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ORIGINAL DATA

High Recovery of Functional Islets Stored at Low and Ultralow Temperatures

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■ Abstract

BACKGROUND: Poor recovery of islets upon cryopreservation is the main hurdle in islet banking. Pancreatic islets have a poor antioxidative defense mechanism, and exposure of islets to low temperature leads to oxidative stress. **AIM**: We aimed to investigate whether known compounds such as metformin, γ aminobutyric acid (GABA), docosahexanoic acid (DHA), or eicosapentaenoic acid (EPA) alone or in combination are capable of reducing oxidative stress for better islet recovery upon storage at suboptimal temperatures. **METHODS**: Islets isolated from mouse pancreas were stored at low temperature (4°C) for 15 days and at ultralow temperature (-196°C) for 30 days with or without additives. After revival from cold storage, islets were assessed by using three methods: (1) specificity by dithizone (DTZ), (2) viability by fluorescein diacetate/propidium iodide (FDA/PI) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and (3) functionality by glucose-stimulated insulin secretion (GSIS). The oxidative status of the islets stored at suboptimal temperatures was determined by both intracellular free radical release (fluorometric analysis) and lipid peroxidation (enzymatic determination). **RESULTS**: Supplementation with additives led to an improvement in islet survival upon storage at suboptimal temperatures, without depletion of insulin secretory activity, which was comparable to that of controls. The additives acted as cryoprotectants and antioxidants as revealed by high recovery of viable islets and reduction in total reactive oxygen species (ROS) and malonidealdehyde (MDA), respectively. **CONCLUSIONS**: Our results demonstrate for the first time that supplementation with EPA, DHA, and metformin may lead to higher islet recovery from -196°C storage, enabling proper islet banking.

Keywords: diabetes · pancreatic islets · insulin secretion · storage · oxidative stress · reactive oxygen species · transplantation · cryopreservation · metformin

1. Introduction

Diabetes mellitus is one of the most prevalent metabolic diseases characterized by high blood glucose due to inadequate insulin production and/or insulin function. Islet auto- or allotransplantation has been the proven line of treatment for type 1 diabetes. However, the scarcity of islets and the transportation hazard are major obstacles in their usage for clinical purposes.

There are several reports on successful cryopreservation of islets. However, the recovery of viable islets was around 60-70% only [1-3]. There are a few reports on storage of islets at low temperature claiming survival of up to 48 hours only [4, 5]. Different concentrations of taurine in the freezing mixture have been shown to improve the viability of cryopreserved islets [6]. The decrease in survival rate of islets is due to cellular injury mediated by cryopreservative dimethyl sulfoxide (Me_2SO) and enhanced oxidative stress that occurs during cryopreservation. Since the islets possess poor antioxidant defense [7], molecular damage mediated by oxidative stress during cryopreservation is substantial.

Glucotoxicity is one of the causes for β-cell apoptosis *in vivo* [8]. There is also an enormous increase in endoplasmic reticulum stress due to the high demand of secretory insulin. In combination

with oxidative stress, these factors eventually lead to β-cell dysfunction and destruction [9]. However, oxidative stress-induced damage to islets differs from species to species [10]; accordingly the treatment needs to be different.

In this study, we identified a couple of additives that play a key role in maintaining the integrity of mouse pancreatic islets at ultralow temperatures. These additives include:

- Metformin, an insulin sensitizer [11].
- GABA, a neuroprotective agent [12].
- Polyunsaturated fatty acids such as eicosapeexaenoic acid (EPA) and docosahexanoic acid (DHA) [13].

Metformin is a commonly prescribed antidiabetic drug, and has a direct beneficial effect on β-cells [14]. GABA has been shown to exert protective and regenerative effect on islet β-cells leading to a reversal of diabetes [15]. A recent study demonstrated that GABA regulates survival and replication of β-cells [16]. Omega 3 fatty acids are polyunsaturated fatty acids, which are essential for normal metabolism [17]. EPA and DHA are known to reduce adiposity, prevent high fat-induced obesity [18], exert anti-inflammatory action [19], and reduce cardiovascular complications [20]. Their role as cryoprotectants in islet storage has not yet been explored.

The aims of the present study were to evaluate the impact of the aforementioned additives on the survival of islets and retention of their functionality at low (4°C) and ultralow (-196°C) temperatures, and to decipher the possible mechanism of islet survival. Furthermore, we examined the insulin secretagogue activity of the additives on freshly isolated islets. Our data revealed that most of these additives act as insulin secretagogues, and their supplementation in cryo-mixture significantly decreased the oxidative stress of cryopreserved islets, leading to enhanced survival and functionality.

2. Materials and methods

2.1 Animals

Eight to ten week old Swiss albino male mice weighing 20-25 g were procured from the Department of Livestock Production and Management, Veterinary College, Bangalore. All animal experiments were performed in accordance with the institutional ethical committee for animal experiment guidelines and regulations. Animals were kept in animal cabinets and allowed to have free access to water and food.

