Original Article

Comparative Study of The Effects of Confounding Factors on Improving Rat Pancreatic Islet Isolation Yield and Quality

Maedeh Moazenchi, M.Sc.[1, 2#], Anavasadat Sadr Hashemi Nejad, M.Sc.[1, 2#], Mahmoud Izadi, M.Sc.[1, 2#], Maedeh Khalaj, M.Sc.[1], Zakieh Samsonchi, M.Sc.[1], Pouya Tavakol Rad, H.S.D.[1], Payam Amini, Ph.D.[3], Yaser Tahamtani, Ph.D.[1, 3], Ensiyeh Hajizadeh-Saffar, M.D., Ph.D.[2, 3, 4*]

1. Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
2. Advanced Therapy Medicinal Product Technology Development Center (ATMP-TDC), Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran
3. Reproductive Epidemiology Research Center, Royan Institute for Reproductive Biomedicine, ACECR, Tehran, Iran
4. Department of Regenerative Medicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran

**Corresponding Address: P.O.Box: 16635-148, Department of Regenerative Medicine, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, ACECR, Tehran, Iran*
Email: en.hajizadeh@royan-rc.ac.ir

#These authors contributed equally to this work.

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Abstract

Objective: Isolated pancreatic islets are valuable resources for a wide range of research, including cell replacement studies and cell-based platforms for diabetes drug discovery and disease modeling. Islet isolation is a complex and stepwise procedure aiming to obtain pure, viable, and functional islets for *in vitro* and *in vivo* studies. It should be noted that differences in rodent strains, gender, weight, and density gradients may affect the isolated islet's properties. We evaluated the variables affecting the rat islet isolation procedure to reach the maximum islet yield and functionality, which would be critical for further studies on islet regenerative biology.

Materials and Methods: The present experimental study compared the yield and purity of isolated islets from non-diabetic rats of two different strains. Next, islet particle number (IPN) and islet equivalent (IEQ) were compared between males and females, and the weight range that yields the highest number of islets was investigated. Moreover, the influence of three different density gradients, namely Histopaque, Pancoll, and Lymphodex, on final isolated islets purity and yield were assessed. Finally, the viability and functionality of isolated islets were measured.

Results: The IEQ, IPN, and purity of isolated islets in 15 Lister hooded rats (LHRs) were significantly (P≤0.05) higher than those of the other strains. Male LHRs resulted in significantly higher IEQ compared to females (P≤0.05). Moreover, IPN and IEQ did not significantly vary among different weight groups. Also, the utilization of Histopaque and Pancoll leads to higher yield and purity. *In vivo* assessments of the isolated islets presented significantly reduced blood glucose percentage in the transplanted group on days 2-5 following transplantation.

Conclusion: Based on these results, an optimal protocol for isolating high-quality rat islets with a constant yield, purity, and function has been established as an essential platform for developing diabetes research.

Keywords: Insulin Secreting Cells, *In Vitro* Techniques, Pancreatic Islets, Rodent, Type-1 Diabetes

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Introduction

According to the International Diabetes Federation Diabetes Atlas, the number of worldwide diabetic patients has been estimated to be 536.6 as of 2021, and it has been projected to be increased to 783.2 million by 2045, making this condition a significant threat to human health (1).

Insulin therapy is recommended for patients with type-1 diabetes and even some with type-2 diabetes, although it is not considered a cure. Insulin injections can cause weight gain, edema, refractive changes, fat atrophy, and other long-term side effects (2). Also, it may cause diabetes-induced life-threatening complications such as severe hypoglycemic events following exogenous insulin administration (2, 3). Recently various studies showed that pancreas and islet transplantation (including live cell transplantation) are potentially ideal therapies for patients diagnosed with type-1 diabetes. Pancreas transplantation is relatively a mature treatment method; however, main issues such as major surgery complications and lifelong usage of immune-suppressive drugs remain to be addressed. Nevertheless, islet transplantation through minor surgery, limited immune suppressive regimens, and the possibility of using immunoisolation strategies have fewer complications (2, 4-7). Due to the minor

side effects associated with islet transplantation, it is considered a promising therapy for type-I diabetes (8).

Therefore, researchers worldwide have focused on pancreatic islet and beta-cell transplantation since late 1900. Also, many studies were conducted on islets of Langerhans as a key platform for developing various diabetes research (2, 9, 10). Although animal model-based studies are not entirely predictive of human responses, isolated islets from animal models provide more opportunities to study the mechanisms of diabetes, potential drug discovery, and genetic-based research (9, 10). Based on the previous reports, the quantity and quality of isolated islets play an essential role in successful islet transplantation in experimental studies (2). In an effort to develop a standard and effective method, researchers have modified and improved the initial isolation and purification methods, such as the Edmonton protocol (8, 11).

Although animal studies are not considered a replacement for human trials, they can mimic human diseases and provide a neat way to study similar pathways in human and animal models (12). Also, preclinical studies are important in the safety assessment of different therapeutic products and methods. From understanding the function of the pancreas and the discovery of insulin to new concepts such as stem cell therapy, islet isolation, and transplantation, animal studies have played a significant role in diabetes research. The primary function of islets is insulin production, which is pretty similar between humans and animals, and the restoration of normoglycemia can be achieved by transplantation of mouse/rat islets in diabetic mice/rats (13).

