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Short Paper

Isolated bovine pancreatic islets as an alternate *in vitro* model for diabetes research

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***Background & objectives*: Isolation of functional pancreatic islets for diabetes research and clinical islet transplantation stands as a big challenge despite the advancements in the field. In this context, the non-availability of human/animal tissues is one of the major impediments to islet-based research, which has tremendous scope for translation. The current study explores the feasibility of using the bovine pancreas as an alternative source to isolate pancreatic islets and assess its functionality for *in vitro* studies.**

***Methods*: The bovine pancreas was collected from a registered slaughterhouse and transported in an ice-cold medium – Hank's Balanced Salt Solution (HBSS) to the laboratory. Islets were isolated by sequential collagenase digestion followed by a two-step filtration and purification by density gradient separation method. After isolation, islets were identified with dithizone staining and the islet function was assayed *in vitro* for assessing the dynamic insulin secretory function by monitoring the glucose-stimulated insulin secretion (GSIS), in response to low and high glucose. Staining techniques were also used to understand the cytoarchitecture of the bovine pancreas.**

***Results*: The islet yield was 157±23 islets per gram of pancreas and was viable. The cold ischaemia time was reduced to 60-75 min. The islets released insulin with glucose stimulation. The insulin release was observed more with high glucose (28 mM) than with low glucose (2.8 mM). Dithizone staining confirmed the presence of islets after isolation and the size of islets ranged from 50 to 600 µm size. The mantled islets (islets with acinar tissue) were also noted with the pure islets in culture. Hematoxylin and eosin (H&E) and aldehyde- fuchsin showed islets interspersed in the acinar tissue of the bovine pancreas. Special stain defined the islets better than regular staining. Fluorescent and diaminobenzidine (DAB) staining with insulin, glucagon and somatostatin revealed the arrangement of the cells in each islet. The beta cells were majorly found in the islet core with alpha cells interspersed with the delta cells in the periphery.**

***Interpretation & conclusions*: The isolation procedure described in this study yielded viable islets for *in vitro* studies which showed a differential response to glucose challenge, confirming their viability. We provide a simple and reproducible method for small-scale isolation of functional islets from the bovine pancreas. This model proffers the beginner a hands-on in islet experiments and helps to re-iterate the process that could be extrapolated to other pancreatic tissues as well as to expand on diabetes research.**

Key words Bovine pancreas - collagenase - insulin - islets - transplantation

Diabetes mellitus (DM), a global health problem, with millions affected by this chronic and potentially life-threatening disease[1]. Type 1 DM is caused by the auto-immune destruction of the pancreatic beta cells[2] and type 2 is due to insulin resistance and decreased beta cell secretory function[3]. The discovery of insulin and the commercialization of its production changed diabetes management[4]. The advancements in therapeutic options, technology and clinical research continued to improve the management of diabetes[5]. Research models (*in vitro* as well as *in vivo*) are essential to understand islet biology and the impact of the microenvironment contributing to beta cell dysfunction and also to explore the strategies for improving beta cell function[6-9]. The conventional *in vitro* method is a monolayer culture rather than a 3D model (islets are cell clusters)[10,11]. Various methods have now been developed to design *in vitro* 3D islet models to mimic the *in vivo* pancreatic islets[12,8]. The reaggregated *in vitro* islet model is an advancement in the field[13]. These studies highlight that the islets have to be studied as an organ by itself rather than as individual cells. The *in vitro* islet models are a great tool to understand islet behaviours under physiological as well as pathological conditions which could be used for biomedical applications including disease modelling, drug screening and islet transplantation[9,14].

