Cryopreservation of insulin-secreting INS832/13 cells using a wheat protein formulation

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A B S T R A C T

Diabetes is a global epidemic that affects about 285 million people worldwide. For severely-ill patients with type I diabetes, whole pancreas or islet transplantation is the only therapeutic option. Islet transplantation is hindered by the scarce supply of fresh functional islets and limitations in cryopreservation procedures. Thus, improved cryopreservation procedures are needed to increase the availability of functional islets for clinical applications. Towards this goal, this work developed a cryopreservation protocol for pancreatic cells using proteins that accumulate naturally in freezing-tolerant plants. A preincubation of cells with 1% lecithin-1% glycerol-1% N-methylpyrrolidone followed by cryopreservation with partially purified proteins from wheat improved the viability and insulin-secreting properties of INS832/13 cells, compared to cryopreservation with 10% dimethyl sulfoxide (Me2SO). The major factor that enhanced the cryoprotective effect of the wheat protein formulation was preincubation with the lipid lecithin. Expression profiles of genes involved in metabolic and signaling functions of pancreatic cells (Ins, Glut1/2/3, Pdx1, Reg1x) were similar between fresh cells and those cryopreserved with the plant protein formulation. This novel plant-based technology, which is non-toxic and contains no animal material, is a promising alternative to Me2SO for cryopreservation of insulin-secreting pancreatic cells.

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Introduction

Diabetes, the world’s 4th leading cause of death, is a global epidemic that affects an estimated 285 million people worldwide [Canadian Diabetes Association, 2012; www.diabetes.ca]. With 7 million new cases diagnosed worldwide each year, the total number of people affected is expected to reach 438 million by 2030. There are two main types of diabetes: type I and type II. Approximately 10% of diabetics have type I diabetes while 90% are afflicted with type II diabetes. Type II diabetes can often be controlled by lifestyle management, whereas juvenile onset type I diabetes is an unpreventable disease that requires daily insulin injections. The causes of type I diabetes are unknown, but genetic and environmental factors appear to be involved. It is an autoimmune disease that results in the destruction of insulin-secreting beta cells of the Islets of Langerhans. This renders the pancreas unable to produce insulin and results in glucose accumulation in the blood, which leads to multiple health problems. Life expectancy for people with type I diabetes may be shortened by about 15 years.

There is no cure for type I diabetes and treatment with insulin is often not sufficient to prevent long-term complications of the disease such as blindness, cardiovascular disease, stroke, nerve problems and kidney disease. For severely-ill patients, alternative therapies involve the transplantation of either whole pancreas or Islets of Langerhans [20,40,41,51]. Islet transplantation is preferred over organ transplantation because it is less invasive and safer. However, a major limitation is that islet transplantation requires a large number of human islets (usually from two or three donors), which are in scarce supply. The long-term storage of islets in cell and tissue banks is therefore essential for transplantation, blood type and histocompatibility matching, testing of viability, sterility and islet function, as well as transportation to distant sites. Cryopreservation is one of the options for long-term storage of islets. However, currently used islet cryopreservation protocols are suboptimal since they use toxic chemicals such as dimethyl

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disulfide (Me₂SO), which cause extensive loss of islet mass (~50% survival) and inhibit glucose-induced insulin secretion [29,38,46,50].

To find more effective and less toxic alternatives to Me₂SO, scientists have long considered using natural substances produced by organisms that survive freezing conditions, such as freezing tolerant hardy plants. To ensure survival, several hardy plants have evolved efficient mechanisms to tolerate extreme freezing winter conditions. Hardy winter wheat varieties are inherently more freezing tolerant compared to their less hardy, spring counterparts, even without exposure to the inductive low temperatures. This is due to the presence of substances known to protect plants against cold and freezing stresses, such as freezing tolerance associated proteins, antifreeze proteins (AFPs), sugars (glucose, fructose, sucrose and trehalose), glycine betaine (an organic osmolyte), antioxid- ids, and phenolic compounds (reviewed in [47]).

To mimic the strategy of nature that winter hardy wheat use to tolerate freezing, we developed a novel protocol using soluble proteins from winter wheat plant extracts (WPE) as a cryopreservative agent for mammalian cells. WPEs from winter wheat were shown to efficiently cryopreserve primary cultures of rat hepatocytes [13,15]. In view of the promising results obtained for hepatocytes, it was therefore of interest to test the efficacy of these plant proteins for cryopreservation of other cell types. Given that cells and tissues are complex and dynamic structures with a wide range of morphological, biochemical and physiological characteristics, customized cryopreservation strategies need to be designed for each biological system to ensure maximum cell viability and functional capacity. This study investigates the ability of a mixture of winter wheat proteins to cryopreserve insulin-secreting INS832/13 pancreatic cells, while allowing them to maintain their metabolic functions. Lipids appear to play a beneficial role in cryoprotection [10,17]; therefore we determined whether plant-derived lipids could enhance the cryoprotective effect of plant proteins.