Rodents have many advantages, including high reproductive capacity, pure strains, accessibility, repeatability, short experiment times, and low cost, enabling them to be widely used in various research projects (8). Thus, we have established Royan Institute Rodent Pancreatic Islet Processing Facility (RI-RPIPF) to promote research projects toward improving islet transplantation outcomes and facilitate studies focused on different aspects of islet nature and transplant. This study described our improved rat pancreatic islet isolation method in RI-RPIPF. Moreover, two rat strains, Lister hooded rats (LHR) and Wistar albino rats (WAR), were compared in terms of islet equivalent (IEQ) and islet particle number (IPN). Then, the best strain was used to compare gender, weights, and different density gradients. The optimal procedure for rat pancreatic islet isolation was set up in view of islets yield, purity, and functionality.

Materials and Methods

The materials and methods used in this experimental study are as follows:

Animals

Following the Royan Institute Ethics Committee approval (IR.ACECR.ROYAN.REC.1396.29 and IR.ACECR.ROYAN.REC.1397.227), LHRs and WARs, weighing 250-450 g, were bought from Animal Core Facility, Reproductive Biomedicine Research Center, Royan Institute for Biotechnology. Rats were housed in cages in a room with controlled temperature (24°C), humidity, and 12 hours/12 hours light and dark cycle. Also they were given ad libitum access to food and water. All protocols conformed with Guide Laboratory Animals for The Care and Use (eighth edition). The total number of rats used in this study was 95 LHRs and 15 WARs. Strains were selected based on the previous rat islet isolation studies and in-house protocols. The number of rats used in each experiment is mentioned; however, it should be noted that the sum of rats in different sections is more than the number provided above since islets isolated from some rats were used for more than one experiment.

Rat islet isolation procedure

Pancreas isolation

The pancreas isolation method used in this study is based on in-house protocols and what was previously described (14). In brief, the digestion solution was prepared using Hanks' Balanced Salt Solution (HBSS) 1x without calcium and magnesium (Gibco-14170) with 1 mg/ml collagenase type V (Sigma-Aldrich, Germany, C9263, ≥1 FALGPA units/mg solid, >125 CDU/mg solid). Then, 20 mg of collagenase was completely dissolved in 20 ml HBSS to isolate islets per rat pancreas; next, the enzyme solution w filtered through a 0.22 μm filter (TPP, Switzerland, 99722). The collagenase solution should be prepared freshly and placed on ice to achieve optimal enzyme activity.

Healthy, non-diabetic rats were anesthetized and sacrificed in a CO_2 chamber. Following abdominal skin sterilization, the abdomen was cut with a V-incision. The duodenal papilla was horizontally clamped with a surgical hemostat to block the bile pathway toward the intestine. The 10 mL cold collagenase solution was injected into the Common Bile Duct (CBD). The whole pancreas was bulged from head to tail due to digestion solution flow in all regions. The pancreas was rapidly removed, and fat and connective tissues were trimmed and subsequently transferred into a 50 ml conical tube containing a 5 ml cold collagenase solution.

Pancreas digestion

Pancreas digestion is also performed using in-house protocols and methods described previously (14). In brief, the conical tube was placed in a 37°C water bath for 5 minutes, and the mixture of pancreas and collagenase was transferred to a culture dish to cut the tissue into small pieces. An extra 5 ml pre-warmed (at room temperature) collagenase solution was added to the culture dish, and the mixture returned into a conical tube placed in a 37°C water bath. After 15

minutes, samples were taken each 2 minutes, and the number of free islets that appeared spherical, and golden-brown from acinar tissue was estimated. When over 50% of islets became free of exocrine cells, the suspension volume was immediately doubled using the stop buffer to terminate the digestion process. Stop buffer and culture media containing Roswell Park Memorial Institute (RPMI) 1640 (Corning, USA, 17-105-CV) with 10% fetal bovine serum (FBS, Gibco, Brazil, 10270), 1% L-glutamine (L-GLU, Gibco, Scotland, 25030024), and 1% Penicillin-Streptomycin (Pen-Strep, Sigma-Aldrich, Germany, P4333). The suspension was centrifuged at 900 RPM for 2 minutes at 4°C, and the supernatant was decanted into a waste container. The pellet was washed with 10 ml washing buffer containing RPMI 1640 with 5% FBS and 1% L-GLU and 1% Pen-Strep and centrifuged at 900 RPM for 2 minutes at 4°C (repeated two times).

Islet purification

In this study, three different density gradients, including Histopaque, Pancoll, and Lymphodex, were used for purification to determine the most efficient density gradient to be used in the rat islet isolation process, without islet hand-picking or filtration.

The pellet was purified by discontinuous density gradient centrifugation immediately after discarding the supernatant. First, the pellet was suspended in 10 ml density gradients Histopaque-1077 (Sigma-Aldrich, Germany, 10771) or Pancoll 1.077(PAN-Biotech, Germany, P04-601000) or Lymphodex 1.070 (Inno-train, Germany, 002041600), and subsequently, 10 ml washing buffer was slowly added. The two-layer suspension was centrifuged at 2000 RPM for 18 minutes at 4°C. Islet cells were retrieved from the interphase between density gradient and washing solution.

Purified islets were washed with a washing buffer and centrifuged at 1000 RPM for 2 minutes at 4°C (repeated two times). The supernatant was discarded and replaced with 10 mL of culture media.

Islet culture

The suspension was transferred to a sterile petri dish in a laminar airflow cabinet using a stereomicroscope; then, the dish containing islets was placed at 37°C in a 5% CO_2 incubator. Figure 1 (created by Adobe InDesign version 6) demonstrates a brief schematic presentation of the stepwise rat pancreatic islet isolation procedure.

