Despite their hopeful potential for islet transplantation, it is still facing several difficulties to obtain successful islet transplantation. One of the important obstacles is a lack of islet viability inside capsules/ devices. To overcome this limitation, we explored a new venture for islet encapsulation through *in vitro* along with *in vivo* pancreatic islet encapsulation study. The rationale for conducting *in vitro* islet encapsulation study followed two approaches: (1) The study evaluated the effect of direct exposure of bioactive molecules during the islet encapsulation process. (2) The response and behavior of encapsulated islets *in vitro* to diabetic serum for mimicking *in vivo* diabetic

condition. Although *in vivo* diabetic conditions are multifactorial involving various organs, our approach of using diabetic serum would provide the first line information regarding the performance of encapsulated islets along with bioactive. However, it is highly challenging to mimic *in vitro* diabetic situations because *in vivo* diabetic conditions are complex and multi-organ involvement.

In the present investigation, we have demonstrated that exposure of encapsulated islets *in vitro* to the diabetic serum for 48 hours, affected their viability. This loss of viability was contributed to the presence of a higher concentration of various stressful conditions such as glucose, cytokines and oxidative stress- thus resembling the *in vivo* diabetic profile. Several studies have suggested cytokines, fatty acid, nitric oxide, etc., involved in oxidative stress formation eventually leading to apoptotic death of pancreatic β-cells (Rabinovitch 1998, Tejedo *et al.* 1999, Shapiro *et al.* 2000). Some studies have also demonstrated a reduction in islets viability & function, to be influenced by transcriptional changes due to diabetic serum/high glucose concentration (Eizirik *et al.* 1992, Jackson *et al.* 2012). The contribution of coagulation factors in these experiments can be excluded due to the use of serum rather than plasma. Thus, other soluble ingredients present in diabetic serum/non-diabetic serum gaining access to islets even after encapsulation led to a reduction in encapsulated rat islet viability. Additionally, in our experiment, we employed mice serum without heat-inactivation for considering complement activation components for generating an appropriate xenograft *in vitro* model system (Soltis *et al.* 1979). Isabel Maestre *et. al.*,2003 demonstrated a spontaneous increase in superoxide production over 36 hours with remarkably reduced mitochondrial function leading to oxidative stress in pancreatic islets even in absence of external stressors such as serum. The same group also indicated that elevated concentration of glucose and fatty acid also accelerated the rate of apoptosis in the pancreatic β cell line model (INS-1) (Maestre *et al.* 2003).

Thus, having established that pancreatic β cells are highly susceptible to oxidative stress & islets viability being one of the major challenges faced after encapsulation, along with the challenge of insufficient nutrient & blood supply (Scharp and Marchetti 2014). Thus, in the present study, to enhance the islets viability of encapsulated islets (Rat islet/ILCCs), three bioactive molecules were selected based on their antioxidant and cell survival properties (Oh 2015). Exposure of rat islets encapsulated in HFM along with swertisin, curcumin & genistein (bioactive molecules) to diabetic mice serum, showed less PI staining (a marker for dead cells/necrosis), indicating that, bioactive compounds prevented necrosis of islets & enhanced

cell viability. Several studies have demonstrated that curcumin could prevent cytokine-induced islet death *in vitro* and *in vivo* as well as improve islet recovery after cryopreservation. (Kanitkar and Bhonde 2008, Kanitkar *et al.* 2008). Several experiments also proved dexamethasone along with curcumin as more efficiently anti-inflammatory agents, which was incorporated in combination with alginate encapsulation techniques to mitigate the fibrotic response during pancreatic rat transplantation (Fritschy *et al.* 1994, Chun *et al.* 2003, Dang *et al.* 2013). Additionally, few researchers established a detailed study on the diffusion influence of the curcumin release kinetics from the non-degradable calcium-alginate encapsulation. They concluded that curcumin persisted in the calcium-alginate encapsulation for a longer period of time due to slower release of curcumin because of poor solubility of curcumin and hence prolonged its potency in mitigating the apoptosis response (Kurien *et al.* 2007). Recently, Sabri Sudirman *et. al.*, 2019 reveled that chitosan encapsulated curcumin significantly improved encapsulation protocol (Sudirman *et al.* 2019). The previous report from our lab demonstrated that swertisin is an insulin secretagogue and has anti-apoptotic and islet protective properties against oxidative stress (Srivastava *et al.* 2016). Oh *et. al.*, 2015 have reported that genistein increases insulin secretion & islets protective action against apoptosis (Oh 2015). The bioactive molecule's precise mode of action involved in rescuing, apart from the above-mentioned properties may be multifactorial. Thus, we demonstrated that elevated glucose markedly enhances ROS production and eventually apoptosis in encapsulated pancreatic β-cells stressed by incubation in the presence of diabetic mice serum and is much less toxic in presence of bioactive molecules.

