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Reviving hope: unlocking pancreatic islet immortality by optimizing a trehalose-based cryopreservation media and cell-penetrating peptide

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Abstract

Background Diabetes mellitus remains a pervasive global health concern, urging a deeper exploration of islet transplantation as a potential enduring solution. The efficacy of this therapeutic approach pivots on the precision of cryopreservation techniques, ensuring both the viability and accessibility of pancreatic islets. This study delves into the merits of cryopreserving these islets using the disaccharide trehalose, accompanied by an inventive strategy involving poly L proline (PLP) as a cell-penetrating peptide to overcome the cryoprotectant limitations inherent to trehalose.

Methods In our experiments with rat islets, we conducted meticulous viability assessments for fresh and frozen samples. We employed a spectrum of methods, including live/dead staining, insulin/glucagon staining, and measurement of reactive oxygen species (ROS) levels. To gauge functional integrity, we executed glucose-stimulated insulin secretion tests. Subsequently, we transplanted thawed islets into diabetic mice to scrutinize their performance in clinically relevant conditions.

Results Our study yielded compelling results, affirming the successful cryopreservation of pancreatic islets using trehalose and PLP. Viability, as corroborated through live/dead and insulin/glucagon staining, underscored the sustained preservation of frozen islets. Moreover, these preserved islets exhibited functional integrity by releasing insulin responsively to glucose stimulation. Significantly, upon transplantation into diabetic mice, the thawed islets proficiently restored euglycemia, evidenced by a substantial reduction in fasting blood glucose and an enhanced glucose tolerance.

Conclusion Our findings accentuate the potential of trehalose and PLP as sophisticated cryoprotectants for preserving pancreatic islets. Beyond highlighting viability and functionality, the preserved islets demonstrated a remarkable capacity to restore euglycemia post-transplantation. This research holds promise in addressing the inherent limitations of islet transplantation, particularly in the realm of Type 1 diabetes treatment.

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Introduction

Diabetes mellitus remains a serious global health concern affecting millions worldwide through hyperglycemia caused by insufficient insulin secretion [1]. Despite various treatment methods available today, there exists no definitive cure for this chronic metabolic disorder. However, progress in medical science has brought reason for optimism, mainly through advances in islet transplantation. This innovative method involves isolating a cluster of cells containing beta cells that secrete insulin from one person's pancreas and transplanting them into another person with diabetes [2]. Ultimately, the approach aims to restore the recipient's natural ability to regulate blood glucose levels and produce insulin [3]. What makes islet transplantation an exciting option for patients seeking relief from diabetes is its quick recovery times [4, 5]. Despite all the advantages mentioned above, islet transplantation has some limitations. One of the most prominent challenges with islet transplantation is the limited availability of donor pancreas and efficient and effective storage of islets before transplant, during donor-recipient cross-matching, as the procedure requires a deceased donor [6–11]. To surpass challenges arising from a scarcity of individual donor organs during transplantation procedures, scientists have developed an innovative solution with potential benefits [12].

In the process of islet transplantation and preservation of cells, the cryopreservation technique would be the central pillar. Cryopreservation describes preserving biological materials at low temperatures (− 196 °C) to prevent degradation and keep their structural and functional integrity [13, 14]. However, the success rate of cryopreservation may be influenced by various factors, such as the choice of cryoprotectant and cooling and thawing procedures. And the overall preservation technique. Nevertheless, selecting a highly efficient cryoprotectant is pivotal to ensuring successful cryopreservation of pancreatic islets. Cryoprotectants are substances that prevent ice crystal formation and reduce osmotic stress. Thereby protecting cells during freezing and thawing. The ideal cryoprotectant should possess properties that enable it to penetrate cells and safeguard cellular structures effectively. And maintain both cell viability and functionality after thawing [15]. Also, shifts in ambient atmospheric oxygen concentrations cause ATP metabolite depletion, leading to compromised viability and functionality of islets [16].

The rate of the freeze–thaw process at which the cooling and warming occurred was the most crucial factor in determining the survivability of the cells. Instantaneous cooling rates can lead to cellular harm, while slow rates can induce ice crystal formation and contribute to foreign body response (FBR)-mediated caused rejection upon transplantation [17]. Remarkably, minimizing the number of freeze–thaw cycles to maintain the integrity of the samples is essential. While there are benefits to preserving biological materials through cryopreservation, there are also limitations that must be considered [13]. Nonetheless, achieving successful outcomes through this method largely depends on factors such as the type and concentration levels chosen for each substance [18]. Specifying authentic cryoprotectants is critical to process attainment [19].

In terms of preserving biological material through freezing, there are two kinds of cryoprotectants: those that penetrate cells and those that don't. Penetrating options like glycerol and dimethyl sulfoxide (DMSO) and non-penetrating choices such as sugars and polyethylene glycol (PEG) don't infiltrate cells but still shield the extracellular environment from these harmful crystals [20].

Trehalose is a naturally occurring disaccharide with cryoprotective properties [21]. Trehalose acts as a non-permeating cryoprotectant and forms a glass-like matrix around the cells or tissues during freezing, which prevents ice crystal formation and preserves the integrity of the biological material. Trehalose protects against membrane damage when cells undergo freezing or thawing [22, 23]. Compared to synthetic compounds, its natural composition provides better compatibility with living organisms, making it an ideal choice while being cost-effective and easily accessible [22]. However, trehalose, as a cryoprotectant, still has some limitations. Preserving sensitive cells or tissues from freezing damage requires thorough protection with Trehalose, which may not always suffice [22]. Several methods, such as the microinjection [24], ion channel stimulation [25], pore formation using mutant bacterial toxins [26], fluid-phase endocytosis [27], internal trehalose synthesis via genetic engineering [28], and membrane permeabilizing biopolymers can assist in facilitating Trehalose penetration into cells [29]. Among all the techniques mentioned above, membrane permeabilizing biopolymers are the safest and non-invasive alternative.