Abbreviations:
ANOVA – analysis of variance
DHA – docosahexanoic acid
DMSO – dimethyl sulfoxide (Me_2SO)
DNA – deoxyribonucleic acid
DTZ – diphenylthiocarbazone
EPA – eicosapentaenoic acid
FDA – fluorescein diacetate
GABA – gamma aminobutyric acid
GSIS – glucose-stimulated insulin secretion
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
KO-DMEM – knockout Dulbecco's modified Eagle's medium
KRBH – Krebs-Ringer bicarbonate HEPES
MDA – malondialdehyde
MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NO – nitric oxide
OD – optical density
ROS – reactive oxygen species
PBS – phosphate buffer saline
PI – propidium iodide
SD – standard deviation

2.2 Isolation of islets

Islets were isolated following a previously described protocol [8]. Mice were sacrificed and the pancreas obtained was washed twice with phosphate buffer saline (PBS) containing penicillin streptomycin (GIBCO, Life technologies, Darmstadt, Germany). The pancreas was then minced thoroughly in a glass petridish and digested using a cocktail of 0.1% of collagenase-IV, soyabean trypsin inhibitor 0.1% (STI), and 1% BSA (Sigma-Aldrich, St. Louis, USA). The digestion was carried out for 5-7 minutes with frequent shaking; 0.5 ml of fetal bovine serum (FBS; GIBCO, Life Technologies) was added to neutralize the collagenase. The digested tissue was centrifuged at 800 rpm for 10 minutes. Then, the pellet was seeded in a T-25 flask and cultured for 48 hours at 37°C in CO_2 incubator (Hera Cell 240 CO_2 Incubator, Thermo Scientific, Carlsbad, CA, USA) in KO-DMEM (GIBCO, Life Technologies) with 10% FBS. At the end of a 48 h culture, the islets were obtained [21].

2.3 Additives and storage at suboptimal temperatures

In order to investigate the effect of additives on islet survival and retention of their viability following cold storage, the additives were added to the cryopreservation mixture either alone or in

combination. The final concentration of additives was optimized before selection. The concentration used for each additive was: metformin (100 µg/ml), GABA (50 µM and 100 µM), EPA and DHA (1 µM) [22, 23]. In the control set, the islets were stored in complete medium without any additive. For low temperature storage, islets were kept at 4°C, and for ultralow storage at -196°C [24], for 15 and 30 days, respectively. The islets were stored at low temperature in 15 ml falcon tubes with 3 ml of complete medium (KO DMEM + 10%FBS) for each set of additives. The islets at 4°C were spun at 800 rpm for 5 minutes, and the pellet obtained was re-suspended in fresh medium and seeded in 60 mm uncoated petri dishes (BD Falcon) for the experimental studies.

2.4 Cryopreservation and revival of islets

The islets were cryopreserved following a protocol described earlier [25]. The islets were transferred into cryovials (2 ml) and resuspended in 200 µl of KO DMEM and 10% FBS for the control set. Similarly, separate vials were made with additives, either alone or in combination. For each set of treatment, 100 islets were used. Thereafter, Me_2SO was added stepwise, starting with 100 µl for 5 minutes at 0°C, followed by 100 µl of Me_2SO for 5 minutes at 0°C, and finally 400 µl 3.1M DMSO for 5 minutes at 0°C. After this pre- freezing phase, the cryovials were chilled by a programmable biofreezer at a rate of 1°C/min.

On completion of the freezing program, the cryovials were transferred into liquid nitrogen. After storage in liquid nitrogen for 30 days, cryovials were rapidly thawed at 37°C in a water bath until the sample reached 0°C [26]. One ml of 0.75M sucrose was added to remove the effect of Me_2SO at 0°C in ice slush for 30 minutes. KO DMEM was added to the tubes serially every 5 minutes for the removal of sucrose (1 ml, 2 ml, and then 1 ml). The tubes were then centrifuged at 800 rpm for 5 minutes, and the pellet obtained was transferred to a 60 mm uncoated Petri dish. The revived islets were tested for their specificity, viability, and functionality.

2.5 Diphenylthiocarbazone (DTZ) staining

Islets were stained with dithizone (DTZ) for their specificity [27]. The stock solution of DTZ (10 mg/ml) was prepared in Me_2SO. Ten µl of the stock was dissolved in 1 ml of Krebs-Ringer bicarbonate buffer (pH 7.4) with 10 mM N-2-hydroxyethylpiperazine-N1-2-ethanesulfonic acid, then incubated at 37°C for 5-10 minutes, washed thrice in phosphate buffer solution (PBS), and examined under the microscope for morphological evaluation and positive staining.

2.6 Fluorescein diacetate/propidium iodide staining of revived islets

To determine the viability of freshly isolated and cryopreserved islets with or without additives, islets were stained with cell-permeable esterase substrate, fluorescein diacetate (FDA; Sigma Aldrich, St. Louis, MO, USA) and the cell-impermeable nucleic acid stain, propidium iodide (PI; Sigma Aldrich). The FDA stock (0.67 µM) was made in acetone; 1 mg/ml stock of PI was made in PBS and wrapped with aluminium foil as it is light-sensitive. Islets were seeded in 24 well plates, and incubated with FDA for 2 minutes followed by PI for 30 seconds. Islets were washed with PBS thrice. Then, digitized microscopic images (Nikon Eclipse TE 200-S, Chiyoda-Ku, Japan) were captured at 100 X magnification.

2.7 Colorimetric assessment of viability

To confirm the viability of revived islets, we performed an MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [28] with some modifications. The assay was standardized with freshly isolated islets. MTT stock (5 mg/ml) was made in 1X PBS along with 0.1% Triton X for the permeabilization of islets. The working stock of MTT used was 0.5 mg/ml in each well, and the plates were then incubated for 5 hours in a CO_2 incubator. After incubation, the islets were washed with PBS. Then, 200 µl of DMSO was added to dissolve the formazan crystals, and the islets were incubated for 1 hour in dark.

The plate was read at 560 nm using a spectrophotometer, and a standard curve was plotted (number of islets vs. OD). The standard graph obtained determined the viability and number of islets seeded. The revived islets were transferred into 96 well plates and incubated with MTT (0.5 mg/ml). The viability of islets was normalized by standards of freshly isolated islets.