Interestingly, our results demonstrated that encapsulated rat islets showed lesser apoptotic and necrotic status as compared to stem cell-derived differentiated ILCCs in presence of diabetic serum condition, suggesting that rat islets are relatively resistant towards elevated glucose condition. This result was in accordance with several research groups. They illustrated that the susceptibility of pancreatic islets to dysfunction and apoptosis is dependent on species as well as genetic background and experimentally proved that human islets in *in vitro* culture were highly vulnerable to dysfunction and apoptosis when exposed to high glucose as compared to rodent islets (Federici *et al.* 2001, Maedler *et al.* 2001, Donath *et al.* 2005). The present data indicated that the islet encapsulation by calcium alginate along with bioactive molecules cocktail in HFM reinforces the islet viability, thus generating a novel strategy for diabetes therapy. After the accomplishment of our *in vitro* islet encapsulation study, in *vivo* islet

transplantation study has been performed. Nowadays, there is a lot of pancreatic islet encapsulation associated research to get rid of the utilization of immuno-suppressant in patients with type-I diabetes. This investigation is generally uncoordinated due to complex and complicated experimentation and lack of well documented organized study of the divrse encapsulation methods. The correlation between animal model (including non-human primates) and human subject in the biocompatibility of device and function of the encapsulated islet transplantation is poorly demonstrated. The success rate of several pre-clinical and human clinical trials implemented by various scientific institutes/university and biopharmaceutical companies using encapsulated pancreatic islet transplantation has not been very promising, leaving still room for improvement (Calafiore *et al.* 2006, Tuch *et al.* 2009, Jacobs-Tulleneers-Thevissen *et al.* 2013, Dolgin 2016, Bochenek *et al.* 2018). However, an attempt to allow xeno-islet transplantation to survive the harsh xeno-immune response has shown some success in the field of transplantation. In the xenografting experiment, we exploited the possibility of encapsulated rat islet longevity in a diabetic mouse model using the hollow fiber membrane (HFM) as an immune isolation device. "Rat to Mouse" xeno-islet-transplantation evokes a xeno-immune response which is not comparable to that of other existing xenograft models, like "Pig to Human" xenotransplantation (Pan *et al.* 2007, Chen *et al.* 2010, Muller *et al.* 2010, Niclauss *et al.* 2011).

In the current study, we evaluated clinically relevant approaches for transplantation in combination with encapsulation, to find a synergic outcome that may translate pre-clinical to clinical studies. Thus, we attempted to solve these problems using HFM as an immune-isolating device and conducted a systematic study of encapsulated rat islets transplantation into the STZ diabetic mouse model. Jayesh Bellare *et. al.,* and its groups synthesized, fabricated and optimized HFM for hemodialysis application (Dahe *et al.* 2011, Verma *et al.* 2018) and performed *in vivo* biocompatibility and graft acceptance study in rats (Dahe *et al.* 2011). Subsequently, they explored HFM as an immune-isolation device for islet transplantation study and improved polysulfone (PSF/TPGS) composite HFM, in such a way that it showed maximum permeability and minimum adsorption of insulin by HFM. Additionally, they successfully achieved normoglycemia in STZ diabetic mice when transplanted with HFM encapsulated porcine or differentiated islets from human umbilical cord Wharton's jelly (Teotia *et al.* 2017). Other researchers used "Theracyte" as an immune isolation device which although ameliorates hyperglycemia (Sasikala *et al.* 2013, Boettler *et al.* 2016), but it cause central

necrosis and allows antibodies to pass through it. In the present study, we utilized an above-mentioned HFM for islet transplantation study, to improve xeno-islet transplantation strategy. Severe hypoxia caused by a lack of vascular supply and an inability to retrieve encapsulated islets transplanted inside the peritoneal cavity for biopsy and subsequent evaluation are obstacles to pre-clinical and clinical study of various encapsulation strategies for pancreatic islet transplantation. In the present study, we didn't observe any negative effect of HFM containing encapsulated rat islet when transplanted in the intra-peritoneal cavity. Previously, few scientists reported that severe hypoxia caused by a lack of blood supply in post retrieval evaluation of encapsulated islets, when transplanted in the peritoneal cavity (McQuilling *et al.* 2011, Bartholomeus *et al.* 2013). However, in our study, we found plentiful vascularization developed in the peritoneal cavity, indicating acceptance of HFM by the diabetic mouse.

It is estimated that near about 5,00,000 IEQ may be required to cure a one human type-1 diabetic patient (Robertson *et al.* 2001). To deliver such a demanding number of islets, the transplantation device storage capacity is critical. Due to the narrow thin cylindrical geometry, HFM can be scaled up in the longitudinal direction to a larger space and can still be easily retrieved through minimally invasive surgical procedures. To prove this concept, we assembled 3 cm (centimeter) long three HFM devices for storing approximately 1000 encapsulated rat islets in peritoneal cavity per one diabetic mouse model. Based on scientific report, the certain number of rat pancreatic islets that are transplanted to STZ induced diabetic mouse model to achieve normoglycemia most commonly ranges between 250 to 1000 (Kahraman *et al.* 2011). We noticed that transplantation of 1000 rat islets successfully achieved normoglycemia and the graft was stable up to 3 months. Another important limitation is cellular and collagen deposition on the outer surface of transplanted devices which cause delayed insulin diffusion in response to blood glucose changes decreased nutrient transport and eventually reduced graft survival and function (Gotfredsen *et al.* 1990, de Vos *et al.* 2002). It is interesting to note that our rat encapsulation with HFM devices protocol for "Rat to Mouse" xenotransplantation study, we didn't recognize any fibrotic or pericapsular overgrowth and long-term survival of the encapsulated graft. Unfortunately, there is a considerable irregularity in results of biocompatibility testing *in vivo* pre-clinical models with various encapsulation devices and experimental approaches. However, we found consistent results with previously published HFM and experimental approaches with the different animal model systems (Teotia *et al.* 2017).