Using non-toxic biopolymers, pores formation occurs in the cell membrane, and trehalose will be simplified to penetrate the cell. Poly-L-lysine isophthalamide (PLP) is a synthetic polymer utilized as a permeabilizing tool for cell membranes. The mechanism of PLP cell-permeabilizing is due to hydrophobic interchanges with the phospholipid bilayer of the cell membrane. It permits the delivery of molecules such as drugs, DNA, RNA, or other biomolecules into the cell. PLP enhances the cellular uptake of trehalose by increasing cell membrane permeability [30, 31]. Studies have shown that trehalose complexed with PLP can improve cell survival under various stress conditions, including oxidative stress, heat shock,

and dehydration. In addition, trehalose complexed with PLP improves the cryopreservation of the cells, which can have consequential matter for the storage and transportation of islet cells [32, 33]. While using PLP for trehalose cell permeabilization has shown advantageous impacts, it also has toxic effects on the cells at high concentrations [32]. Optimization of the PLP concentration and direction time is required to minimize toxicity while performing optimal trehalose uptake and cell protection. The thorough purpose of this study is to optimize the freezing media to enhance the survival and function of frozen islets after thawing and transplantation. In this regard, we developed a modified and clinically relevant technique for the cryopreservation of pancreatic islets. This study investigated the efficacy of trehalose as a non-toxic cryoprotectant in combination with poly (L-lysine isophthalamide) (PLP) as a membrane-permeabilizing biopolymer to improve intracellular uptake of Trehalose. Also, we used Curcumin as an antioxidant to decrease cryopreservation-induced oxidative stress in vitro and in vivo.

Materials and methods

Materials

Sodium hydroxide (106,462), trehalose (6138-23-4), 2′,7′-Dichlorofluorescin diacetate (4091-99-0), DAPI (D8417), fluorescein diacetate (F7378), dimethyl sulfoxide (D2650), tris-buffer (T6066), alloxan monohydrate (A7413), collagenase from Clostridium histolyticum type V (C9263), propidium iodide (P4864), phosphate-buffered saline (P4417), dithizone (D5130) and anti-glucagon antibody (G2654) were obtained from Sigma-Aldrich. Hank's buffered saline solution (14,025,076), fetal bovine serum (10,270,106), and penicillin/streptomycin were purchased from Gibco. Acetone (100,014) and ethanol (100,983) were from Merck. Insulin antibody was supplied by Abcam (ab7842). Roswell Park Memorial Institute (RPMI)−1640 medium (17–105-CV) was purchased from Corning. Pancol human density (P04-60100) was obtained from PAN-biotech, and rat/mouse Insulin ELISA was purchased from Millipore: l-lysine methyl ester dihydrochloride, potassium carbonate, isophthaloyl chloride, poly(L-lysine isophthalamide.

Synthesis of PLP

PLP was synthesized according to our previous work [34] employing a single-phase polymerization technique (S1.a); briefly, the procedure commenced by dissolving L-lysine methyl ester hydrochloride (5 mmol) and potassium carbonate (15 mmol) in 10 ml of deionized water. Stirring of the aqueous solution at 25 °C was facilitated using a Teflon-coated magnet until the appearance of an ice crystal signaled cooling. Subsequently, a pre-cooled acetone solution (10 ml) containing isophthaloyl chloride (5 mmol) was swiftly introduced. Over 30 min, the solution underwent a reaction, yielding a precipitate of poly L-lysine methyl ester hydrochloride, evident by the formation of a powdered cream white gum within 2–5 min. The resulting polymer was then dried overnight in a vacuum oven set at 60 °C. The dried poly L-lysine methyl ester was then solubilized in dimethyl sulfoxide to prepare the sodium form of poly L-lysine isophthalamide. Subsequent treatment with a 5% ethanol solution induced precipitation of the hydrolyzed polymer, which dissolved once more in deionized water. Dialysis against deionized water for approximately 2 days ensued to eliminate unreacted and excess components. The material was acidified to pH 3 in sodium to achieve a neutral polymer using 1 M hydrochloric acid. Finally, the resulting precipitate was collected, washed, and subjected to lyophilization to obtain a white powder. Structural elucidation of the synthesized PLP molecule was accomplished via NMR spectroscopy in d6-DMSO using a Bruker Avance 500 MHz spectrometer at room temperature. Also, gel permeation chromatography (GPC) performed molecular mass distribution analysis.

Animals

All animals were housed in cages in a temperature-controlled environment with a regular 12/12h light/dark cycle and had ad libitum access to food and water. Experiments were performed following the NIH Guidelines for Institutional Animal Care and Use (Eighth edition) after the Royan Institute Ethics Committee (IR.ACECR.ROYAN.REC.1397.227) approval. The Listar Hooded rats were used for islet isolation, and NMRI mice as the recipient. For anesthesia, we used a mixture of ketamine (80–100 mg/kg) and xylazine (5–13 mg/kg) through intraperitoneal injection. In addition, NMRI mice were injected with alloxan to induce diabetes. The mice were classified as diabetic when two consecutive blood glucose levels ≥ 18 mmol/L occurred outside fasting.

Pancreatic islet isolation

The islets were isolated based on our previous study [35, 36]. Briefly, after injection of cold collagenase solution into the rat`s common bile duct and completely bulged pancreas from head to tail via a perfusion phase, the pancreas was cut, trimmed from extra fat and connective tissue, and digested in a water bath (37 °C). For purification, Ficoll density gradient centrifugation was used, and then islets were cultured in RPMI-1640 containing 10% v/v FBS, 2% v/v penicillin/streptomycin, and 1% v/v L-glutamine (Fig. 1a).

Live/dead staining

Fluorescein diacetate/propidium iodide (FDA/PI) staining was performed to assess islet viability. Then, 24 h after thawing, islets from all groups were suspended in 480 µL DPBS and stained with DPBS (10 µL) containing FDA (0.46 µM) and PI (14.43 µM). Islets were immediately assessed by fluorescence microscopy.

Glucose-stimulated insulin secretion test

The GSIS test was performed to investigate the in vitro functionality of islets. Short islets were incubated in Krebs bicarbonate buffer (KRBB) with low and high glucose concentrations (2.8 and 28 mM) for 1 h. Stimulation index (SI) was measured with a rat/mouse insulin ELISA kit (Millipore, EZRMI-13 K) and calculated by dividing the amount of insulin released at high glucose concentration by the amount released at low glucose concentration.