Materials and methods

Preparation of plant protein extracts

Winter wheat plants (Triticum aestivum L. cv Clair) were germinated and grown in water-saturated vermiculite for 10 days at 20 °C, 70% relative humidity, and a 16 h photoperiod under an irradiance of 100 μmol m⁻² s⁻¹. Partially purified wheat protein extracts (WPE) were prepared as described previously [11], except that overnight acetone precipitations of proteins were performed. Protein integrity was validated and quantified by SDS–PAGE.

Cell culture

The insulin-secreting INS832/13 cell line (kindly provided by Dr. Marc Prentki, Montreal Diabetes Centre) was used in this study. This cell line was generated by transfection of INS-1 cells to stably express the human proinsulin gene [16]. The INS-1 cell line was established from cells isolated from an X-ray-induced rat transplantable insulinoma [4]. INS-1 cells retain a high degree of differentiation, and they stably mimic the function of β cells within the normal pancreatic islets of Langerhans. INS832/13 cells exhibit markedly enhanced and stable responsiveness to glucose compared to INS-1 cells. INS832/13 cells (passages 30–70) were grown in monolayer in tissue culture plates in RPMI-1640 medium (pH 7.4) supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS Premium; Wisent, Montreal QC, Canada), 2 mM L-glutamine, 1 mM sodium pyruvate, 50 μM β-mercaptoethanol (hereafter called “complete RPMI”) at 37 °C and 5% CO₂ in a humidified atmosphere. The doubling time of INS832/13 cells is around 100 h and these cells are passaged every 8 days [16]. At 80% confluence, cells were washed twice with PBS and detached using trypsin–EDTA (Sigma, St. Louis MO, USA). After centrifugation, the pellet was resuspended in complete RPMI.

Cell cryopreservation

Cells (0.75 × 10⁶) were aliquoted into cryovials in 0.5 mL of complete RPMI without antibiotics, and then supplemented with the WPE [13]. Cells cryopreserved with 10% Me₂SO-50% FBS served as a reference, and complete RPMI without antibiotics as a negative control. For optimization of the cryopreservation protocol, cells were also preincubated for 1 h at 37 °C with mixtures of lipids (lecithin, glycerol, glycerin) and/or N-methylpyrrolidone (NMP) at 1% each (see figures for details), before addition of the WPE. When present, the final concentrations of lecithin, glycerol, glycerin and NMP in the cryopreservation mixture were 0.4% (except for Fig. 2). The rationale for using these substances is described in the results section. The WPE was resuspended in ice-cold RPMI medium before addition to the cell suspension. Tubes containing cells were frozen at a cooling rate of 1 °C/min in a freezing container (“Mr. Frosty”; Nalgene, Rochester NY, USA) in a −80 °C freezer for 24 h, and then transferred to liquid nitrogen for at least 7 days prior to thawing. Frozen cells were thawed quickly by gentle agitation in a 37 °C water bath and then analyzed for viability, cellular functions and gene expression.

Cell viability

Viability was determined immediately after thawing by staining cells with 9 μM SYTOX Green (Molecular Probes, Eugene OR, USA) in RPMI medium for 5 min. SYTOX Green is a nucleic acid stain that readily enters cells with compromised cytoplasmic membranes. Cells (10,000) were analyzed by flow cytometry (excitation at 488 nm) using a FACScan (Becton Dickinson, Oxford, UK). The number of cells showing green fluorescence was determined with the Cell Quest software (Becton Dickinson).

Cell adhesion

For adhesion, thawed cell suspensions were washed twice with cold PBS containing 0.1% Tween-20 (unless otherwise specified; see Tables S2 and S3) and centrifuged at 1,500g for 2 min at 4 °C. Cells (3.75 × 10⁵) were then plated and cultured in 6-well culture dishes in complete RPMI medium containing 100 U/mL penicillin, 100 μg/mL streptomycin and 2.5 ng/mL amphotericin B. Cell adhesion was observed 48 h after plating in dishes using an Eclipse Ti 3000 inverted microscope (Nikon, Montreal QC, Canada) with a 40X Hoffman objective. In general, cells were either adherent on plastic dishes or completely non-adherent (-) where they floated in the culture medium. Adhesion was qualified as strong, medium or weak for degrees of confluence of 100%, 75% and 50%, respectively.