2.8 Glucose-stimulated insulin secretion (GSIS) assay

Insulin secretion function of freshly isolated and cryopreserved islets was assessed by performing GSIS according to a previously published protocol [29]. The medium-sized islets (4-6 in number)

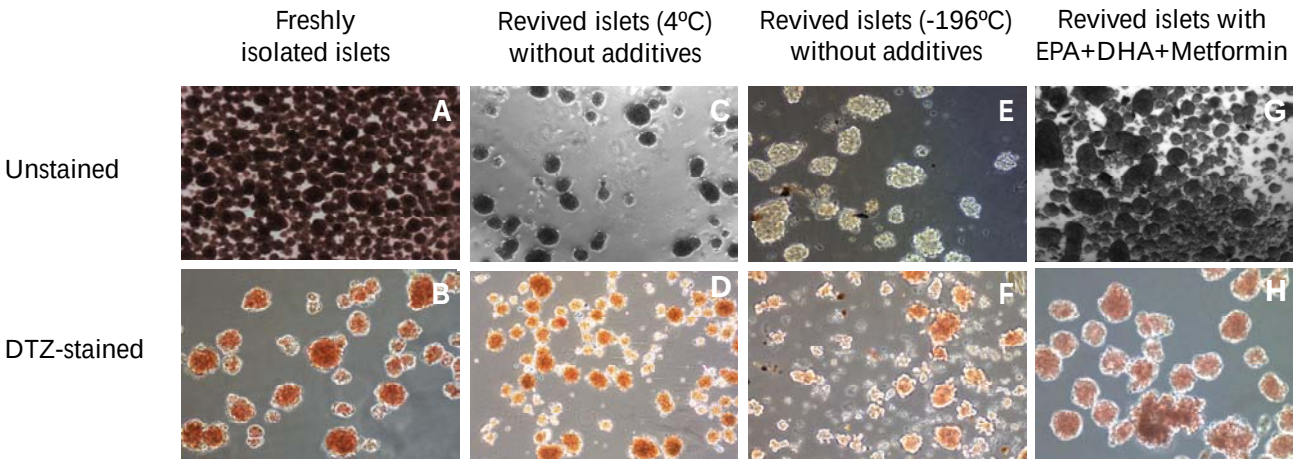

Figure 1. Morphology and purity of freshly isolated and revived islets. The figure shows unstained (upper row) and stained islets (lower row). Islets revived from suboptimal and ultralow temperatures without additives (**C**-**F**) had different morphology and were decreased in number compared with freshly isolated islets (**A**, **B**). Images **G** and **H** show islets revived from suboptimal temperature (4°C), where additives (EPA, DHA, and metformin) were included in the storage mixture. Morphology and population size of the revived islets were similar to that of freshly isolated islets.

ranging from 100-150 µm, were handpicked and added to 24 well plates (Nunc A/S) in triplicate. They were then washed with 250 µl PBS thrice and glucose-starved in Krebs-Ringer bicarbonate buffer (pH 7.4), supplemented with 10 mM HEPES (now called KRBH, without glucose) at 37°C in a CO_2 incubator for 1 hour. This procedure induced the stabilization and glucose starvation process.

The supernatant was discarded, and the islets were subjected to low glucose (2.5 mmol/l) for basal stimulation for 1 hour. Subsequently, the supernatant was collected and stored at -20°C. The same islets were then subjected to high glucose (16.7 mmol/l) for 1 hour in a CO_2 incubator. The supernatant was collected again and stored at -20°C until further use. Furthermore, the insulin secretagogue function of the additives was assessed in the presence of low (2.5 mmol/l) and high glucose (16.7 mmol/l) using the commercially available mouse insulin ELISA kit (Mercodia, AB, Sweden).

2.9 Fluorescence-based intracellular free radical detection and lipid peroxidation

The status of oxidative stress in cryopreserved islets was determined and compared with that of freshly isolated islets. The oxidative stress of the islets stored at suboptimal temperatures with or without additives was assessed using a fluorescence-based intracellular ROS detection method and by estimating the concentration of malondialdehyde (MDA), a by-product of lipid peroxidation. The formation of total ROS, superoxide ion, and nitric oxide was determined using a reactive oxygen species detection kit (ENZ-51011, Enzo Life Sciences, Farmingdale, New York, USA). The kit was applied according to the manufacturer's protocol.

Digitized microscopic images (Nikon Eclipse TE 200-S, Chiyoda-Ku, Japan) were captured at 100 X magnification in six different fields, and the islets' fluorescent intensity was measured following the protocol by Sundar *et al*. [30]. Lipid peroxidation of freshly isolated and stored islets was measured using the Bioxtech MDA-586 assay kit (Oxis International Inc., Foster City, CA, USA); the concentration of MDA was estimated according to the manufacturer's instructions.

2.10 Statistical analysis

All experiments were conducted thrice, and each set of experiments was performed in triplicate. Data were noted as mean ± standard deviation (SD) as an average of three individual experiments. For statistical comparisons between the different groups, one way analysis of variance (ANOVA) was used followed by Student's unpaired *t*-test. P-values <0.05 were considered to be significant. For statistical analysis, Graph Pad Prism version 5 was used.

3. Results

3.1 Islet morphology and DTZ staining

The isolated islets were of similar size and shape, as can be seen from **Figure 1A**. The total yield of islets per mouse pancreas was around 1000 islets (IC 1000/pancreas). The freshly isolated islets stained crimson red when treated with DTZ, indicating the presence of insulin (**Figure 1B**). In contrast, islets revived from low and ultralow temperature without additives were morphologically dissimilar to the freshly isolated islets and weakly stained with DTZ (**Figure 1C-F**). However, addition of EPA, DHA, and metformin in combination retained the purity of the islets, which stained positively with DTZ (**Figures 1G** and **1H**).