Cryopreservation and thawing of rat islets

In each group, 500 Islet Equivalent (IEQ) of rat pancreatic islets were transferred to a 1.8-mL cryovial and suspended in 1 ml of RPMI-1640 consisting of 2 M DMSO. Compounds such as trehalose (200 mM) and PLP (200 µg) were added and incubated for one hour. In all groups, 10% FBS was added to the freezing medium. The islets were brought to −7.4 °C to nucleate the samples and ensure uniform cooling. The islets were then slowly frozen to −40 °C at a rate of −1°C/min and immersed in liquid nitrogen. The vials were then stored in the liquid phase of liquid nitrogen until thawing. Cryopreserved pancreatic islets were removed from liquid nitrogen, rapidly thawed in a 37 °C water bath at 200 °C/min, and diluted with culture medium (RMPI + 10% FBS). At the end of the dilution phase, the cryoprotectant was reduced to 10% of the original one. The islets were transferred to a fresh Petri dish containing culture medium and cultured at 37 °C and 5% CO2 (Fig. 1b). The dilution procedure and removal of the cryoprotectant were performed according to the table shown in Fig. 1c.

Immunohistochemistry

Immunofluorescence staining was performed according to the following protocol. First, the slides were placed in an oven at 60 °C for 30 min. The slides were deparaffinized and rehydrated with a graded alcohol series, then incubated in the antigen retrieval (2100 retriever, England) solution at 121 °C for 20 min. After the solution cooled, the slides were washed twice in PBS-T %0.05, and then 0.5% Triton X 100 was added for 10 min to permeabilize the membrane. In the next step, the blocking solution was added to the slides for one hour to block the reaction of the secondary antibody with the samples. The slides were incubated with the primary antibodies for 24 h at 2–8 °C. After 24 h, the sections were washed thrice for 5 min each in PBS-T %0.05. The secondary antibody was added, and the slides were incubated at 37 °C for one hour in the dark condition. Specimens were placed in a dark room and rinsed with PBS-T %0.05, and then a DAPI solution was poured onto the slides. The slides were then washed with PBS-T %0.05 for one minute two times, and a solution of PBS and glycerol was added. Finally, the slides were viewed under fluorescence microscopy (Olympus).

Intracellular oxidative stress

DCFDA (2′, 7′-dichlorofluorescein diacetate) dye was used to investigate intracellular oxidative stress. Thawed islets were handpicked and centrifuged at 400g for 3 min. The islets were washed with PBS and resuspended in 990 mL of PBS containing 10 mL of 10 mg/mL DCFDA dye for 20 min at room temperature. After centrifugation, the islets were washed twice with PBS and resuspended in 50 mL of PBS. Finally, the slides were viewed under fluorescence microscopy (Olympus), and the intensity of the green color was measured using ImageJ software.

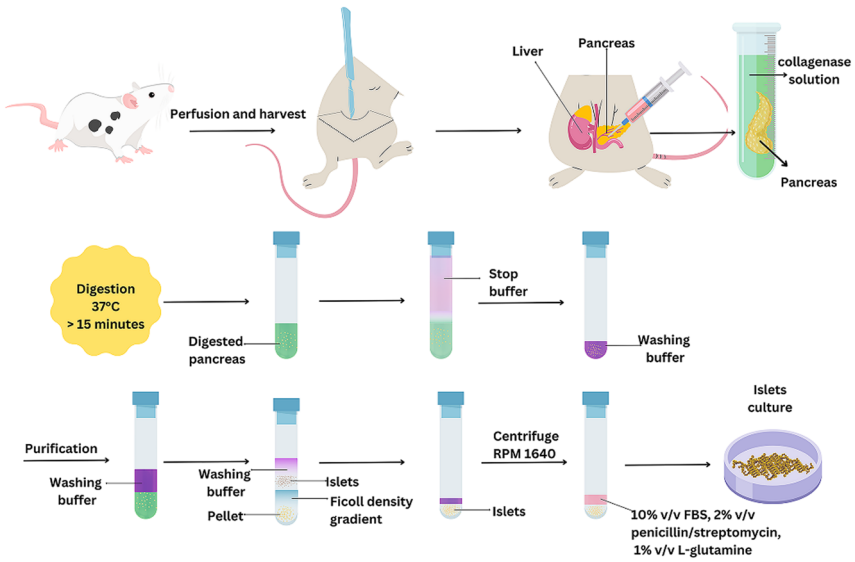
Islet transplantation in diabetic mice

A single injection of alloxan (120 mg/kg) intraperitoneally into 8–10-week-old male NMRI mice was used for diabetes induction. Diabetes was confirmed by three consecutive fasting blood glucose levels greater than 250 mg/

(See figure on next page.)

Fig. 1 Islet isolation and freeze/thaw procedure. **a** Schematic illustration of islet isolation from rat pancreas. The method for isolation of rat pancreatic islets consists of three main steps: perfusion, digestion, and purification. A collagenous enzyme is injected into the common bile duct in the perfusion phase. Then, the pancreas is removed from the rat's body and transferred into a conical tube. The digestion step began when the enzyme solution reached 37 °C to remove islets from the exocrine tissue and was stopped by adding a stop buffer and washing steps to prevent excessive digestion of islets, which could degrade the islet capsules. Finally, the islets were purified using density gradients and multiple washing steps to separate the islets from the non-islet tissue and cultured to maintain cell viability. **b** Schematic of islet freeze/thaw. The cryopreservation media was added to the isolated islet, cooled to − 20 °C, and then transferred to a liquid nitrogen tank. **c** Table of thawed process. Cryopreserved mouse, pancreatic islet samples, were removed from liquid nitrogen and transferred to a water bath on dry ice. Rapid thawing in a 37 °C water bath was performed until the last ice crystal was visible in the vial, and dilution of the cryoprotectant with culture medium (RMPI + 10% FBS) was conducted over 24 min as described in the table

(a)



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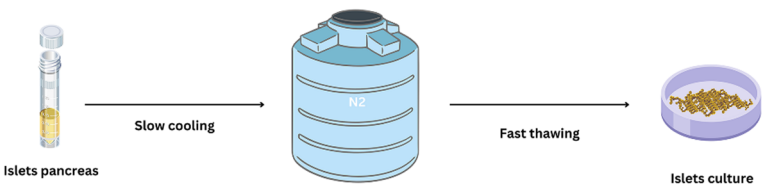

(c)