Cellular morphology

Thawed cells were washed and cultured for 1 and 14 days on poly-D-lysine-coated coverslips. Morphology was evaluated by microscopy and photographs were analyzed using Image J software (National Institutes of Health, USA).

Glucose-Induced insulin and C-peptide secretion

Thawed cells were washed and cultured for 5 and 14 days prior to secretion tests. The 14-day time point refers to cells that had been passaged only once after thawing. Cells were incubated in
complete RPMI with low glucose (1 mM) for 2 h in 48-well plates. They were washed and preincubated for 1 h in Krebs–Ringer bicarbonate buffer containing 10 mM HEPES pH 7.4 (KRBH) and 0.07% bovine serum albumin (BSA). Cells were then incubated in fresh KRBH containing 1 or 15 mM glucose for 1 h. The concentrations of insulin [31] and C-peptide [33] released into the media were determined by ELISA (Mercodia, Salem, NC, USA), following the manufacturer’s instructions. DNA was extracted using phenol-chloroform and quantified by spectrophotometry. Values were normalized to the DNA content (ng insulin/ug DNA and pmol C-peptide/ug DNA), and then glucose-induced stimulation was calculated as the 15/1 mM ratio for both insulin and C-peptide release.

Glucose uptake

Cells were thawed and washed, and then cultured in RPMI medium deprived of serum and glucose for 2 h in 96-well plates, and then preincubated for 2 h in KRBH. The glucose uptake assay was initiated by replacing the KRBH with 200 μl of KRBH containing 0.4 μCi of 2-deoxy-[3H]-D-glucose (Perkin-Elmer, Woodbridge ON, Canada) [26]. As a negative control, 20 μM of cytochalasin B was added to the assay medium. After 15 min, transport was blocked by washing the cells 3 times with ice-cold PBS containing 1 μM cytochalasin B. The cells were solubilized with 0.4% SDS and the radioactivity was detected using a liquid scintillation counter (Tri-Carb 2800TR, PerkinElmer, Shelton, CT, USA). The net values for glucose uptake were obtained by subtracting the non-specific uptake in the presence of cytochalasin B. Results are expressed as pmol [3H]-D-glucose/ug DNA/min.

RNA extraction and semi-quantitative RT-PCR

Total RNA was extracted from cells using the RNeasy Mini Kit and reverse transcribed using the Omniscript RT Kit (QiAGEN, Mississauga ON, Canada). Primers used for gene expression analysis are given in Table S1. The resulting cDNAs were PCR-amplified for 35 cycles with specific primers for 18S rRNA, insulin (Ins), regenerating islet-derived 1 alpha (Reg1α), pancreatic and duodenal homeobox 1 (Pdx1), and glucose transporters 1, 2 and 3 (Glut1, Glut2, Glut3). Control amplifications of RT reactions performed without reverse transcriptase confirmed that there was no DNA contamination in the RNA samples.

Statistical analysis

Comparison between groups and analysis for differences between means of control and treated groups were performed using ANOVA followed by the Bonferroni post hoc test (P < 0.05 significance level).

Results

Wheat protein extracts can efficiently cryopreserve INS832/13 cells

The cryopreservation efficacy of the WPE was evaluated in insulin-secreting INS832/13 cells and compared to the classical cryoprotectant Me2SO. We first confirmed that the WPE was non-toxic to fresh (non-cryopreserved) cells. This was shown by the lack of adverse effects on glucose-induced insulin secretion properties after 30 min of incubation of cells with the WPE (Fig S1). Subsequently, the cryoprotective ability of the WPE was determined by measuring cell viability. Post-thaw viability increased as a function of protein concentration from 0.5 to 20 mg of WPE proteins/7.5 × 10^5 cells [Fig. 1]. Viability of cells cryopreserved with the WPE at 5 mg of protein/7.5 × 10^5 cells was about 70% and equivalent to that of cells cryopreserved with Me2SO (10% Me2SO-50% FBS). At a higher concentration of the WPE (20 mg protein/7.5 × 10^5 cells), viability was similar to that of fresh cells (>90%). When BSA was tested as an alternative protein for cryopreservation (20 mg/7.5 × 10^5 cells), post-thaw viability was very low (<10%), indicating that high cell viability following cryopreservation with the WPE is specific to the wheat proteins (Fig 1). The viability of cells cryopreserved with RPMI medium alone, without proteins, was also very low (<10%) (Fig 1). An important factor for cryopreservation of cells for clinical purposes is to avoid animal products for biosafety reasons. These results show that wheat proteins can effectively and specifically maintain high viability in insulin-secreting cells during cryopreservation without animal serum or Me2SO.