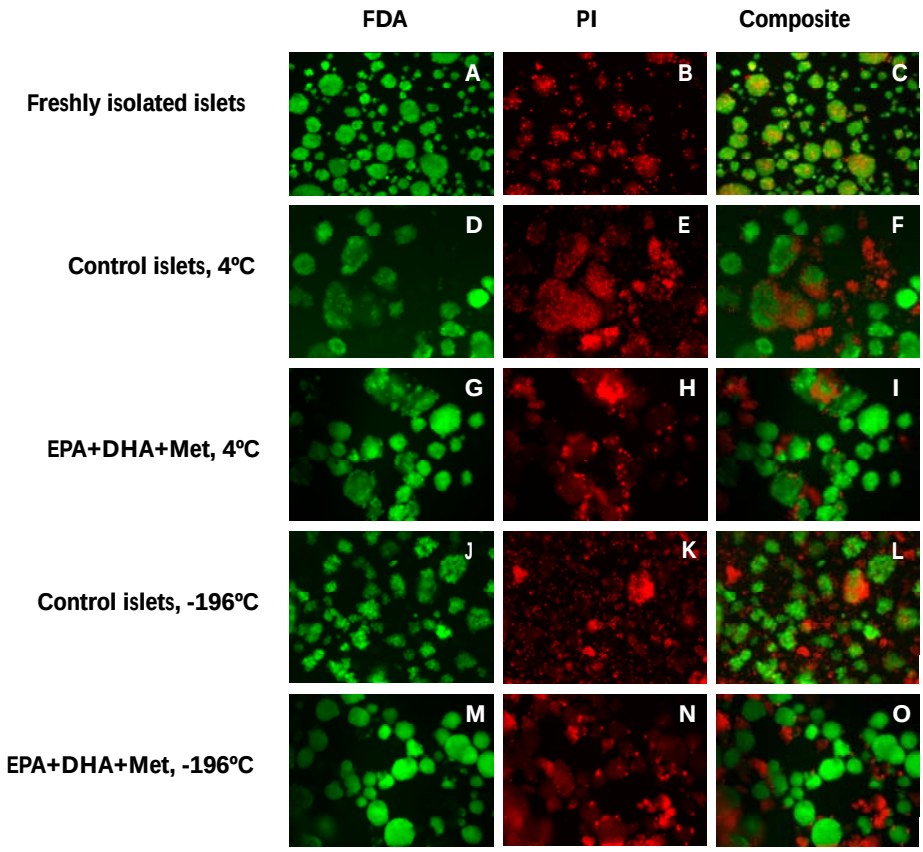

Figure 2. Viability of islets stored at suboptimal and ultralow temperature evaluated by FDA/PI staining. Green fluorescent color denotes viable, red fluorescent color dead islets. Merged images show both viable and dead cells. Images **A-C** represent the staining pattern in freshly isolated control islets. The islets were almost all viable indicated by the higher intensity of green compared to red staining. Images **D-F** illustrate the viability of islets stored at 4°C. The number of dead cells was higher than in freshly isolated islets. The viability of islets stored in culture medium with EPA+DHA+Metformin (**G-I**) was improved islets stored at 4°C without additives; the cell death was reduced. **J-L**: Control islets revived from -196ºC had atrophied (shrunken) and lost their morphology. Single cells were more common. The viability of islets stored at -196ºC under addition of EPA+DHA+Metformin (**M-O**) was optimal, with similar morphology and viability to freshly isolated islets.

3.2 Additives preserved the viability of islets stored at suboptimal temperatures

FDA/PI staining. FDA/PI dual staining was carried out to evaluate the viability of freshly isolated and cryopreserved islets with or without additives. Freshly isolated islets stained with FDA/PI showed a high level of green fluorescence and a low level of red fluorescence indicating high viability (Control 1; **Figure 2A-C**). Islets stored at 4°C without any additives (Control 2; **Figure 2D-F**) showed prominent PI-positive cells in the center of the islets indicating that more non-viable cells originated from the core of the islets following cryopreservation. However, when islets stored at suboptimal temperatures were supplemented with EPA+DHA+Metformin, they exhibited similar morphology and viability to those of freshly isolated islets.

MTT assay. To confirm the survival of islets in all treatment groups, MTT was performed. The standard graph was plotted against optical density, and was used for normalization of the treated islets (**Figure 3A**). The untreated control islets showed only 20% viability after revival from low and ultralow temperatures (**Figures 3B** and **3C**). Metformin storage showed 55% ($p < 0.05$) viability,