Time after thawing (min)	Addition of CM (µl)	Concentration of CPA (% original concentration)
-	-	100
0	100	80
2	〃	66.7
4	〃	57.1
6	〃	50
8	200	40
10	〃	33.3
12	〃	28.6
14	〃	25
16	400	20
18	〃	16.7
20	〃	14.3
22	〃	12.5
24	800	10

Fig. 1 (See legend on previous page.)

dL measured with a glucometer (Accu-Chek, Roche, USA). The diabetic mice were divided into five groups: 1) untreated control, 2) fresh islets, 3) trehalose + PLP frozen islets, and 4) DMSO frozen islets. Islets (fresh and frozen) were transplanted into the epididymal fat pad of mice after anesthetization with ketamine (90 mg/kg) and xylazine (4.5 mg/kg). An incision was made through the peritoneal wall in the midline, closed to the genital area. Grasped the EFP from the abdominal cavity with forceps and removed them. At least 500 IEQ rat islets were transplanted (n = 6 recipient mice per group). Subsequently, the muscle layer was closed with 5–0 vicryl, and the skin with silk suture. Xenograft function was evaluated by monitoring fasting blood glucose levels in each recipient during 28 days post-transplantation.

Transplanted islets functionality tests

Fasting blood sugar (FBS) levels were measured for four weeks after transplantation by drawing blood drops from the tail veins of the mice and analyzing them with an Accu-Chek blood glucose meter. On day 14 after transplantation, the intraperitoneal glucose tolerance test (IP-GTT) was performed in all groups, and blood glucose levels were measured at 0, 15, 30, 60, and 120 min after injection of 2 g/kg D-glucose following a 6-h fasting period. The area under the curve (AUC) for IP-GTT was measured. Serum C-peptide levels were measured four weeks after transplantation using a rat/mouse C-peptide ELISA kit. At the end of the experiment and the follow-up period, we killed all mice (experimental and control mice) involved in the experiment by humane euthanasia. The mice were transferred to the carbon dioxide chamber: Carbon dioxide is introduced very slowly into the chamber for 5–10 min. This leads to respiratory arrest.

Statistical analysis

Data were presented as mean ± standard deviation (SD). Statistical significance was tested by GraphPad Prism 6.0 (GraphPad Software, Inc., San Diego, CA) using an unpaired t-test or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc analysis. Differences were considered significant at *P*-values < 0.05.

Arrive guidelines 2.0

All aspects of this animal research have been reported in line with the ARRIVE guidelines 2.0.

Results

Chemical synthesis and characterization of PLP

The characterization results are fully presented in our previous study [34] and S1.The PLP polymer in acidic form was identified by ^{1}H NMR spectroscopy in d6-DMSO, as shown in S1.b. ATR-FTIR analysis was done for preliminary chemical characterization of the synthesized PLP. The presence of the functional group that appears in the region of 2500–3700 confirms the active group. On the other hand, the stretching C–N, bending N–H, stretching C = C, and stretching N–H groups are observed in the regions 1222, 1641, 1641, and 3412, respectively. The molecular weight obtained in this study was 23.6 ± 5.6.

Morphology and viability of islet following cryopreservation

Pancreatic islets were thawed one month after freezing and examined by light microscopy (Olympus). The collagen capsules around the islets are rounded and indiscernible from fresh islets. Some hypoxic centers were observed in the DMSO group, which were much less in the groups with modified cryopreservation media (Fig. 2a). The viability of thawed cryopreserved islets was determined compared to fresh islets. The viability of islets in the fresh islet, DMSO, and trehalose groups was 96%, 80%, and 96.25%, respectively. The viability of islets in the trehalose group was significantly higher than that in the DMSO group ($p < 0.05$). There was also no significant difference between fresh islets and the trehalose group (Fig. 2b and c).

Optimized cryopreservation media improved in vitro insulin release in thawed islet

The GSIS test was performed to determine the islet functionality in vitro. The stimulation index (ratio of secreted insulin in high glucose to secreted insulin in low glucose) in fresh islet, DMSO, and trehalose group was 1.57, 1.12, and 1.26, respectively. These data confirm that cryopreserved islets in both groups are viable and functional

(See figure on next page.)

Fig. 2 In vitro studies on freeze/thaw islets. **a** Light microscopy of the thawed islets after one month of freeze. It is in three groups: I) fresh islet, II) DMSO, and III) trehalose + PLP. **b** fluorescent microscope images of the thawed pancreatic islets stained with fluorescein diacetate/propidium iodide (FDA/PI), I) fresh islets, II) DMSO and III) trehalose + PLP. **c** Quantified live/dead staining in all three groups. **d** The glucose-stimulated insulin secretion (GSIS) assay. The stimulation index (SI) showed that modified cryopreservation media could increase the functionality of islets as much as fresh islets, compared with the DMSO group. Data points were presented as mean ± SD for a six number per group in a single measurement. *$p < 0.05$, **$p < 0.01$. Scale bar: 100 µm