The major constraints for doing animal/human tissue-based research are the non-availability of tissue and the ethical issues involved. The facilities to house the experimental animals (rodents/large animals) under optimal conditions are lacking in many institutions within the country. The possible sources for the human pancreas are small tissue bits (<1 g) from surgical specimens (tumour resection surgeries/distal pancreatectomy procedures done for chronic pancreatitis)[15]. However, these specimens are formalin-fixed for histopathology, from which islet isolation is not possible. The best option would be a whole pancreas from a brain-dead donor. The islets could be harvested in larger numbers for *in vitro* and *in vivo* transplantation studies among others[16,17]. However, it is an expensive, resource-intensive task in addition to ethical constraints which limit its applicability. There are several publications on the isolation of islets from animal pancreas, including bovids, pigs, canines, rodents and monkeys[18-23]. Most of these explain the large-scale isolation of islets using the whole organ, which is resource-intensive. The basic steps include preparation of the organ, digestion, purification of islets and culture/transplantation. There are innumerable descriptions of the modification of each step involved in the entire process customized to research laboratories to achieve the same objective[24-26]. Our objective was to develop cost-effective resource-stratified protocols to standardize a doable islet model for research. Therefore, in the current study, we decided to use the bovine pancreas, an easily available tissue from a local slaughterhouse.

Material & Methods

This study was undertaken at the department of Physiology, Christian Medical College, Vellore, Tamil Nadu between April 2019 and April 2022. The study was approved by the Institutional Review Board. Figure 1 depicts the study design.

Isolation of bovine pancreatic islets:

Collection and preparation of pancreas: Bovine pancreas tissue (*Bos taurus*, cow, adult) was procured from a registered abattoir and transported in ice-cold, oxygenated HBSS (Hank's Balanced Salt solution, Sigma). The distal part of the pancreas was identified and processing was done immediately after removing the fat and the extraneous tissue. About 3-5 g of tissue were dissected and transferred to a glass bowl, and washed multiple times with ice-cold HBSS. Tissue was chopped into small pieces to hasten the digestion process.

Enzymatic digestion of the pancreas: The fresh collagenase type XI (2.5 mg/g, Sigma) in HBSS (1 ml/g) was prepared, filtered with a 0.22 µm syringe filter (Minisart) and stored at 4°C. The tissue with the enzyme solution was transferred to a Petri dish and kept in a CO_2 incubator for 30–40 min at 37°C to allow static digestion. Then, the contents were transferred to a 50 ml tube placed in a shaking water bath (37°C; 120 rpm) for five minutes to continue the enzymatic plus mechanical digestion. The contents of the tube were mixed well by gentle shaking in between to enhance the tissue dissociation. A few syringe pushes were done to disperse the tissue. The final undigested tissue was less than 10 per cent of the total tissue used.

Arresting of enzymatic digestion: The digestion was arrested by adding thrice the amount of ice-cold HBSS, quickly filtered through 500 µ SS mesh and a 40 µ cell strainer. The digested tissue was flushed with ice-cold HBSS through the inverted strainer placed on a wide-mouthed glass beaker or by shaking the strainer immersed in 25-30 ml of HBSS held with