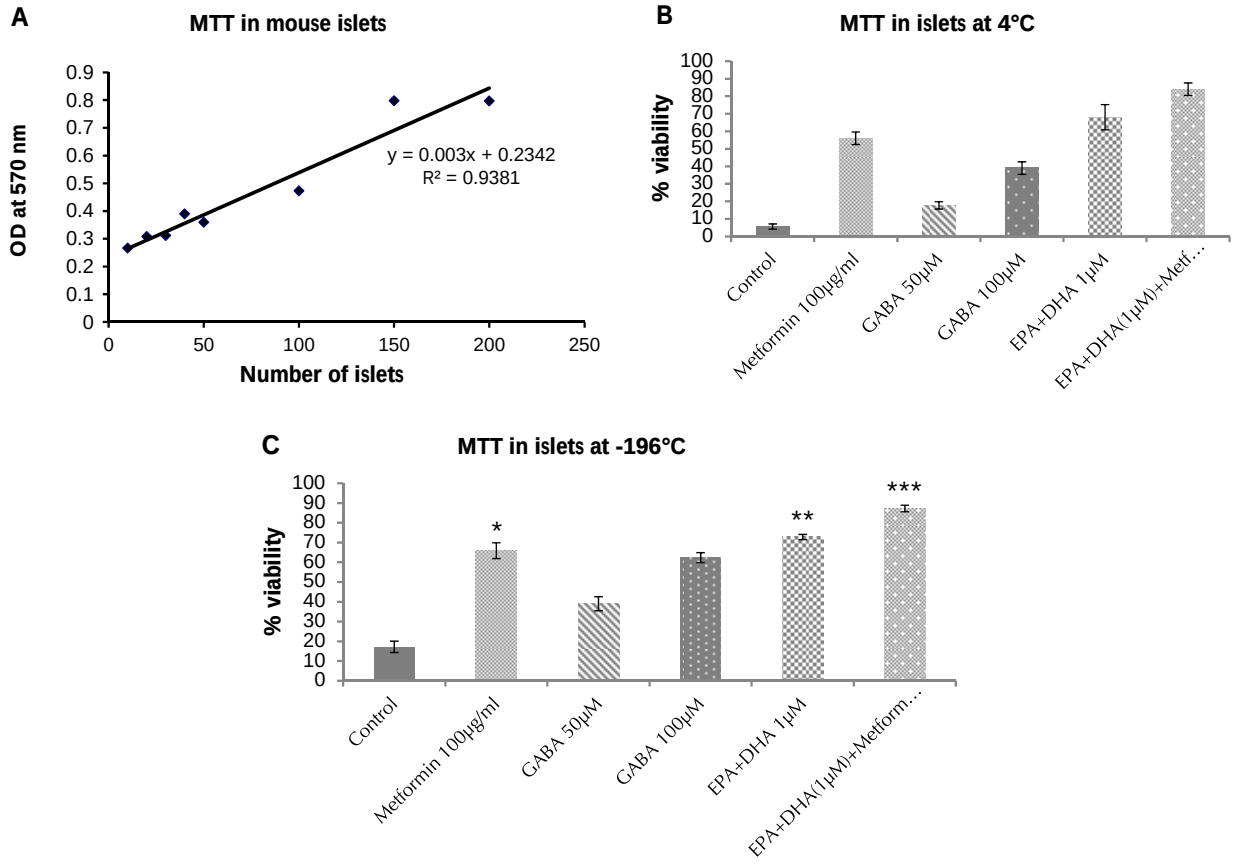

Figure 3. Viability of freshly isolated and stored islets evaluated by MTT. A: MTT standard curve for freshly isolated islets. **B**, **C**: Viability of islets revived from low and ultralow temperatures in all treated and untreated groups. All revived islets stored with additives at low and ultralow temperatures showed an increased viability compared to untreated control islets. The combination EPA+DHA+Metformin achieved the highest viability among all additives tested. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

while GABA supplementation at 50 and 100 µM did not exert any significant effect on islets stored at 4°C (**Figure 3A**). The combinations of EPA+DHA and EPA+DHA+Met significantly increased viability to 70% ($p < 0.01$) and 88% ($p < 0.001$), respectively (**Figure 3A**). Metformin alone enhanced the viability by 65% ($p < 0.05$), while GABA at 50 and 100 µM did have a significant effect on the viability. EPA+DHA and the combination of EPA+DHA+Met increased the viability to 70% ($p < 0.01$) and 85% respectively ($p < 0.001$) (**Figure 3B**).

3.3 Additives preserved insulin-secretory function in islets stored at suboptimal temperature

GSIS was carried out to assess the insulin-secreting function of freshly isolated islets. The islets exhibited normal GSIS and secreted 10 ng/ml of insulin when stimulated with high glucose ($p < 0.001$) (**Figure 4A**). Freshly isolated islets stimulated with metformin (100 µg/ml) had the same response to high glucose as GABA (100 µM), with an insulin secretion of 9 ng/ml ($p < 0.001$). In contrast, GABA (50 µM) and EPA+DHA resulted in a release of 3 ng/ml insulin only, which was statistically not significant when compared with the response to low glucose (**Figure 4A**).

To evaluate the functionality of islets revived from storage at low and ultralow temperatures we stimulated them with low and high glucose. Islets stored without additives responded differently to low and high glucose, indicating their status to be functional. Islets revived from 4°C suboptimal storage showed significantly increased insulin re-