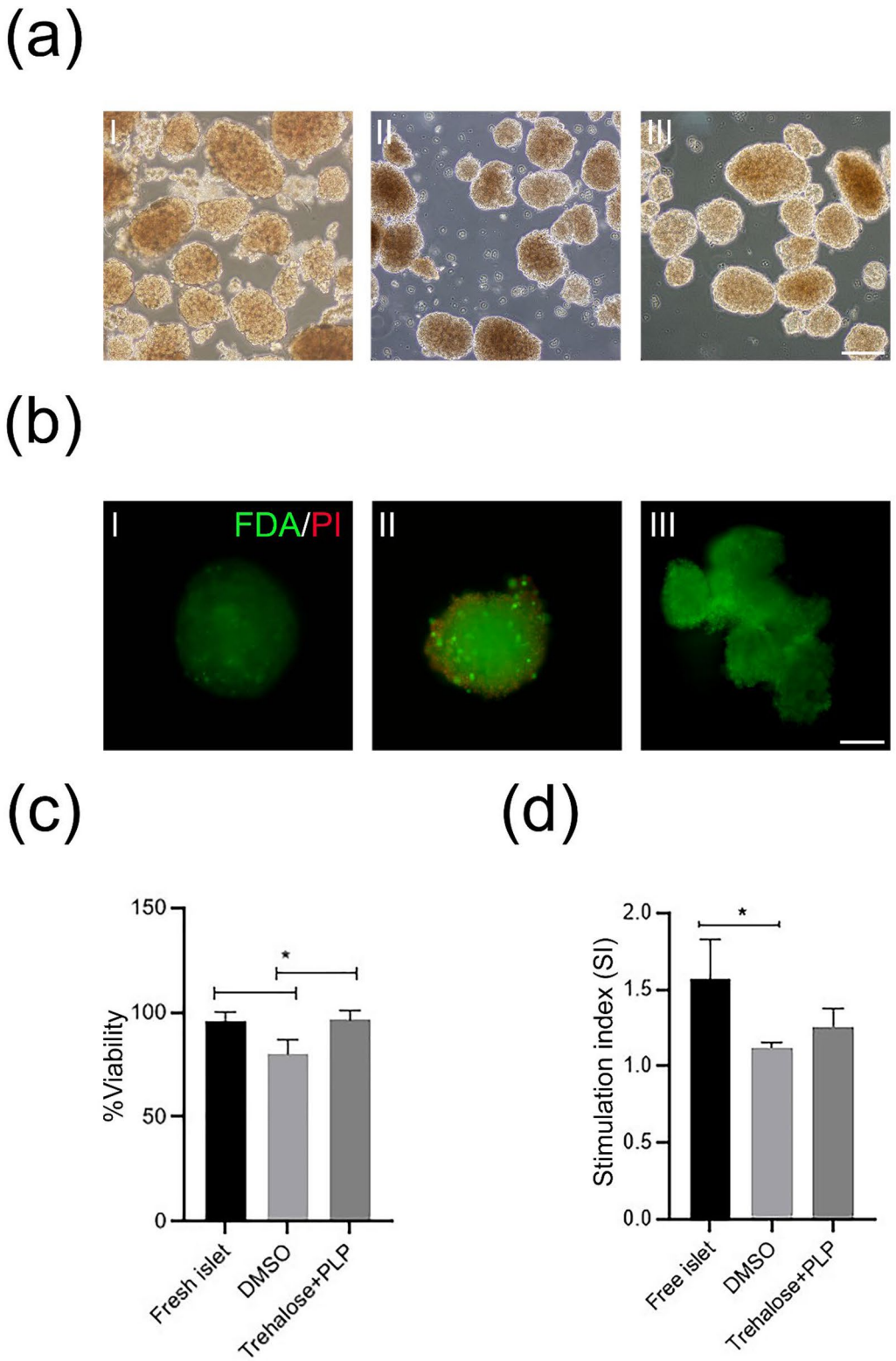

Fig. 2 (See legend on previous page.)

in vitro. The SI of the trehalose group was similar to the fresh islet, but there was a statically meaningful difference between the DMSO group and the fresh islet ($p < 0.05$) (Fig. 2d).

Insulin/glucagon immunostaining and DCFDA staining show the superiority of modified cryopreservation media compared with DMSO

To study the presence of α- and β-cells in thawed islets, the immunostaining of insulin and glucagon was performed. Islets were stained with indicated antibodies that recognize insulin (red) and glucagon (green). The β-cells are localized in the periphery and core of the islets in comparison with α- α-cells scattered in the periphery region (Fig. 3a). For manual quantification of pixel intensities of images, we used the analysis platform ImageJ. No significant differences were found for each cell type between the fresh and trehalose group, as shown in Fig. 3b. Even though the insulin/glucagon-positive cells were observed in the DMSO group, the difference between insulin-positive cells in DMSO and fresh islet was statically meaningful ($p < 0.05$). It shows that the islet function was preserved in both groups after freezing and that the optimized freezing medium was superior to the DMSO group.

DCFDA-stained islets in the trehalose group showed lower levels of intracellular oxidative stress, demonstrated in Fig. 3c, as the green color's intensity increased (Fig. 3d). The quantified differences between the fresh islet/DMSO and trehalose group /DMSO are significant ($p < 0.01$). Estimating intracellular ROS levels demonstrated the potential of modified cryoprotectants by trehalose and PLP in reducing reactive oxygen production, similar to that of fresh islets.

Transplantation of thawed islets could control the blood glucose in the normal range

The islets (fresh and thawed) were transplanted into the epididymal fat pad of alloxan-induced diabetic mice. This site is well-vascularized, and it is easily accessible in comparison with other sites such as portal vein and omentum. These advantages make it suitable for islet transplantation. Fasting blood sugar (FBS) levels were monitored in all transplant recipient mice for one month. The FBS level of diabetic animals treated with fresh and thawed islets in the DMSO and trehalose group decreased to normoglycemia three days after transplantation (greater than 250 mg/dL to 80–110 mg/dL). In both groups of receiving thawed islets (DMSO and trehalose), FBS was in the normal range until the end of the follow-up (Fig. 4a).

The results confirm that freezing does not adversely affect islet function in vivo, and the FBS in the transplanted mice is within the normal range. This test was performed in the fourth week after transplantation to evaluate the response of transplanted islets to the glucose used by the body. The IP-GTT graph results confirm the reaction of transplanted islets to the injection of high amounts of glucose (Fig. 4b). However, the blood glucose levels returned to the normal range after 120 min, which was the same as the healthy mice. These data showed the transplanted thawed islets were functional. The AUC graph of the IP-GTT data showed no significant difference between the DMSO and trehalose groups in this regard (Fig. 4c).

Discussion

At present, diabetes represents a worldwide problem, and relief from this chronic disease is, hence, very urgent. In the case of diabetes, the transplantation of pancreatic cells has been tried due to the deficiency of insulin production. The front runner in such attempts is represented by the transplantation of pancreatic islets. Major challenges include attacks from the immune system and the rather limited availability of these cells. While numerous studies have concentrated on overcoming strategies for immune responses, these are further exacerbated by shortages of organs and cold-chain restrictions that hinder the storage of cellular products. [35–38]Further complications arise due to time constraints between islet isolation and transplantation since there is a narrow window to efficiently match donors and recipients.

[12]. One new technique involves pooled islet transplantation, wherein islets from multiple pancreases are combined. This approach avoids the organ shortage problem and improves the success of grafting, but it also requires very specific islet isolation and purification [39].

CPAs are indispensable in islet preservation for transplantation, and there are two major kinds: permeating and non-permeating. Permeating CPAs, such as EG and DMSO, are smaller in size and can penetrate cells,

(See figure on next page.)