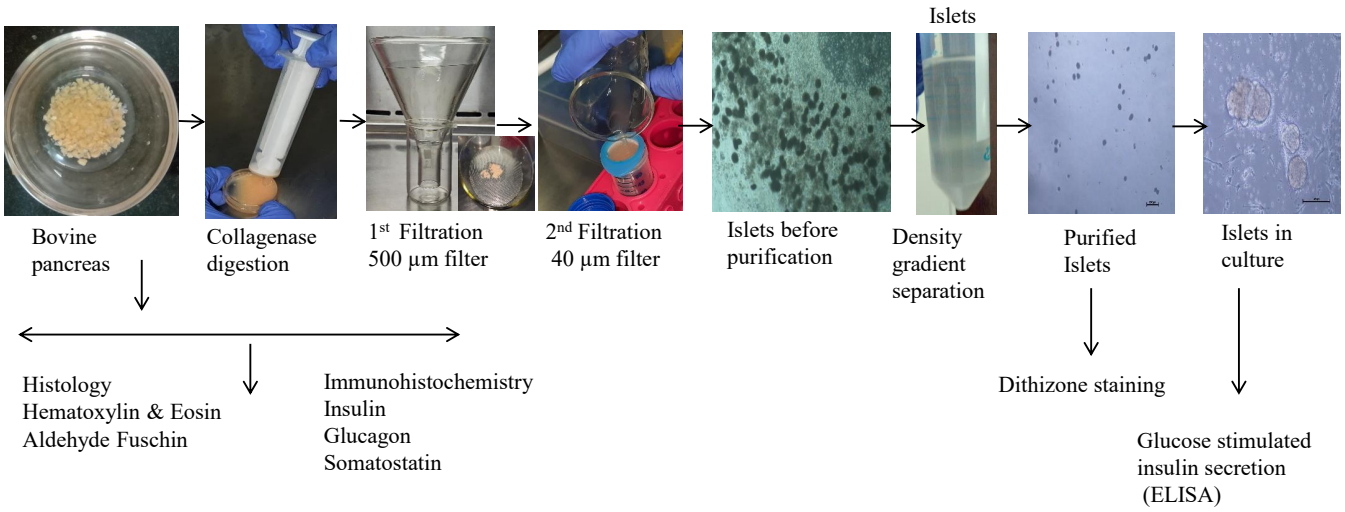

Fig. 1. Experimental design. ELISA, enzyme-linked immunosorbent assay.

forceps. The cell suspension was then decanted into a 50 ml centrifuge tube, allowed to settle for 2 min on ice and then centrifuged at 100 g for one min at 4°C temperature. Fresh cold HBSS (5 ml) was added to the supernatant collected and centrifuged again to form a pellet.

Purification of islets & culture of the isolated bovine pancreatic islets: For purification, the pellet was thoroughly mixed with 10 ml of Histopaque 1077 and carefully layered with 10 ml of HBSS containing 20 per cent foetal bovine serum (FBS) in a 50 ml centrifuge tube. This was centrifuged at 170 g for 8 min at 4°C. The islets seen at the interface of the two layers were gently collected using a Pasteur pipette and washed with HBSS containing 2 per cent FBS and centrifuged at 100 g for a minute. A 100 µl sample was taken for dithizone staining. The rest of the isolated islets were re-suspended in culture media (Thermo Fisher Scientific, USA) supplemented with 10 per cent FBS and 4 mM glutamine. Islets were cultured for up to two weeks with the culture medium replaced every two days.

Dithizone staining of islets: Isolated islets were identified and confirmed with dithizone (DTZ, Sigma) staining. The DTZ stain was prepared as per manufacturer's instructions. 10 µl of DTZ were added to 100 µl cell suspension and incubated for 10-15 min in the dark to visualize the islets. Islet count and size distribution were assessed with the calibrated grid attached to the light microscope.

Glucose induced insulin secretion: After overnight culture, islets were first washed with PBS and then incubated in 1 ml of Kreb's Buffer containing 2.8 mM glucose or 28 mM glucose at 37°C in an incubator for 1 h. The conditioned medium was then collected, centrifuged and stored in a −20°C freezer for insulin estimation. Insulin was estimated with an ELISA kit (Mercodia, Sweden) and was expressed per islet equivalent, which is equal to a 150 µm sized islet.

Haematoxylin and eosin & Gomori's aldehyde fuchsin: Pancreatic tissue samples were fixed in 10 per cent buffered formalin for 18-24 h. Five-micrometre tissue sections were stained with H&E as per the standard protocol. Modification of the staining protocol published by Jackson's histology lab was used to stain the islets with a special stain[27].

Fluo-4 AM and PI staining for viability: Fluo-4 AM (Life Technologies), a membrane-permeant AM ester, is often used to perform live cell calcium imaging. It is readily hydrolysed by endogenous esterase to Fluo-4, which binds calcium (green fluorescence), reflecting cell viability. The cells were incubated with Fluo-4 AM (4 µl/ml cell suspension) for 30 min in an incubator, centrifuged and the supernatant was discarded. It was followed by a wash with calcium-free Krebs buffer. The islets were stimulated with 1 mM calcium and the images were taken pre- and post-calcium stimulation. Finally, propidium iodide (PI) (2 µl) was added to confirm the presence of dead cells and the images were captured with Leica CTR 6000 fluorescent microscope.