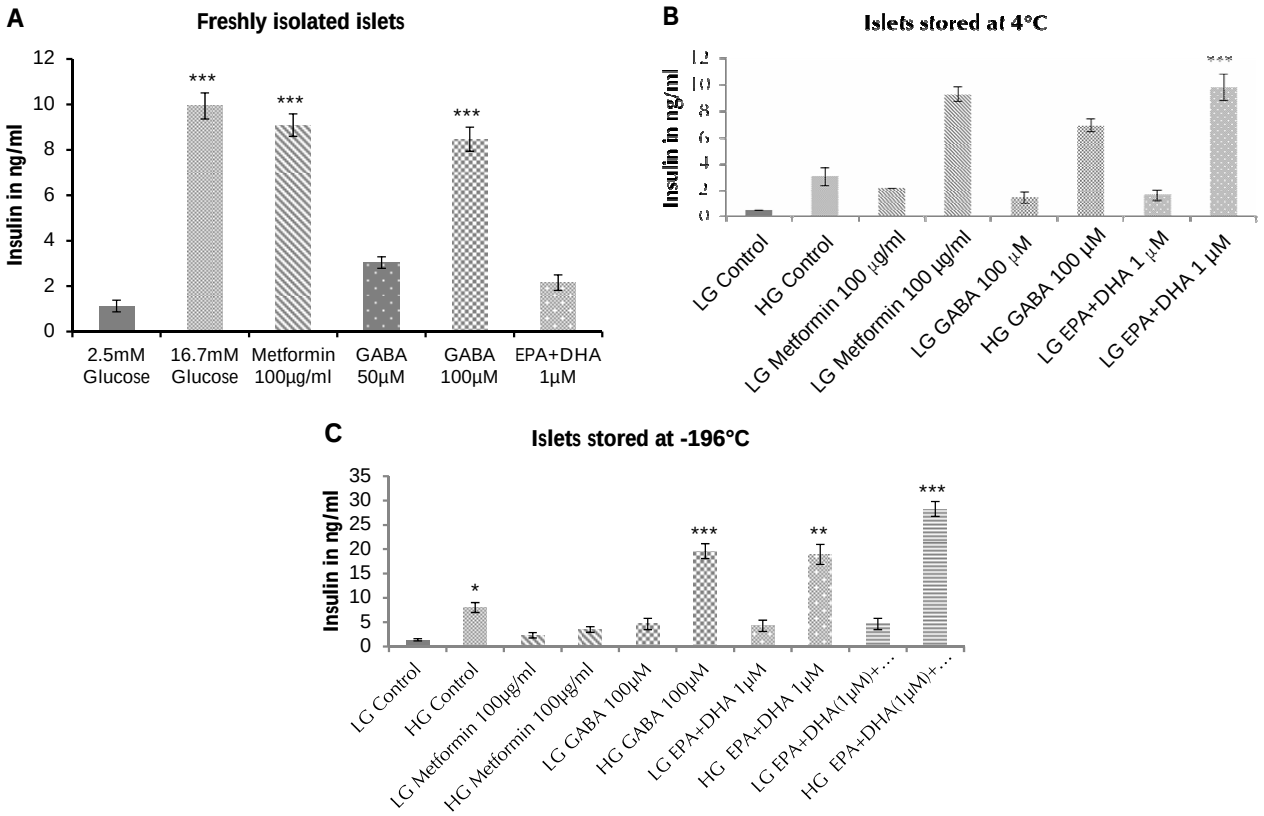

Figure 4. Insulin secretion of freshly isolated and revived islets stimulated with glucose and additives. A: Freshly isolated islets responded well to low and high glucose, with a moderate response to low glucose and a strong response to high glucose. The insulin-stimulating effects of the additives were evaluated by comparing them with the response to low glucose. It was found that the additives acted as insulin secretagogues, with metformin and GABA 100 µM reaching significant response levels similarly to high glucose. **B**, **C**: Islets revived from cold storage were stimulated with low (LG) or high glucose (HG) alone (control) and in combination with the different additives. The insulin-stimulating effects of the additives were evaluated by comparing them with the response of the islets to high glucose alone. Additives significantly increased insulin secretion under high glucose stimulation compared with the control insulin secretion. The stimulation index for freshly isolated islets was >5, while that for islets stored at low and ultralow temperature was >6.

sponses when metformin, GABA 100 µM, or EPA+DHA was added to the storage mixture, with 8 ng/ml ($p < 0.001$) for metformin, 5.5 ng/ml ($p < 0.01$) for GABA 100 µM, and 11 ng/ml ($p < 0.001$) for EPA+DHA (**Figure 4B**). The islets supplemented with different additives either alone or in combination exhibited a stimulation index >5, indicating high glucose sensitivity and high functionality.

Islets revived from storage at -196°C were processed and evaluated equivalently to those stored at 4°C. These islets were also well functional after revitalization. Generally, they showed better response to glucose than those stored at 4°C due to the more reasonable storage temperature. Metformin as cryo-additive did not exert significant effects on insulin secretion, while storage with GABA 100 µM and EPA+DHA resulted in high insulin secretions of 20 ng/ml ($p < 0.001$) and 18 ng/ml ($p < 0.001$), respectively (**Figure 4C**). The combination of EPA+DHA+Metformin even increased insulin secretion to 25 ng/ml ($p < 0.001$) (**Figure 4C**). The stimulation index of the islets stored with the different additives either alone or in combination was >6.

3.4 Additives reduced free radical formation in islets stored at suboptimal temperature

Intracellular ROS levels. The ROS production in untreated control islets was higher than in freshly