Islet quality control tests

Light microscopy and islet purity evaluations

Following the isolation procedure, pancreatic islets were observed and assessed using light microscopy regarding morphology, integrity, and capsule intactness. The purity of isolated islets was reported as the percentage of free islets obtained from the acinar tissues, estimated by two independent investigators after observation under a light microscope.

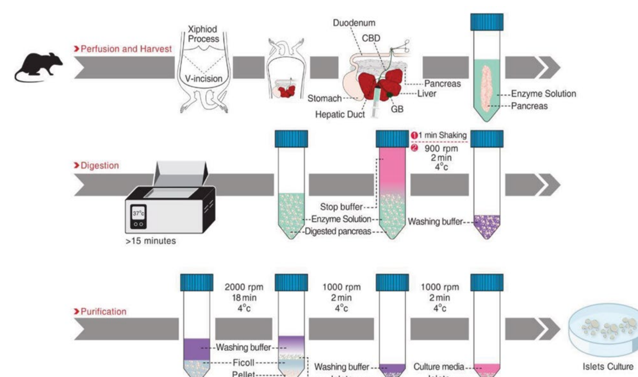

Fig.1: A schematic representation of rat pancreatic islet isolation procedure. The rat pancreatic islet isolation procedure consists of three major steps. First, using surgical techniques, the common bile duct is approached, and the collagenase solution is injected to bulge the pancreas. The pancreas is then extracted from the rat body and transferred to a conical tube with the rest of the enzyme solution. The digestion step starts when the enzyme solution reaches 37°C to remove islets from the exocrine tissue and stops by adding a stop buffer and washing steps to prevent over digestion of islets which could degrade islet capsules. Finally, islets are purified using density gradients and several washing steps to separate islets from non-islet tissue and cultured to maintain cell viability.

Islet yield

Islet yield is defined as the number of islets isolated from one pancreas (14). During the process, islets are isolated with other cells, such as acinar cells, making it hard to detect them under the microscope. So, there should be a specific stain to ease the detection of islets among other cells. Dithizone (1,5-diphenylthiocarbazone, DTZ, Sigma-Aldrich, Germany, D5130) binds with zinc in insulin granules in beta cells of pancreatic islets, causing the islets to stain with a red color while the rest of the cells remain unstained. To identify the islets and determine their quantity and purity, DTZ staining was performed as previously described (15). In brief, 1 mg DTZ was dissolved in 200 µL dimethyl sulfoxide (DMSO, Sigma-Aldrich, Germany, D2650) and then diluted in 800 µL Dulbecco's Phosphate-Buffered Saline (DPBS, Gibco, USA, LS14190250), and filtered through a 0.45 µm nylon filter (TPP, Switzerland, 99745). In a 6 cm Petri plate, 30 µL of DTZ solution was added to islet samples (100 µL), incubated at room temperature for 1-2 minutes, filled up with DPBS to half of the plate height, and visualized under the microscope, using a 10X eyepiece and a 4X objective to magnify 40X. The number of islets in the sample was counted, and the total IPN was estimated after calculation of dilution factor using the following equation according to Table 1.

Dilution Factor = Total volume of preparation that sample taken from (mL)×1000/Volume of sample taken (µL)

Based on the number of isolated islets in each diameter range (IPN, second column in Table 1), the IEQ (last

column in Table 1) was calculated, following mathematical correction for islet diameter (IEQ conversion factor), as shown in Table 1.

Table 1: Equations for total IPN and total IEQ

Islet diameter range (µm)	IPN	IEQ conversion factor	IEQ per range
50-100		×0.167	
101-150		×0.648	
151-200		×1.685	
201-250		×3.500	
251-300		×6.315	
301-350		×10.352	
>350		×15.833	
Σ IPN		Σ IEQ	
Dilution factor [(mL total volume/µL sample volume)×1000]			
Total IPN=Σ IPN×dilution factor			
Total IEQ = Σ IEQ×dilution factor			

IPN; Islet particle number and IEQ; Islet equivalent.

Islet viability and functionality

Fluorescein diacetate/Propidium iodide staining

In the fluorescence staining method for assessment of islet viability, fluorescein diacetate (FDA) and propidium iodide (PI) are used simultaneously to determine cell viability by distinguishing viable and nonviable cells, as previously described (16). In brief, 1.99 mg FDA (Sigma-Aldrich, USA, F7378) was dissolved in 200 mL acetone (Merck, Germany, 100014) in a glass bottle, fully covered by an aluminum foil, and subsequently aliquoted in 10 mL tubes and stored at -20°C. Also, 12.5 mg of PI (Sigma-Aldrich, USA, P4170) was dissolved in 25 mL of DPBS (Gibco, USA, LS14190250), covered by an aluminum foil, and subsequently aliquoted in 5 mL tubes and stored at 2-8°C. Four hundred sixty µL DPBS was added to a new culture dish to adjust the final concentrations of FDA, and PI to 0.46 and 14.34 µM, respectively. Islet suspension was mixed well, and a 100 µL sample was taken and transferred into an empty tube. From the bottom of the tube, 43 µL of settled islets were transferred to 460 µL of DPBS in the culture dish. The islet suspension was then treated with 10 µL of PI followed by 10 µL of FDA. The suspension was assessed by fluorescent microscopy, in which viable cells were considered as those with bright green fluorescence produced by FDA and dead cells as those with red fluorescence emitted by PI. The percentage of viable cells versus total cells was estimated after complete assessment.