Fig. 3 In vitro studies on freeze/thaw islets. **a** Immunostaining of insulin/glucagon in a thawed islet in I) fresh islet, II) DMSO, and III) trehalose + PLP groups. **b** Percentage of insulin and glucagon-positive cells in islets showed the efficacy of optimized cryomedia. **c** DCFDA staining of thawed islets in all three groups for assessment of ROS and **d** quantified date of staining, which shows the significant differences between DMSO and trehalose + PLP cryomedia. Data points were presented as mean ± SD for a six number per group in a single measurement. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Scale bar: 200 µm

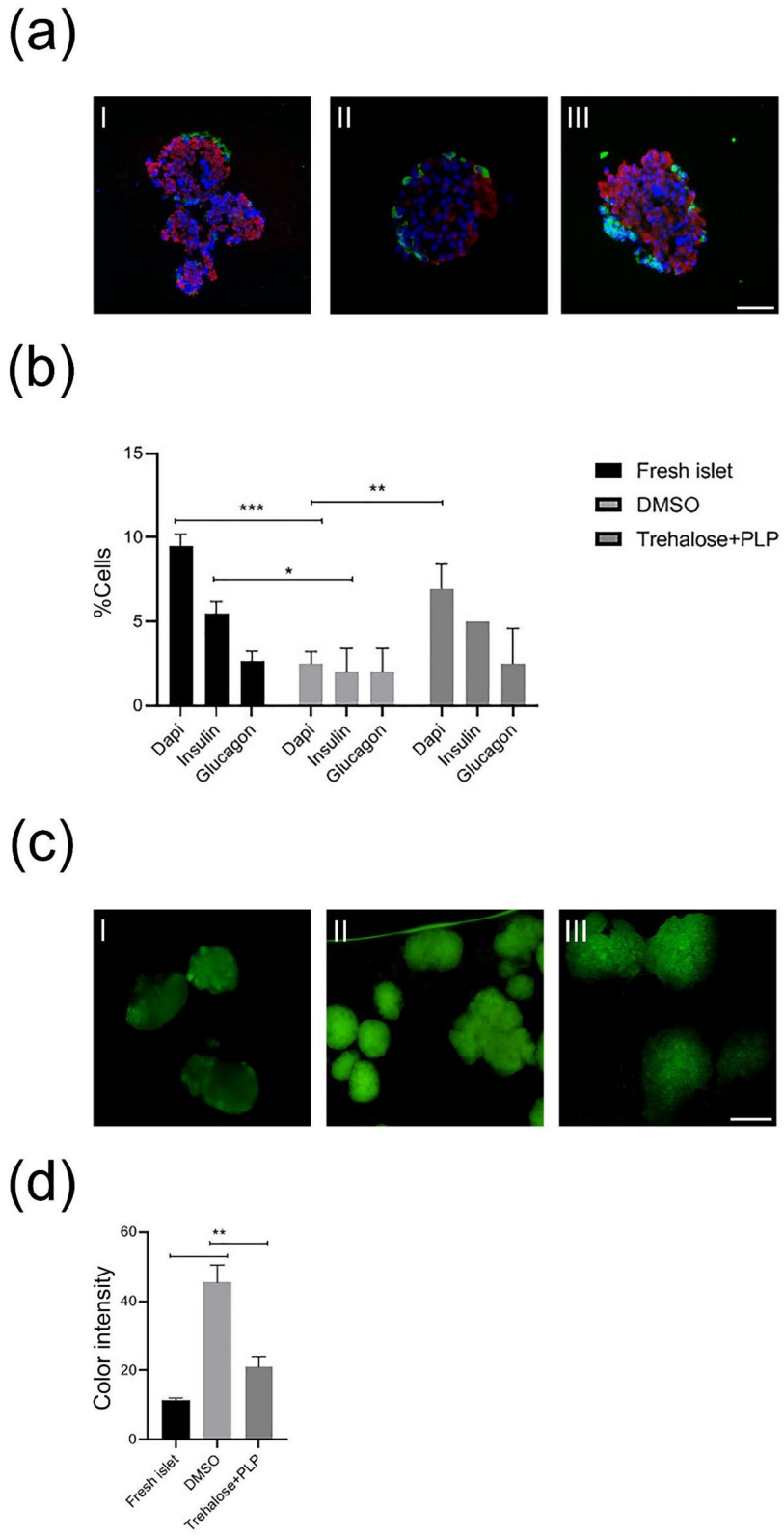

Fig. 3 (See legend on previous page.)