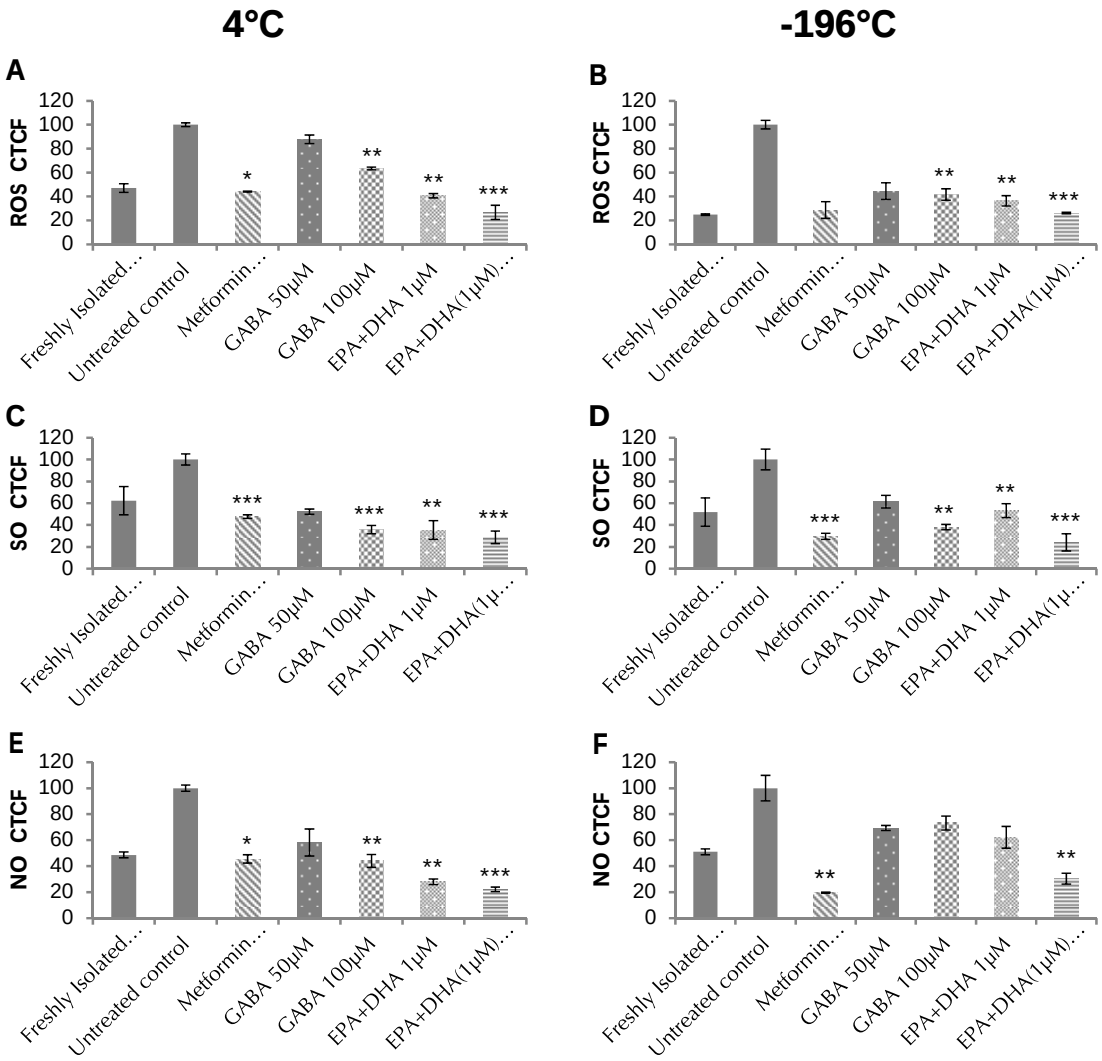

Figure 5. Oxidative stress status of islets revived after storage at suboptimal and ultralow temperature. A, **B**: ROS levels in mouse islets stored at low and ultralow temperatures. **C**, **D**: Superoxide ion levels at low and ultralow temperature. **E**, **F**: Nitric oxide levels of the islets revived from low and ultralow temperature. The additives significantly reduced the oxidative stress in the revived islets. Combination EPA+DHA+Metformin showed maximum reduction in oxidative stress.

isolated islets across all conditions and treatments. When intracellular ROS was assessed, we observed a substantial decrease in ROS formation in the following order (**Figure 5A**):

1. GABA 50 μM: 20%
2. Metformin: 70% ($p < 0.05$)
3. GABA 100 μM: 40%
4. EPA+DHA: 50% ($p < 0.01$)
5. EPA+DHA+Metformin: 80% ($p < 0.001$)

The reduction rates in ROS levels in islets revived from -196°C were as follows (**Figure 5B**):

1. Metformin: 50%
2. GABA 50 μM: 55%
3. GABA 100 μM: 60% ($p < 0.01$)
4. EPA+DHA+Metformin: 60% ($p < 0.01$)
5. EPA+DHA: 70% ($p < 0.01$)

Superoxide anion levels. **Figure 5C** demonstrates the superoxide anion levels in islets stored at low temperature with additives. The following reductions were found:

1. Metformin: 20%
2. GABA 50 μM: 40%

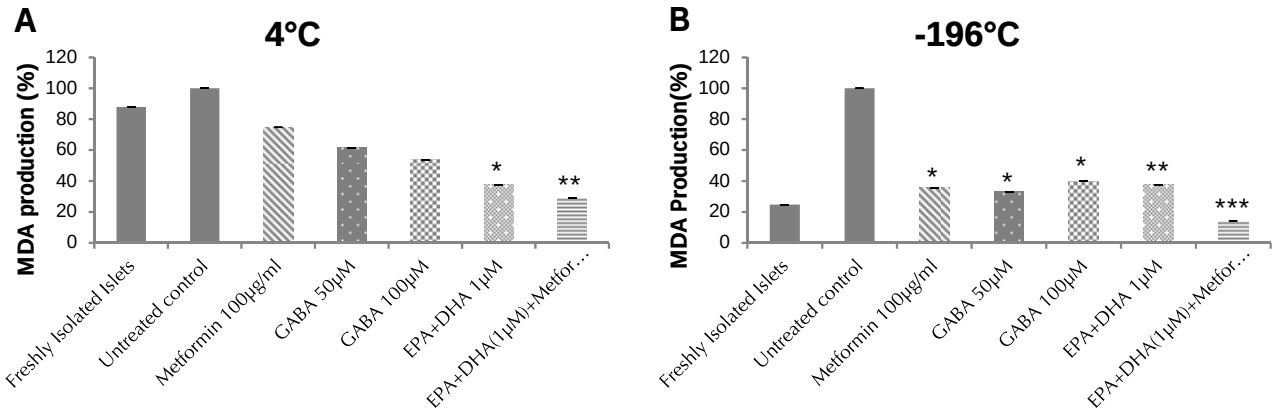

Figure 6. Lipid peroxidation of islets revived from suboptimal and ultralow temperature. A, **B**: MDA levels of islets stored at low and ultralow temperature with the additives. Treated islets revived from low and ultralow temperature showed significantly reduced lipid peroxidation compared with untreated islets.

3. GABA 100 µM 50%
4. EPA+DHA: 50% ($p < 0.01$)
5. EPA+DHA+Metformin: 60% ($p < 0.001$)

Islets stored with different additives at -196°C showed the following reductions in superoxide anion levels (**Figure 5D**):

1. GABA 50 µM: 40%
2. GABA 100 µM: 50%
3. Metformin: 70% reduction ($p < 0.05$)
4. EPA+DHA: 70%
5. EPA+DHA+Metformin: 80%

Nitric oxide. In islets stored at 4°C, nitric oxide levels were reduced by (**Figure 5E**):

1. Metformin: 50%
2. GABA 50 µM: 50%
3. GABA 100 µM: 60% ($p < 0.05$)
4. EPA+DHA: 65% ($p < 0.001$)
5. EPA+DHA+Metformin: 70% ($p < 0.001$)

The reductions in NO levels in islets revived from -196°C were found to be (**Figure 5F**):