Glucose-stimulated insulin secretion test

Glucose-stimulated insulin secretion (GSIS) test is a functional assay that measures the potency of insulin secretion from the islets when different glucose concentrations are applied, as described previously (17). Briefly, after 24-48 hours of islet culture at 37°C, three replicates of 100 IEQ/100 µL culture media were taken from islet suspension. Rat islets were incubated with a low glucose media (2.8 mM), and supernatant samples were taken at time zero and after one hour of incubation at 37°C. Then, another incubation was conducted with the same islets in higher glucose concentrations (28 mM), and supernatant samples were taken at time zero and one hour after the incubation at 37°C. Using a Rat/Mouse Insulin ELISA kit (Millipore, USA, EZRMI-13K), the amount of insulin released was measured in both supernatant samples. The stimulation index (SI) was calculated using the following formula, where insulin concentration from higher glucose concentration is in the numerator, and measurement result from lower glucose concentration is in the denominator. This index measures the ability of purified pancreatic islets to produce insulin in response to increased glucose concentrations.

Stimulation index (SI)=Insulin concentration after stimulation with 28 mM glucose concentration/Insulin concentration after stimulation with 2.8 mM glucose concentration

Islet transplantation*: in vivo* functional test

As we previously described, isolated islets were transplanted in the diabetic mice omentum to evaluate their functionality (18). In brief, male NMRI mice 8-10 weeks old were injected with 90 mg/kg alloxan (Sigma-Aldrich, USA, A7413) via the tail vein to induce diabetes. Two consecutive fasting blood glucose readings of more than 250 mg/dL confirmed diabetes induction. Mice were anesthetized using 90 mg/kg Ketamine 10% (Alfasan, Netherlands) and 4.5 mg/kg Xylazine 2% (Alfasan, Netherlands). Following a median abdominal incision, the greater omentum was spread out onto wet sterile gauze, and a minimum amount of islets (350 IEQ) was transplanted between two omentum layers. Next, the muscular layer was closed by a 5-0 Vicryl suture (VICRYL, Belgium, V2910H), and the skin was sutured with a 3-0 silk suture (MERSILK, W328). For six days following transplantation, non-fasting blood glucose levels were monitored in recipients to measure xenograft function.

Data analysis

SPSS version 20 was used for statistical analysis (SPSS Inc. Chicago, IL). The descriptive statistics of the continuous variables are shown as mean ± standard deviation. Kolmogorov-Smirnov test was utilized to assure the normality of data distribution. Student's t test and one-way analysis of variance (ANOVA) were used to assess the mean difference of continuous variables between two and more than two categories, respectively. Moreover, multiple comparisons were made by the least significant difference (LSD). A two-tailed P value less than 0.05 was determined statistically significant.

Results

Higher islet yield obtained from LHR strain, compared to WARs

Royan Institute Animal Core Facility provided thirty male LHR and WAR strains (15 each). As the first step of the primary endpoint, pancreatic islet yield was assessed in terms of IPN and IEQ and compared between the mentioned strains. As shown in Figure 2, IPN and IEQ for isolated islets from LHR were significantly (P≤0.05) higher than those of the other strains (Fig.2A, B, respectively). The percentage of islet purity, estimated by qualitative analysis, was significantly higher in LHR (61% vs. 75%, Fig.2C).