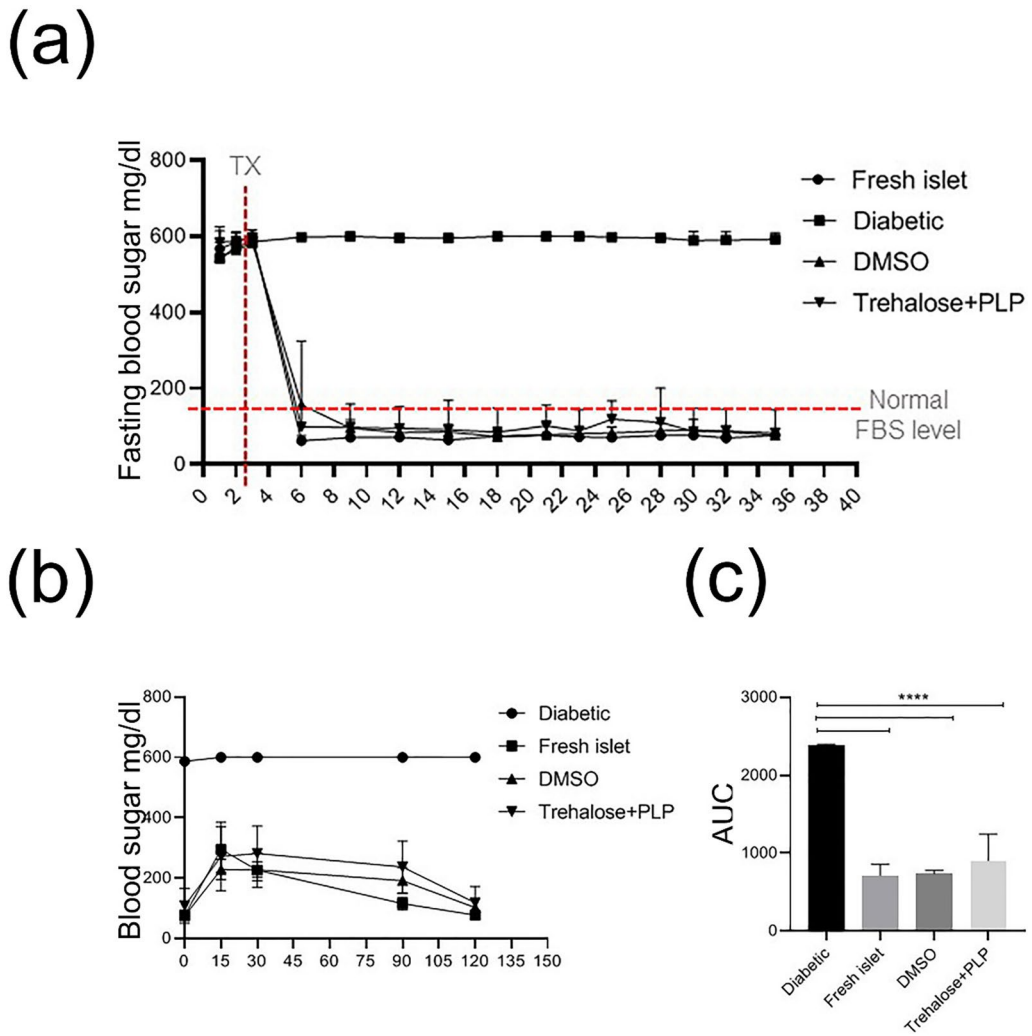

Fig. 4 Biological function thawed islets transplanted in diabetic mice. **a** Blood glucose levels in healthy and diabetic mice. The graphs represent different groups, including healthy mice, surgical operated but non-treated diabetic mice (SHAM) and diabetic mice transplanted either with fresh islets or thawed islets in DMSO and trehalose + PLP cryopreservation media. Data shows normoglycemia in all groups that received islets. **b** Results of the intraperitoneal glucose tolerance test (IP-GTT) from diabetic, healthy and treated diabetic mice one-month post-transplantation. Like the healthy group, treated groups showed no significant delays in blood glucose uptake after injection. **c** Area under the curve (AUC) of the IP-GTT one month after transplantation. Data points are presented as mean ± SD for a minimum of six independent recipients per group at each time point. TX: transplantation, ****$p < 0.0001$

though they may be toxic to them. Non-permeating CPAs include sugars like trehalose and polymers like polyvinylpyrrolidone (PVP) and) Polyethylene glycol (PEG), which act extracellularly and are generally less toxic [40, 41]. Optimum CPA concentrations are of great significance to reduce damage to the cells and preserve the cells effectively [42, 43]. For instance, islet survival can be compromised by stress due to the defense mechanisms present in islets. Similarly, other factors like glucotoxicity and endoplasmic reticulum strain resulting from insulin production demands can contribute to islet apoptosis. Over the past decade, researchers have explored CPAs that benefit against cryopreservation-induced apoptosis in islets [44]. Both permeating and non-permeating substances can harm cells when present in concentrations, although non-permeating substances tend to have an impact. This unfavorable characteristic of cell-penetrating agents increases cell mortality and decreases cell yields. The choice between these two categories depends on what kind of material is being preserved by this method and what it will be used for afterwards. Some options could have toxicity concerns that would rule them out for particular cell or tissue types, while others might be more effective with specific ones [45, 46]. The

concentration of cryoprotectant used is furthermore crucial. Insufficient cryoprotectants may not supply sufficient protection, while excessive cryoprotectants lead to toxicity and generate cellular impairment [18]. The optimal concentration of cryoprotectant must be specified for each type of biological material. Permeating cryoprotectants are mainly applied for cryopreserving cells or tissues susceptible to freezing impairment, such as stem cells and other primary cells [47]. Non-permeating cryoprotectants are constantly used for cryopreserving larger tissues or organs, like blood vessels, heart valves, and whole organs, where ice formation can cause damage to the structure [47].

To ensure consistency in the cryopreservation process, it is essential to standardize the duration of cell exposure to both permeating and permeating agents during the final packaging stage. In this regard, Zhan et al. conducted a project on creating beta-cell islets using porcine, human, and human stem cell (SC)-derived beta cell (SC-beta). The project involved optimizing the composition of cryoprotectant agents and the loading and unloading conditions for vitrification and rewarming. Their data confirm the viability of the islets was found to be 90.5% for mouse islets, 92.1% for SC beta islets, 87.2% for pig islets, and 87.4% for islets, and viability rates remained unchanged after cryogenic storage for at least 9 months [12]. In another attempt, Dolezalova et al. extend a maiden cryopreservation method combining DMSO with trehalose pre-incubation to attain ameliorated cryosurvival which resulted in improved ATP/ADP ratios and peptide secretion from β-cells, preserved cAMP response, and a gene expression profile consistent with improved cryoprotection[48]. LA and colleagues [49] obtained viable cells after cooling in 10% dimethyl sulfoxide and 6% hydroxyethyl starch at 1°C/min to −40°C, storage in liquid nitrogen, rapid thaw, and removal of cryoprotectants by serial dilution, which maintained membrane integrity, transcriptome, and functional phenotype. In this work, we have investigated the efficacy of trehalose associated with PLP as a cryoprotective strategy compared to mainstream usage of DMSO. We did find that both cryoprotectants preserved islet viability and function. However, trehalose has a clear nontoxic profile, at least for possible employment as a safer alternative in clinical uses [50, 51].

The viability of islets cryopreserved with trehalose was significantly higher than that of DMSO (96.25% vs. 80%; $p < 0.05$). Importantly, trehalose-preserved islets showed no significant difference in viability compared to fresh islets (96%). Morphological observations revealed fewer hypoxic centers in trehalose-treated islets than in the DMSO group, aligning with higher overall viability (Fig. 2c). These findings suggest trehalose effectively preserves islet structure during cryopreservation. These results show that trehalose can protect against cryopreservation-induced damage effectively.