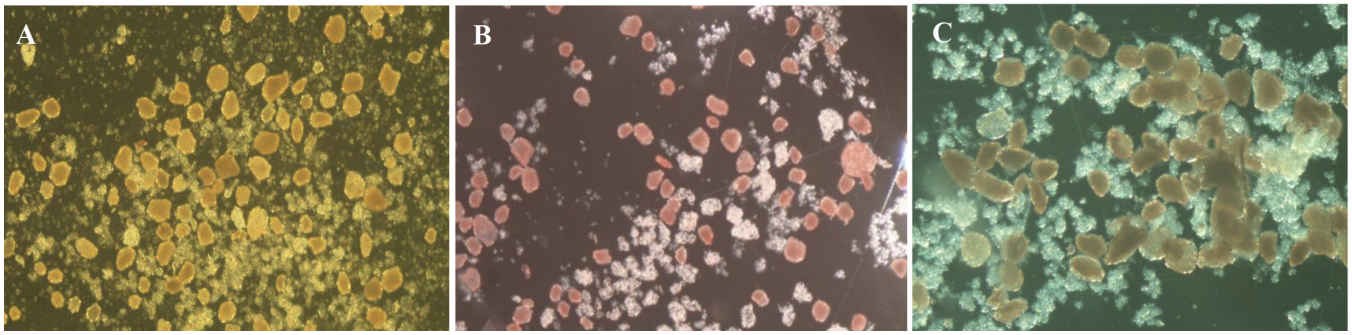

Fig. 2. Microscopic images of isolated bovine pancreatic islets (stained) with acinar tissue (unstained) using a: (**A**) Inverted microscope (4x); (**B**) Stereomicroscope (4x); (**C**) Inverted microscope with external light source (10x).

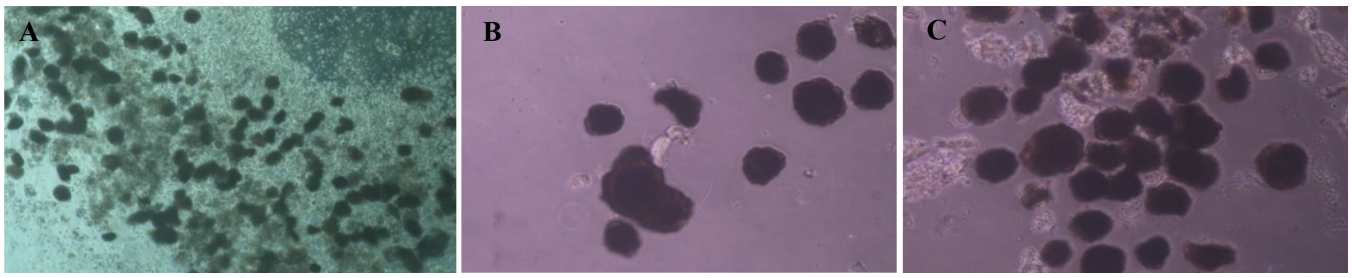

Fig. 3. Isolated islets before and after purification (**A**) before purification (4x); (**B**) before purification (10x); (**C**) after purification (10x).

Sequential double immunofluorescence & chromogen staining of islet hormones: The sections were prepared for antigen retrieval with 0.05M Tris-HCl, and following the protein block for 30 min, the tissue sections were incubated with primary antibodies, anti-glucagon antibody (PA5 88091, 1:200) and anti-somatostatin antibody (PA5 97200, 1:200) at 4°C overnight. On the next day, the slides were incubated with a secondary antibody, goat anti-rabbit IgG, Alexa Fluor® 488 (1:100). After PBST wash, the slides were treated with primary anti-insulin antibody [(K36aC10, 1:500 (Abcam)] for 4 h at room temperature (25°C) and then with secondary antibody goat anti-Mouse, Alexa Fluor® 594, (1:100) for 1 h. Counterstaining was done with DAPI (10 µg/ml) for 5 min and mounted with 90 per cent glycerol. The images were acquired with a Leica CTR 4000 fluorescent microscope. Antigen-retrieved tissue sections were processed as per the standard protocols for the DAB method. The images were taken with the light microscope, OLYMPUS BX43.