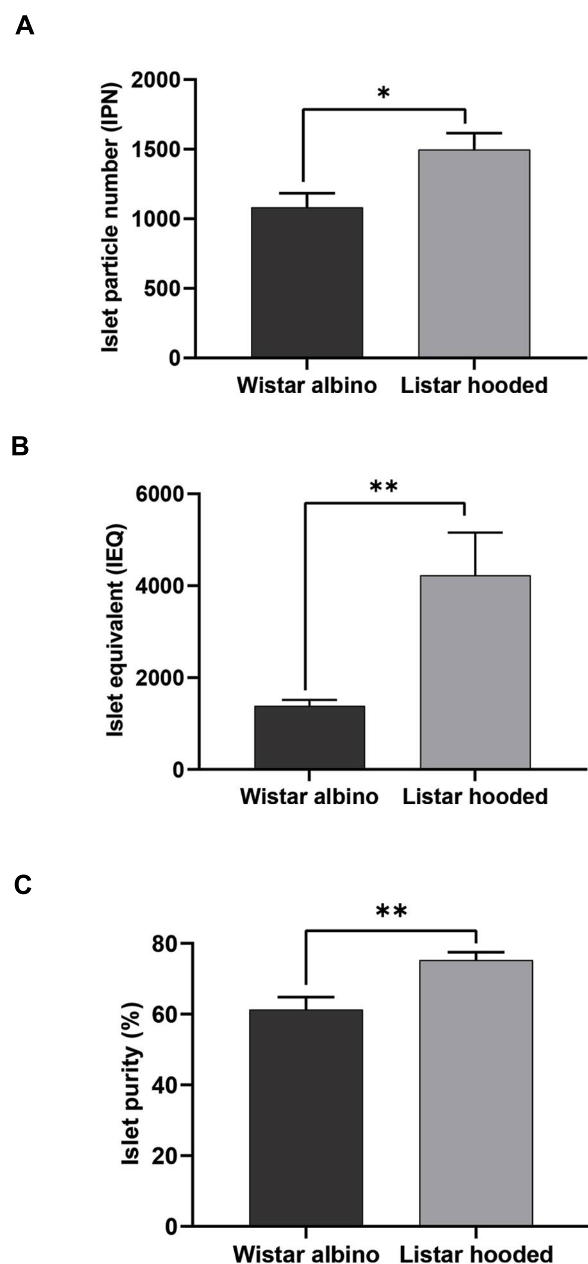

Fig.2: Comparison of IPN, IEQ values, and purity percentages between the two rat strains (n=15 each). **A.** The calculated IPN values were significantly higher in the LHR strain (P=0.013). **B.** The differences in resulted IEQ values in LHR strains were significant (P=0.005). **C.** The purity percentages were significantly higher in the LHR strain (P=0.002). LHR; Lister hooded rats, *; P≤0.05, and **; P≤0.01.

Male LHR yielded higher IEQ

According to the significantly higher yield and purity of LHRs, this strain was selected for further experiments in this study. In the next step, the effects of gender on IPN and IEQ were studied in LHRs (Fig.3A, B). Male and female rats were not significantly different regarding IPN (Fig.3A). However, islets isolated from male LHRs yielded significantly higher IEQ than females (P=0.009, Fig.3B).

No relationship between rat body weight and islet yield

Male LHRs were divided in four weight groups based on their body weights as follows: 250-300 g (n=22), 300-350 g (n=15), 350-400 g (n=13), and 400-450 g (n=9). Here, IPN or IEQ in different weight groups were studied. As shown in Figure 3C, D, there was no significant relationship between body weights and the resulted IPN or IEQ.

Histopaque and Pancoll resulted in high pure islets

Histopaque, Pancoll, and Lymphodex were used as density gradient reagents in the purification step. Comparing these gradient reagents showed no significant difference between Histopaque and Pancoll in IPN, IEQ, and purity. However, results obtained after applying Histopaque and Pancoll significantly differed from Lymphodex (P<0.05). The results demonstrated that the utilization of Lymphodex leads to lower IPN, IEQ, and purity (Figs.4, 5A-C, Table S1, See Supplementary Online Information at celljournal.org) (IPN; Lymphodex vs. two other gradients: both at P≤0.001, IEQ; Lymphodex vs. Histopaque (P=0.003) and Pancoll (P=0.013), Purity; Lymphodex vs. Histopaque (P≤0.001) and Pancoll (P≤0.001).

Isolated islets were viable and functional *in vitro*

The viability and functionality of isolated islets were assessed using FDA/PI staining and GSIS assay, respectively. Purified isolated islets were stained with DTZ (Fig.5D) to differentiate the islets from exocrine tissue and calculate IPN and IEQ. FDA/PI staining was done on purified islets to determine the live/dead ratio, visualized under the fluorescent microscope (Fig.5E). GSIS was performed after a 24 and 48-hour culture of isolated islets. SI was calculated using insulin secretion from islets in response to high versus low glucose concentrations, and results showed no significant differences between 24 and 48-hour islet cultures (Fig.5F).

Isolated islets were able to secrete insulin after transplantation

The *in vivo* function of implanted rat islets into the omentum of alloxan-induced diabetic mice was assessed by their potential to return normoglycemia. During six days post-transplantation, blood glucose level monitoring indicated a significant reduction in the percentages of blood glucose on days 2 (P=0.037), 3 (P=0.021), 4 (P=0.023), and 5 (P=0.044), in comparison with the non-transplanted diabetic mice used as a control group (Fig.5G).