1. EPA+DHA: 35%
2. GABA 100 µM: 35%
3. GABA 50 µM: 40%
4. EPA+DHA+Metformin: 70% ($p < 0.01$)
5. Metformin: 80% ($p < 0.01$)

Lipid peroxidation. The freshly isolated islets and those revived from low temperature (4°C) were further tested for lipid peroxidation status by measuring the concentration of MDA. The following reductions in MDA were observed (**Figure 6A**):

1. Metformin: 30%
2. GABA 50 µM: 40%
3. GABA 100 µM: 50%
4. EPA+DHA: 60% ($p < 0.05$)
5. EPA+DHA+Metformin: 70% ($p = 0.001$)

Islets stored at the ultralow temperature of -196°C were also revived and assessed for MDA concentration. The trend was similar to that of islets stored at suboptimal temperature, namely (**Figure 6B**):

1. GABA 100 µM: 40% ($p < 0.05$)
2. GABA 50 µM: 50% ($p < 0.05$)
3. Metformin: 60% ($p<0.05$)
4. EPA+DHA: 60% ($p < 0.01$)
5. EPA+DHA+Metformin: 90% ($p < 0.001$)

4. Discussion

The main objective of our study was to determine the viability and functionality of islets revived from suboptimal (4°C) and ultralow (-196°C) temperatures using different additives for improving storage. Both freshly isolated and stored islets stained positive with DTZ, indicating the presence of insulin in the β-cells. DTZ staining is highly specific for zinc, which is present in high amounts in the pancreas of animals [22]. Zinc also plays an

essential role in insulin packaging [14]. In earlier studies, 95% of islets recovered after 48 h of storage from 4°C [4]. Our data showed that a similar recovery rate is possible after more than 15 days of storage at 4°C temperature in the presence of different additives.

The viability of the islets was determined by FDA/PI staining. While freshly isolated islets show 100% viability, previous studies reported only 60% survival of cryopreserved islets [31]. In the present study, we could achieve almost 90% viability in a cryomixture containing combinations EPA+DHA+ Metformin or EPA+DHA. These additives are known to act as a membrane stabilizer [9, 17, 18, 21, 32]. They may thus be able to form a protecting shield round the islets to keep them intact. We performed FDA/PI staining to confirm the viability of control and stored islets. This provided evidence for stable membrane integrity and the presence of functional enzymatic activities of the islets [33]. FDA was used to stain live cells with green fluorescence, and to confirm the presence of esterase enzyme [20], and we used PI to fluoresce the dead cells red. We were able to get maximum recovery of islets after short and intermediate term storage at both low and ultralow temperatures, ranging from 15 to 30 days. We employed the MTT assay to test the viability of the revived islets, and found that the additives improved the survival rate of cryopreserved islets [28]

Moreover, we confirmed the functionality of islets by glucose-stimulated insulin secretion [30]. Both metformin and GABA induced insulin secretion comparable to that of high glucose stimulation, whereas EPA+DHA did not show a significant increase in insulin release. It has already been reported that GABA acts as an insulin secretagogue in rat islets [34], while the action of metformin as an insulin secretagogue is still unknown. Our data revealed for the first time that metformin is able to act as an insulin secretagogue in mouse islets similar to GABA. In some animals, such as rabbits and dogs, GABA does not promote insulin secretion, although it is present in pancreatic β-cells [11]. However, it has been reported that oral administration of GABA increases insulin release in humans. Therefore, it is very likely that GABA may be indirectly acting as an insulin-secreting agent by inhibiting glucagon in mouse pancreatic islets [35]. Inclusion of GABA in the storage medium resulted in the maintenance of the insulin secretory function of islets even at suboptimal storage temperature, thus indicating GABA to be a novel cryoprotective agent.

Metformin plays a crucial role as an antidiabetic drug in type 2 diabetes [23]. It has been shown to reverse metabolic abnormalities caused by high glucose and elevated free fatty acids [36]. It is known to inhibit hepatic gluconeogenesis and glycogen synthesis. In the present study, we observed that it also acted as an insulin secretagogue and islet-protecting agent. The cytoprotective role of metformin could be attributed to its action in preventing epithelial to mesenchymal transition [37]. Its ability to maintain the viability of islets may be attributed to the occlusion of the islets' permeability transition pores or to the prevention of their opening [23, 38].

High levels of oxygen free radicals cause apoptosis and necrosis of islets during transplantation [19], leading to a reduced success rate in islet transplantation. Oxidative stress exerts adverse effects on both DNA and proteins [7]. Oral administration of antioxidants may improve the oxidative stress status in islets [2, 39]. Here, we found that the compounds metformin, GABA, EPA, and DHA, used as additives in the cryo-mixture, may act as antioxidants as well, as evidenced by the significant reduction in free radical formation in islets revived from cold storage. Polyunsaturated fatty acids (EPA and DHA) play a beneficial role in humans and animal models of diabetes. This is the first study to show that these compounds may act as antioxidants when added to the storage mixture; they are able to preserve the structural and functional integrity of cryopreserved islets.

This is also the first report showing that islets can be stored at 4°C for 15 days without losing integrity, viability, or functionality. Earlier studies on low temperature islet preservation have shown that islets could not be stored for more than 48 hours [40]. Our data simplifies the technical issues that were faced in storing islets for longer periods of time. All compounds are well known for their effectiveness in various diseases, but their antioxidative property in cryopreserved islets is a novel finding. This discovery could be useful in islet transportation and repeated islet infusion during islet transplantation programs. However, the application of the results presented in this study needs to be further explored in human islets.

In conclusion, our data demonstrated for the first time that the combination of compounds such as EPA, DHA, and Metformin, used as additives in the storage of islets, could improve the survival of islets and their functionality when stored at an ultralow temperature of -196°C and even at a suboptimal temperature of 4°C.

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