The glucose-stimulated insulin secretion (GSIS) assay showed that trehalose-preserved islets had a stimulation index (SI) of 1.26, comparable to fresh islets (1.57), with no significant difference observed between the two. In contrast, the DMSO group demonstrated a lower SI of 1.12, which was significantly different from the fresh islets ($p < 0.05$, Fig. 2d). These results confirm that trehalose maintains β-cell responsiveness to glucose stimulation more effectively than DMSO. The percentage of insulin-positive cells in trehalose-preserved islets closely matched fresh islets, with no statistically significant difference. However, the DMSO group exhibited significantly fewer insulin-positive cells than fresh islets ($p < 0.05$, Fig. 3b). This underscores trehalose's superior preservation of β-cell populations compared to DMSO.

Fasting blood sugar (FBS) levels in diabetic mice normalized to 80–110 mg/dL within three days of transplantation in both the trehalose and DMSO groups. This trend persisted throughout the 28-day follow-up period (Fig. 4a). The intraperitoneal IP-GTT showed similar glucose clearance patterns in trehalose- and DMSO-preserved islets, with no significant differences in the area under the curve (AUC, Fig. 4c). However, it is noteworthy that DMSO-preserved islets demonstrated inferior functional metrics compared to fresh islets, whereas trehalose-preserved islets closely mirrored fresh islet performance.

Also, during cryopreservation, one crucial step is to select the rates at which cooling and thawing occur.

Wakabayashi et al. represented using a volumetric rewarming technology called "nano warming," which was the inductive heating of magnetic nanoparticles under an alternating magnetic field. Nanowarming showed uniform and fast rewarming of vitrified islets in large volumes, and the viability of nanowarmed islets was significantly improved. Their data suggest that nanowarming will lead to a breakthrough in the biobanking of islets for transplantation [52]. It is crucial to prevent the formation of ice inside cells to ensure cryoprotection. This requires paying attention to how water moves across membranes throughout the process.

The current approach for thawing involves shaking the samples in a water bath at 37 °C and removing most of the CPA, as shown in Fig. 1c.

Since ROS can potentially damage pancreatic islets, excessive production of ROS can overwhelm the defense mechanisms in beta cells, resulting in stress. This stress disrupts the processes and can lead to dysfunction in beta cells [53, 54].

Moreover, ROS can hinder insulin secretion by impacting steps in insulin release. They can disrupt ATP generation, interfere with calcium influx, alter the movement of insulin granules, and decrease insulin exocytosis, ultimately leading to insulin release in response to glucose stimulation [55]. Additionally, ROS-induced oxidative stress can trigger apoptosis in beta cells, causing a decrease in their mass. This reduction in beta cell mass significantly contributes to the development and progression of type 2 diabetes, where insulin secretion cannot compensate adequately for insulin resistance. The mechanisms through which ROS-induced damage occurs involve processes. For example, ROS can initiate peroxidation, forming reactive lipid species that damage the membrane [56]. This disruption of membrane integrity affects both the function and survival of beta cells. Also, ROS can directly modify proteins by oxidizing amino acid residues, resulting in protein misfolding, aggregation, and loss of function [57, 58]. In this study, quantitative analysis of reactive oxygen species (ROS) levels using DCFDA staining revealed significantly lower oxidative stress in trehalose-preserved islets compared to DMSO-treated islets ($p < 0.01$, Fig. 3c, d). ROS levels in the trehalose group were comparable to fresh islets. The reduction in ROS underlines the protective effect of trehalose against oxidative damage, a major contributor to islet dysfunction.

The data collectively highlight trehalose as a viable and safer alternative to DMSO for islet cryopreservation. While the stimulation index, insulin/glucagon staining, and IP-GTT AUC values showed no significant differences between trehalose and DMSO-preserved islets, the significant advantages of trehalose in reducing ROS ($p < 0.01$) and maintaining viability (96.25% vs. 80%) emphasize its potential for clinical use. These findings advocate for broader adoption of trehalose in islet cryopreservation, with future studies needed to confirm its long-term efficacy and scalability for clinical applications.

Conclusion

In summary, the current study developed the use of trehalose combined with PLP as a cryoprotectant in rat islets and showed in this method that maintaining the viability and functionality of islets is possible. The results were similar between trehalose and the conventional cryoprotectant DMSO in maintaining islet function and glucose responsiveness, but among all cryoprotectants, trehalose possesses the advantage of non-toxicity. This makes trehalose a safer choice in those specific situations when high cell viability is strictly required along with low cytotoxicity.

Trehalose has been considered one such protector due to its multimodal function in cell membrane protection, reduction in oxidative damage, and prevention of ice crystal growth. Trehalose presents a safer alternative to DMSO, which is cytotoxic at high concentrations, with absolutely no dysfunctioning of islets. These studies highlight the effectiveness of trehalose as an alternative cryoprotectant to DMSO in cryopreservation protocols, especially when clinical applications such as islet transplantation are considered.

Further studies on a larger scale of the trehalose-based cryopreservation protocol have to be elaborated, in fact, together with clinical trials, in sight of verification of long-term safety and effectiveness. Indeed, if this method proves to be valid, it can significantly contribute to the field of islet transplantation as a whole because the quality and availability of preserved islets would improve, and with that, the curing of people suffering from diabetes. This study confirms again the potential of trehalose in non-toxic cryopreservation and opens new horizons for safer and fully effective methods of islet preservation.

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Author contributions

Z.I. and E. H. designed the study. Z.S. performed most of the experiments and Z.S and R.A wrote the manuscript. Z.I., E. H., and H.D., supervised the project, interpreted the data, provided financial and administrative support, discussed the results, and approved the manuscript. L. t. collaborated in the preparation and synthesis of some materials.

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Availability of data and material

The datasets used and/or analyzed are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Title of the approved project; Optimizing the "cryopreservation" environment using the combination of trehalose and PLP biopolymer in order to increase the survival and viability of rat pancreatic islets. Name of the institutional approval committee; Kermanshah University of Medical Sciences. Animal experiments were approved by the ethics committee of the Kermanshah University of Medical Sciences (ethical code: IR.KUMS.REC.1398.257) and performed in accordance with the university's guidelines and the Royan Institute Ethics Committee (IR.ACECR.ROYAN. REC.1397.2.27) approval. Approval number; IR.KUMS.REC.1398.257. Date of approval. February 3, 2020.

Consent for publication

All authors confirm their consent for publication.

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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