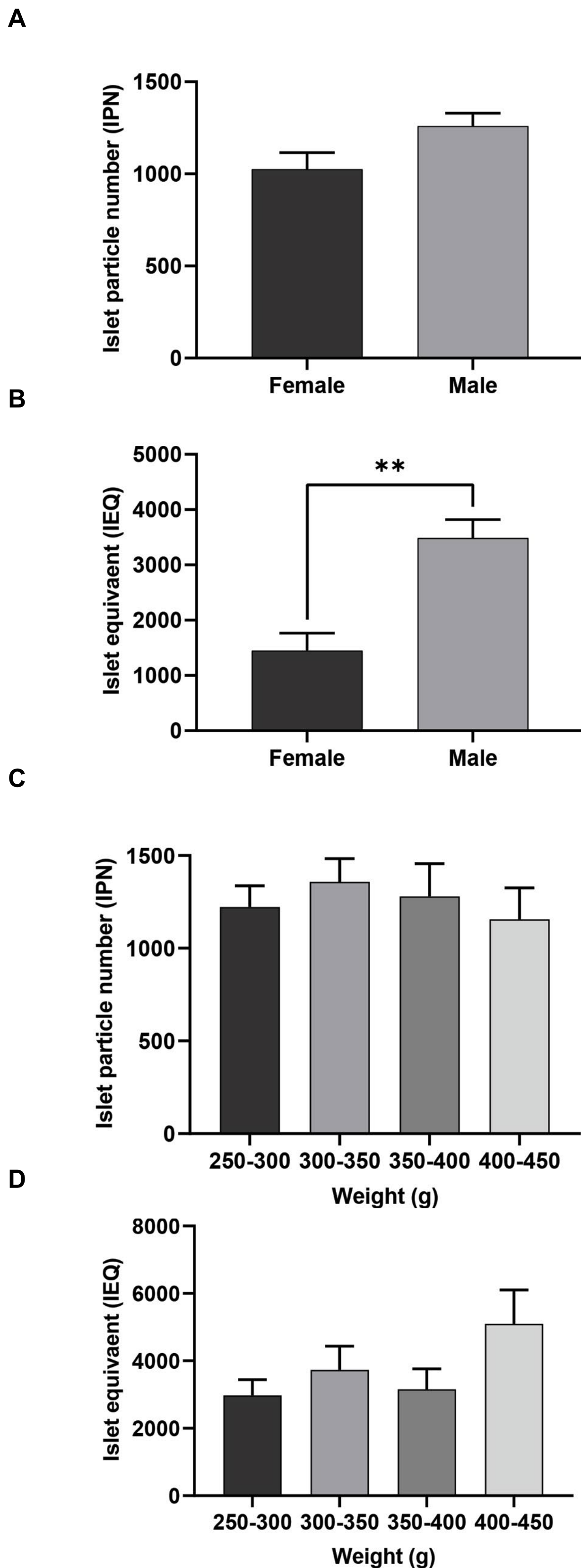

Fig.3: The effects of gender and weight on IPN and IEQ in Lister hooded strain. **A.** The IPN did not significantly vary between male (n=59) and female (n=12) LHRs (P=0.149). **B.** Male LHRs (n=59) had significantly higher IEQ compared to female ones (n=12) (P=0.009). **C.** The IPN did not significantly vary between four male LHR weight groups (P=0.816). **D.** No significant differences were observed among the four weight groups in terms of IEQ (P=0.177). LHR; Lister hooded rats and **; P≤0.01.

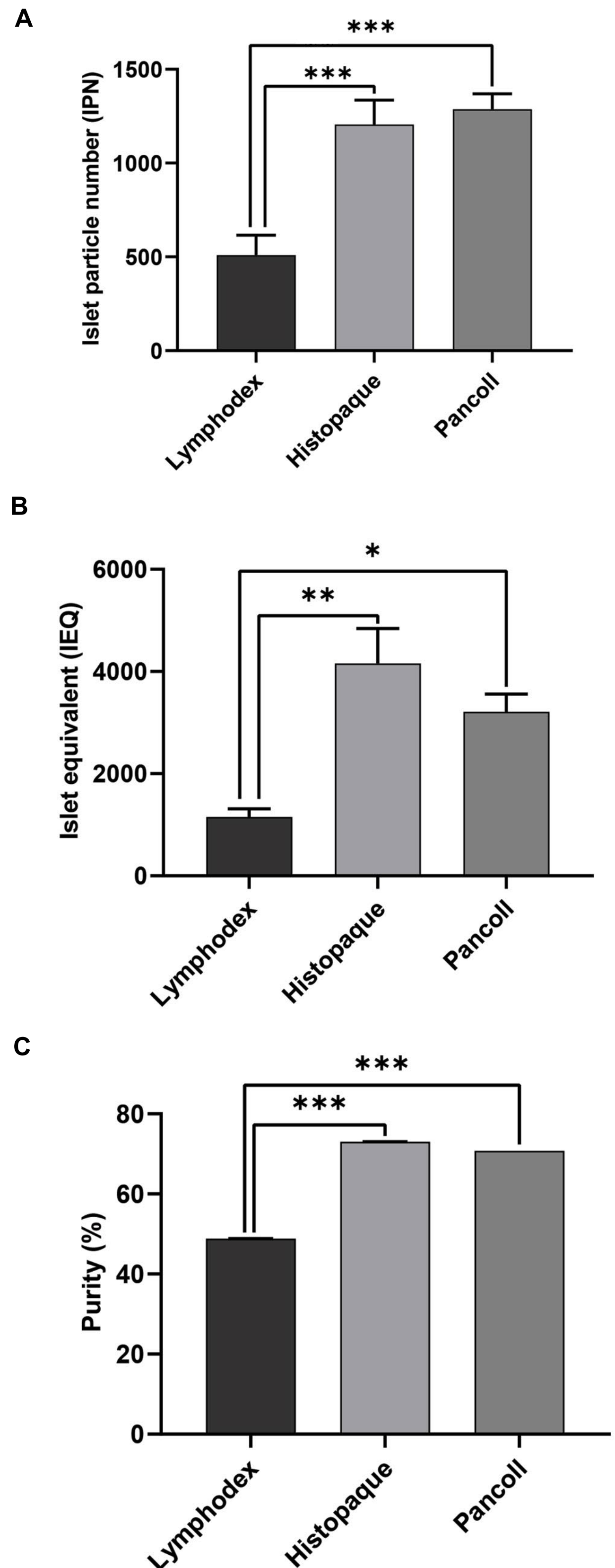

Fig.4: Comparison of different density gradients used in the purification step. **A.** Significant differences were found in IPN between Lymphodex (n=9) and two other gradients (both at P≤0.001). **B.** As compared to Histopaque (n=20) and Pancoll (n=39), the application of Lymphodex led to significantly lower IEQ (P=0.003 and P=0.013, respectively). **C.** Significantly higher purity was achieved when Histopaque (P≤0.001) and Pancoll (P≤0.001) were used as density gradients compared to Lymphodex. *; P≤0.05, **; P≤ 0.01, and ***; P≤0.001.