Results & Discussion

The islet isolations were attempted on the pancreas from older bovine donors (n=7). Isolation from a younger donor was performed twice, although the non-availability of donors afterwards was an impeding factor. Dithizone was taken up by the bovine pancreatic islets and the stained islets were counted to quantify and study the size distribution. The islet yield was around 157±23 islets per g (n=7 isolations) from the distal third of the pancreas, including a few mantled islets (islets surrounded by the acinar tissue). Islet size ranged between 50-600 µm and the islets of 100-200 µm size were seen more. Islets were found in crimson red colour and acinar tissue remained unstained (stereomicroscope). Stained islets were photographed under different light settings (Fig. 2). Mantled islets were separated from the pure islets by density gradient separation (Fig. 3). Mantled islets showed more tendency to attach to the culture plates, whereas completely isolated islets tend to float in the culture dish (Fig. 4). The mantled islets' viability was confirmed with Fluo-4 AM staining (Fig. 5). Isolated islets responded appropriately when treated with glucose*, i.e.,* when treated with a high concentration of glucose (28 mM), higher levels of insulin were produced by the islets. In comparison, islets treated with low glucose (2.8 mM) produced lesser amounts of insulin (Fig. 6). This indicated that the glucose induced secretory function (insulin) of the isolated islets was intact. Islet clusters interspersed within the acinar tissue were identifiable with HE and with the aldehyde fuchsin stain (Fig. 7). With IF, there was a

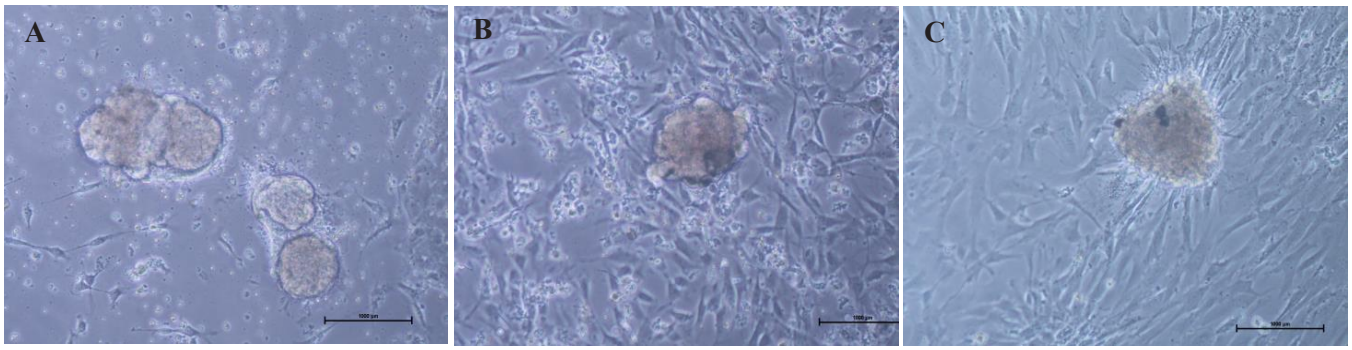

Fig. 4. Islets with acinar tissue components attached to the base of the culture plate (**A**) day 3 islets; (**B**) day 7 islets; (**C**) day 14 islets.

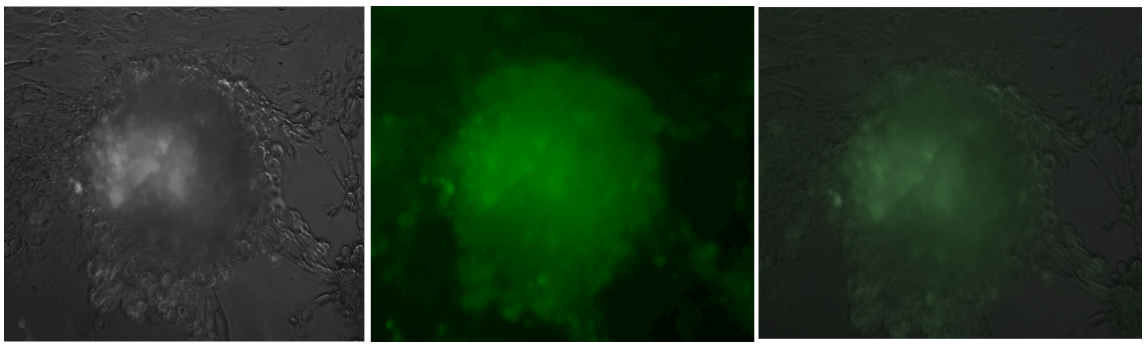

Fig. 5. Mantled islets stained with Fluo-4 AM (20x).