Thus, our study on HFM containing rat islets encapsulated with bio-actives efficiently reduced blood glucose levels in a xeno-transplantation model as a proof of concept for effective islet transplantation therapy for treating diabetes and avoiding the immunosuppression drugs that are normally needed to protect transplanted islets from rejection.

6.6. Summary of chapter-6

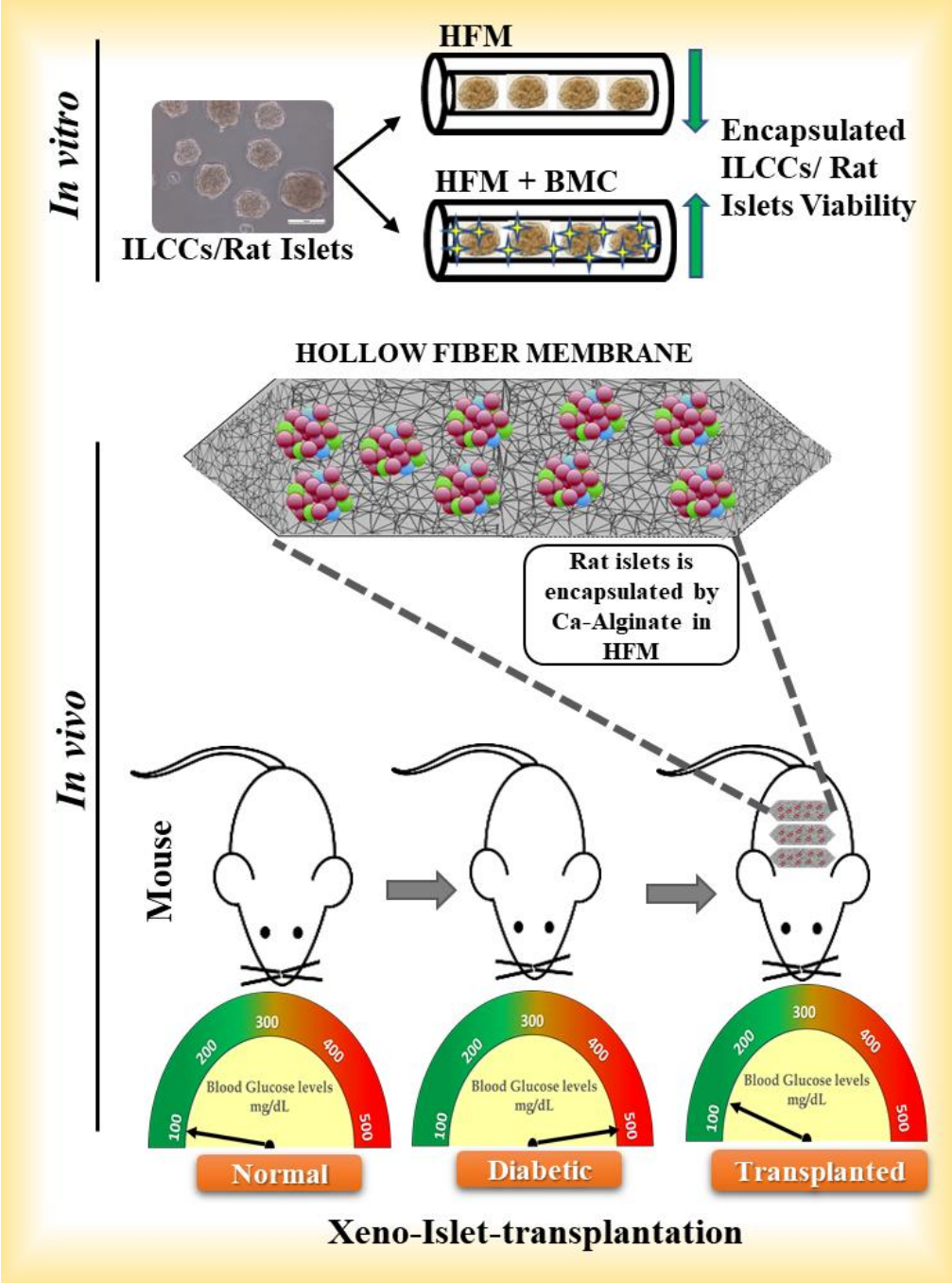

Figure 6. 19: Summary of chapter-6 (*in vitro* and *in vivo* study)