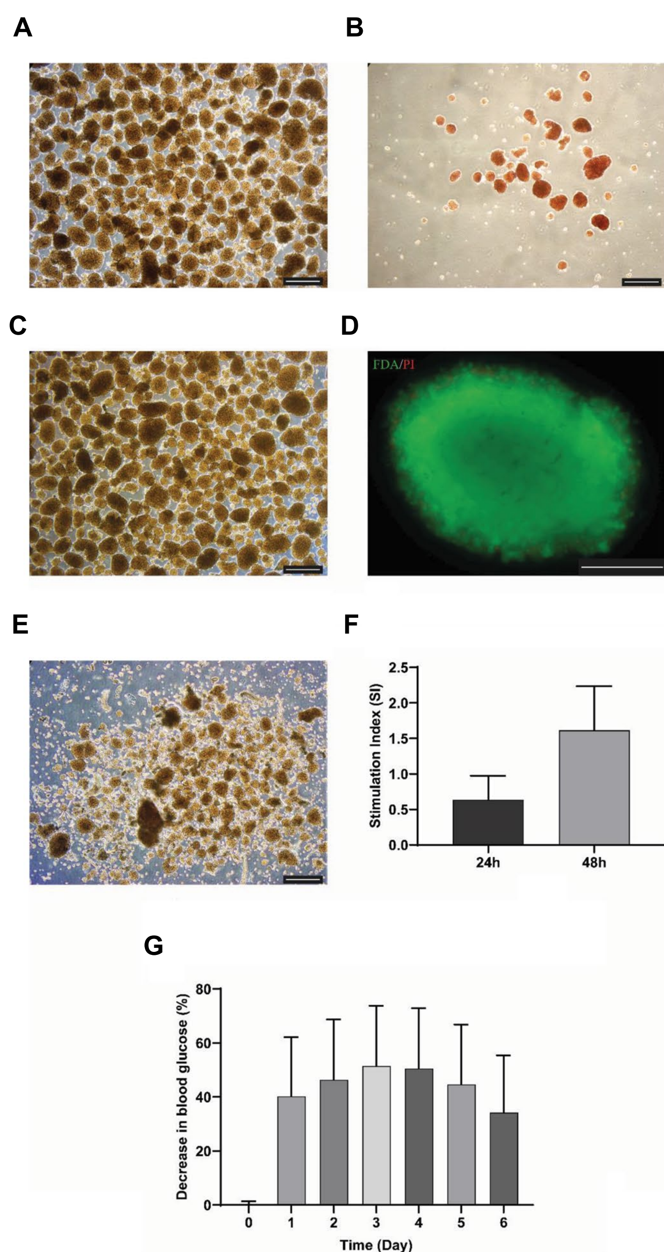

Fig.5: Purity, viability, and functionality assays on isolated islets from male Lister hooded rats. The purity of islets isolated using **A.** Histopaque, **B.** Pancoll, and **C.** Lymphodex (scale bar: 500 μm). **D.** Dithizone (DTZ) staining of isolated islets (scale bar: 500 μm). **E.** Viability estimation in isolated islets stained with Fluorescein diacetate (FDA)/propidium iodide (PI) using fluorescent microscopy (scale bar: 100 μm). **F.** Glucose-stimulated insulin secretion (GSIS). Stimulation index (SI) for 24 and 48-hour cultured isolated islets showed the functionality of isolated islets. SI resulting from a 48-hour culture presented considerable but not significant insulin secretion than 24-hour culture (P=0.074). **G.** Significant reduction in percentages of blood glucose on days 2 (P=0.037), 3 (P=0.021), 4 (P=0.023), and 5 (P=0.044) post islet transplantation (n=5) compared to the control group (n=4). h; hour.

Discussion

Having precise preclinical studies is necessary to start successful clinical trials and elucidate the new signaling pathways in basic research (19, 20). Islet transplantation is now regarded as a promising treatment option for type 1 diabetes; meanwhile, professional isolation of pancreatic islets plays a critical role in this treatment's efficiency (21). Different research groups worldwide have modified the islet isolation process to develop a functional and economical technique in recent decades. The islet isolation process includes digestion, purification, and islet culture steps that have been optimized to produce higher islet yield and purity in order to improve functional results. However, some challenges remain (5, 8, 20, 22, 23).

Nowadays, many researchers focus on improving these steps to achieve highly pure and functional islets. Overcoming isolation-associated obstacles lead to introducing islet transplantation as an efficient, widespread treatment (24). Yet, the shortage of pancreas donors and the high cost of human pancreatic islet isolation limited its availability for research purposes, resulting in a demand for the isolation of islets from animals, especially rodents, which is cost-effective, accessible, and easier (13).

In this study, we showed that variables such as rat strain and gender could affect islet yield and purity. Besides, the effects of rat body weight on IPN and IEQ were evaluated. Then, islet yield and purity were compared after administering different density gradients. Finally, *in vitro* and *in vivo* assays were performed to assess the viability and functionality of isolated islets. Here, we presented a straightforward and transparent protocol for efficient rat islet isolation.

Previous studies reported the dependence of islet isolation yield, purity, and functionality on factors such as strain and gender (11, 21, 25). de Groot et al. (21) showed that islet yield, purity, and function depend on donor rat strain. In comparison to Wistar albino (WA), Lewis, and Sprague Dawley (SD) rat strains, they obtained double amounts of islets from Albino Oxford (AO) rats. The islet purity was also shown to vary among the rat strains, so highly purified islets were isolated from all but SD rats. Moreover, de Haan et al. (26) compared islets isolated from AO, WA, and Lewis rats based on their quantity, size, and function and showed that the size and quantity of the isolated islet from AO rats were significantly larger than those from WA and Lewis. In contrast, Lewis rats had the highest glucose-induced insulin response. They suggested that isolated islets' yield and function were strain-dependent, and interestingly, GSIR does not necessarily increase with islet yield.