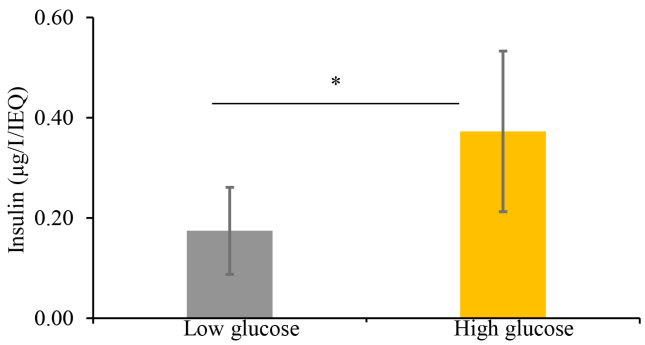

Fig. 6: Glucose stimulated insulin secretion of the isolated islets in culture. Islets were stimulated with 2.8 mM glucose (low glucose, n=15) or 28 mM glucose (high glucose, n=15) (*P* *<0.05). IEQ, islet equivalent.

positive expression of islet markers (Fig. 8). The DAB peroxidase method reconfirmed the localization of cells within islets (Fig. 9).

The development of new *in vitro* models by simple and feasible methods plays a key role in addressing the need, especially in islet research. Hering *et al*[18] used 70-100 g (12-18 months old) of bovine pancreas to isolate islets using two methods such as single-endpoint and the digestion-filtration technique with warm and cold ischaemia times ranging between 20-25 min and 30-35 min, respectively. In the former, the digested tissue was minced and dispersed using a motor-driven tissue macerator, whereas in the latter, the digestion was done by vigorous shaking with continuous recirculation for 10 min followed by which it was minced till islets appeared in the solution. Marchetti *et al*[19] used 34-40 g (12-18 months old) of bovine pancreas to isolate islets with warm and cold ischaemia with times ranging from 8 to 15 min and from 60 to 180 min, respectively. The digestion was done by intra-pancreatic duct cannulation and injection of collagenase, followed by shaking water bath incubation and filtration using a mesh of 300 and 90 µm, respectively. The purification was done with Histopaque-1077 and resuspended in CMRL 1066 medium. Coppelli *et al*[28] also isolated bovine pancreatic islets by enzymatic digestion and density gradient purification.

In this study, we carried out small-scale bovine pancreatic islet isolation (discussed elaborately under methods). It differs from the other methods reported so far. We used 3-5 g of bovine pancreatic tissue and digestion was carried out using collagenase (Type XI) followed by a two-step filtration and density gradient purification with Histopaque. This method effectively reduced the cold ischaemia time to 60-75 min and the

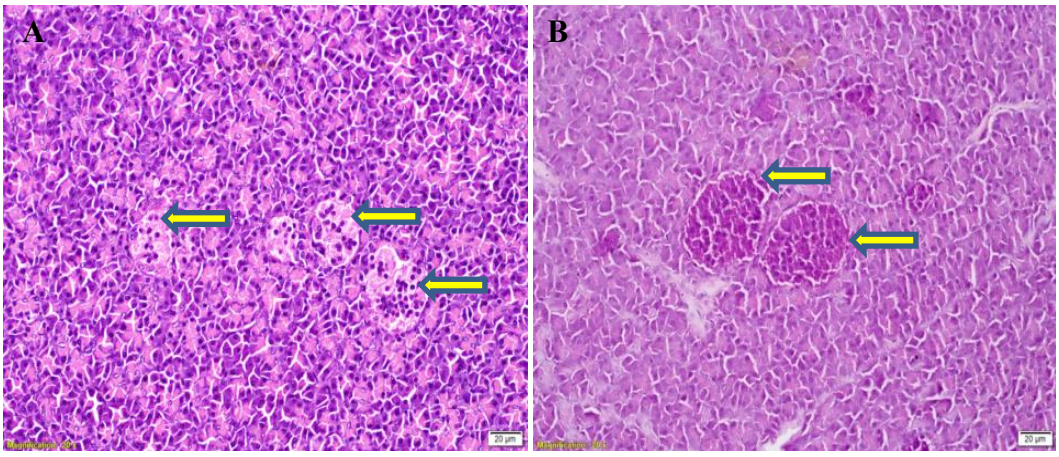

Fig. 7. Histological sections (5 µm) of bovine pancreas stained with (**A**) H&E and (**B**) Aldehyde Fuchsin stain for islets. Arrows indicate the islets. Fuchsin stain clearly distinguishes the islet mass from the acinar tissue. H&E, hematoxylin & eosin.

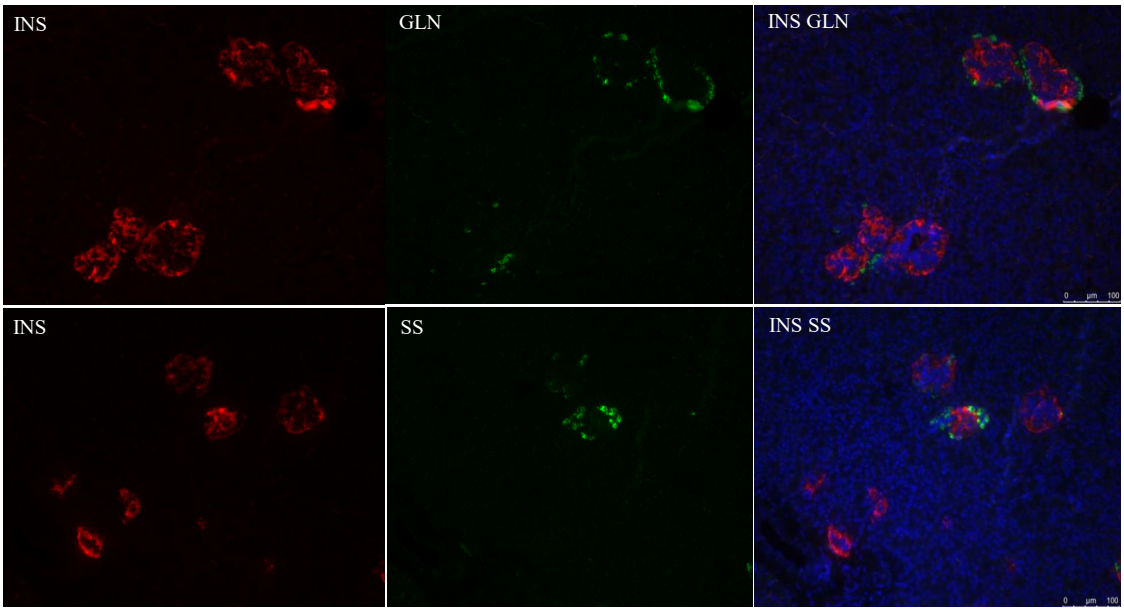

Fig. 8. Immunofluorescence images of the pancreatic section to demonstrate the expression of islet hormones. Beta cells were the predominant cells found in all islet clusters with alpha and delta cells in the periphery of the clusters (INS- insulin, GLN- glucagon, SS- somatostatin). Top panel: Insulin & glucagon; Bottom panel: Insulin & somatostatin.

wastage. The instruments used for the process were autoclavable and reusable. There is a need for a well-equipped laboratory and good funding for performing large-scale isolation. The procedure followed in this study could provide a reasonable number of viable islets (150-200 islets/g) in a short time frame. This optimized process of small-scale isolation of islets is practicable and reproducible. However, this method may not be appropriate for *in vivo* transplantation, for which large-scale islet isolation of islets is recommended. The islet yield with this method could not be compared with methods that use the whole pancreas as there are chances of having only a few islets in the selected tissue[18,19]. This could be addressed to an extent by simple H&E staining to determine an area good for tissue isolation prior to digestion. In this method, the thick bovine pancreas was minced to accelerate the digestion process, which may damage the islets, whereas the large-scale methods use the intra-ductal injection of the enzyme for the digestion process. Digestive enzymes and methods used tend to vary between the species[24,28,29,30]. We propose that a two-step digestion process (static followed by mechanical) improves the islet viability by minimising the effect of cold ischaemia. Marchetti *et al*[19] reported that the islets did not stain with dithizone, whereas when prepared