Our study compared islet isolation parameters between LHR and WAR strains. Islet yield parameters, IPN and IEQ, were significantly different between LHRs and WARs. Islet isolations using LHRs resulted in higher islet yield compared to WARs. Purity was qualitatively estimated and was significantly higher in LHRs. Although there were no significant differences in IPN between male and female rats, IEQ was significantly higher in islets obtained from male rats than in females. According to the strain and gender comparison, where IEQ showed a greater difference than IPN, the results indicated that volume per islet (IEQ/IPN) in LHRs is higher than in WARs. Also, in LHRs, islets isolated from male rats have higher volumes than female islets. Therefore, the difference in diameters and consequently in size of isolated islets led to statistically significant differences in IEQ. Considering

these findings, we used Lister male rats for the rest of this study.

In human islet isolation studies, body mass index (BMI) and weight of pancreas donors could reflect islet isolation outcomes which have been shown that higher donor weight and BMI led to higher isolated islet yield (5, 26-28). Tze and Tai (29) claimed that increased rat body weight could increase IPN. Therefore, in this study, we decided to examine possible correlations between rat body weight and isolated islets' IPN and IEQ. According to our results, the IPN for islets obtained from rats of 400-450 g was not significantly lower than IPN values measured for other weight ranges. Nonetheless, non-significant low IPN and non-significant high IEQ levels measured for 400-450 g rats compared to different weight ranges indicated that rats' body weight might slightly correlate with isolated islets' diameter and size. Altogether, it was concluded that rat bodyweights could not significantly affect islet yields. Likewise, Saliba and Fares (30) evaluated islet yields between mice of different ages (different ages correlates with different weight) and species and concluded that the yields were similar.

Another challenging factor in islet isolation protocols is the density gradient reagent's effect on islet yield and purity. There are different ways for islet separation and purification, including gradient separation, filtration, sedimentation, magnetic separation, and manually hand-picking (30, 31). Several studies focusing on islet isolation protocol development discussed the effects of different density gradients on outcome parameters. Several density gradients, including Ficoll, Histopaque, Dextran, and Iodixanol, have previously been studied for islet isolation that compared their recovery, viability, purity, *in vitro* functionality, IPN, and IEQ and showed that there were no differences in IPN and IEQ among groups. However, regarding purity and viability, Ficoll or Histopaque resulted in the purest and highest number of viable islets compared to Dextran and Iodixanol (11). Another study used Histopaque and Ficoll as gradient density solutions, resulting in no differences between those two, and concluded that both were acceptable and generally usable (14). Therefore, this study examined three density gradients, Histopaque, Pancoll, and Lymphodex, for their effects on islet yield parameters (IPN and IEQ) and purity. There were no significant differences in IPN, IEQ, and purity between Histopaque and Pancoll. Still, utilization of Lymphodex led to significantly lower islet purity and yield (lower IPN and IEQ values). The analysis showed that Lymphodex was not efficient for rat islet isolation, while Histopaque and Pancoll were sufficiently effective in the process. In addition, regarding cost-effectiveness, the prices of 10 ml of Histopaque or Pancoll required for each islet isolation procedure (~ 4.18 $ and 0.95 $, respectively) showed the usage of Pancoll is more cost-effective compared to Histopaque. Notably, in this study, isolated islets utilizing Histopaque and Pancoll were pure enough without the time-consuming procedure of hand-picking and filtration.

As previously reported, FDA/PI and GSIS assays were used after the islet isolation process to determine the viability and functionality of isolated islets, respectively (14). The viable and dead cells in the islets were indicated by green and red fluorescence caused by FDA and PI, respectively. Glucose-stimulated insulin secretion was used to assess isolated islets' functionality, evaluating the insulin-release ability of 24 and 48-hour cultured islets in response to glucose concentrations. While the data showed no significant differences between 24 and 48-hour SI, 48-hour cultured islets showed considerable insulin secretion.

Previously published studies presented transplanted human and mouse islets' functionality to reduce blood glucose levels in diabetic patients and diabetic mice, respectively (32-35). In this study, *in vivo* functional assessments showed a significantly higher reduction in non-fasting blood glucose percentages in transplanted mice than in non-transplanted diabetic one (control group) on days 2 to 5.

To prevent sacrificing more animals for this study, we used rat islets isolated for other diabetes research projects running simultaneously (18, 36, 37).

Conclusion

Given the importance of islet-based studies in developing novel diabetes therapies and drug discovery, a validated, replicable, and enhanced protocol for islet isolation with minimal variation is required. The optimized rat islet isolation protocol using male LHR and a Histopaque/Pancoll density gradient, yielded acceptable results, leading to a standard protocol for isolating high-quality rat islets with constant yield, purity, and functionality, which could meet global criteria to promote diabetes treatment research.

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Authors' Contributions

M.M., A.S.H.N., M.I., E.H.-S.; Contributed to study design, conceptualization, methodology, formal analysis, investigation, and interpretation of the data. M.M., A.S.H.N.; Wrote the original manuscript. E.H.-S., Y.T.; Supervised the project, reviewed writing the draft, and provided financial support. M.Kh., Z.S., P.T.R.; Assisted

with methodology and investigation. P.A.; Contributed to statistical analysis and validation. All authors read and approved the final manuscript.

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