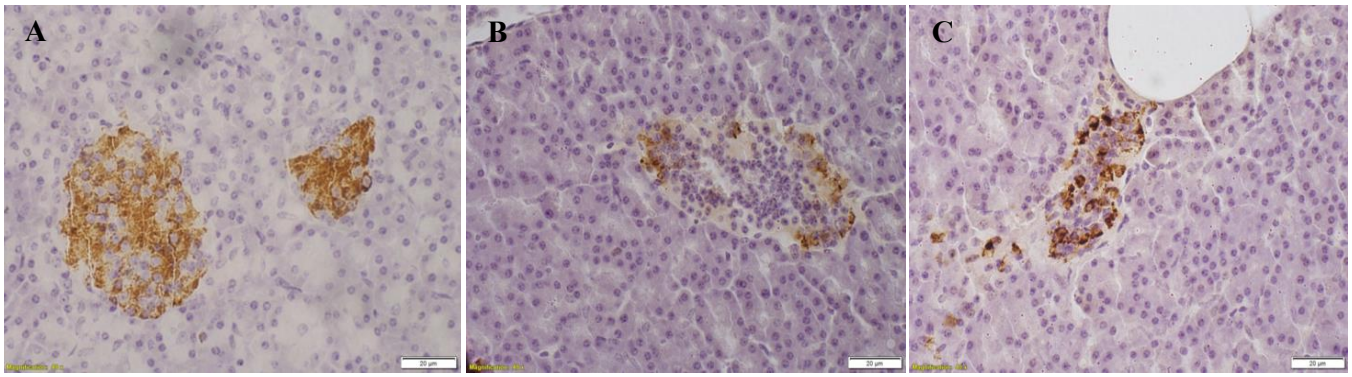

Fig. 9. Light microscopy of bovine pancreatic sections stained (DAB peroxidase method) with (**A**) anti-insulin; (**B**) anti-glucagon and (**C**) anti-somatostatin. Sections demonstrate the localization of beta cells & non-beta cells in an islet.

with DMSO, the islets of varying sizes stained well. We used RPMI medium for the islet culture similar to other standard protocols which could be replaced with a more specific CMRL 1066[28,29,30]. The glucose-stimulated insulin secretion (GSIS) experiments were done by treating the islets, both mantled and pure, with low and high glucose. High glucose treatment increased insulin release, which is expressed as per islet equivalent (IE) rather than the absolute insulin values which is a better index. The same islets were exposed to different glucose concentrations, as it would be better to check the islet efficiency to fluctuating glucose levels. The mantled islets maintained viability long in culture despite their tendency to adhere and the viability was confirmed with Fluo-4 AM staining. We suggest this line of combination experiments; combination of endocrine-exocrine cell model would be good to understand exocrine-endocrine cell interactions to decipher the underlying mechanisms in pancreatic diabetes. There is also a possibility of practising less rigidity on getting pure islets for experimental studies. Increasing the digestion time to obtain pure islets may have negative effects on the viability of islets per se. The double marker staining showed beta cells predominantly in the core, alpha cells in the periphery and delta cells having a more diffuse pattern. However, the proportion of cells in the islets was not determined. Confocal imaging with a triple marker staining reportedly provide a better estimate of the individual cells in the islet[31] however, DAB staining can be an alternative, as it confirmed the findings of fluorescent imaging in the present study. Using two different staining methods helped to confirm the antibody reactivity, as there was no evidence for the same. The aldehyde fuchsin could define the islets better than H&E staining for morphometry studies.

One of the limitations of this study was the utilization of a small amount of tissue, as this would not give adequate numbers for the transplantation studies. However, the method prescribed here could be applied to larger tissues if processed in multiple small batches. The age of the donor is yet another point to be taken care of while considering isolation. The yield of islets upon isolation would be better from young donors rather than older donors. As mentioned, difficulty in achieving pure islets from bovine pancreas was another challenge. It may be argued based on current research discussions which states mantled islets could also be considered for transplantations.

Overall, though this study was not devoid of limitations, the bovine pancreatic islets model could provide a modest number of islets for small scale *in vitro* islets research. This could be a good platform for a beginner to institute and advance in islet research. Further, we surmise that the isolation of actual islets even in smaller numbers would still be a better option for a beginner , even in resource-constrained settings.

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***Conflicts of Interest*:** None.

***Use of artificial intelligence (AI)-assisted technology for manuscript preparation*:** The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing of the manuscript and no images were manipulated using AI.

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