In addition to high post-thaw viability, an important factor for assessing the efficiency of a cryopreservation protocol is whether cells are able to resume their normal metabolic activities once back in culture. Adhesion is critical for allowing cells to resume their growth and to perform metabolic activities. However, fewer INS832/13 cells (7.5% compared to fresh cells) were able to adhere to culture dishes following cryopreservation with the WPE, in contrast to 10% Me2SO (~50%) (data not shown). The loss of cell adhesion had an important impact on insulin-secreting properties, which is a key metabolic function in pancreatic cells. Indeed, there was a pronounced 3–5-fold decrease in glucose-induced insulin secretion in cells cryopreserved with the WPE, compared to fresh and Me2SO-cryopreserved cells (data not shown). Observations by microscopy revealed that after thawing, the wheat proteins appeared to form a gel-like matrix that coated the cell’s plasma membrane. The coating gave cells a blurry appearance and irregular shape (Fig 2A), compared to cells that were cryopreserved with Me2SO (Fig 2B). This matrix resisted extensive washing with PBS and likely explains the low cell adhesion. To improve adhesion properties in INS832/13 cells cryopreserved with the WPE, numerous procedures were tested to remove the membrane coating. These procedures, which included washing cells post-thaw with different buffers, enzymes or detergents (Table S2), and preincubation of cells with different plant oils prior to cryopreservation with the WPE (Table S3), did not remove the protein coating. We subsequently tested other procedures with lipids.

Fig. 1. A wheat protein extract (WPE) can replace Me2SO as a cryoprotectant for INS832/13 cells. Cells were cryopreserved for 7 days with different concentrations of WPE, or with 10% Me2SO-50% FBS (Me2SO). Fresh cells served as control. Cells cryopreserved in complete RPMI culture medium, without proteins, served as negative control. Post-thaw viability was determined with SYTOX Green by flow cytometry. Data (mean ± SEM) represent measurements of at least three different preparations of WPE tested on three independent cell preparations. **(P < 0.001) indicates a significant increase in viability between cells cryopreserved with WPE versus those with Me2SO.
Preincubation with lipids prevents membrane protein coating and improves post-thaw viability and adhesion of INS832/13 cells

To improve cryopreservation properties of the WPE, the next approach that was tested to prevent the membrane protein coating involved preincubating pancreatic cells with lipids such as lecithin, glycerol and glycerin (plant-derived glycerol). Several concentrations of these substances were tested, with or without the WPE (Fig. 3). Post-thaw viability was low (<10%) in cells that were preincubated with 0.25–1% glycerol or glycerin prior to cryopreservation (without the WPE). In contrast, post-thaw viability was high in cells cryopreserved with lecithin alone, at 48–90% for 0.25–1% lecithin, respectively. Addition of the WPE (5 mg) for low temperature storage caused a pronounced increase in post-thaw viability of cells that had been preincubated with glycerol or glycerin (Fig. 3). The highest viability was obtained when cells were preincubated with 1% lecithin and then cryopreserved either with or without the WPE (5 mg) (Fig. 3). In addition, preincubation with each of these lipid substances partially prevented protein coating of membranes and allowed increased adhesion of cells (about 50%). Despite the high viability with 1% lecithin, metabolic function was suboptimal (see next section).

Subsequently, combinations of lecithin and glycerol or glycerin were tested with the WPE to determine if they could improve post-thaw viability and metabolic functions. Post-thaw viability of cells was improved by more than 25–30% when INS832/13 cells were preincubated for 1 h with 1% lecithin prior to cryopreservation with the WPE (Fig. 4). Viability was similar to that of fresh cells (>90%, no significant difference), and significantly higher than that of Me2SO-cryopreserved cells (approx. 75%). Moreover, the addition of glycerol or glycerin (1% each) to lecithin in the preincubation mixture provided an advantage by preventing the protein coating, and consequently, cell adhesion was restored to about 100%. Therefore, the best cryopreservation medium for INS832/13 cells consisted of 5 mg of WPE protein/7.5 × 10^5 cells, preincubated for 1 h with a combination of 1% lecithin and 1% glycerin (Fig. 4).

Curiously, post-thaw viability (Fig. 4) and stimulation ratios for glucose-induced insulin secretion (Fig. 5A) were significantly lower for the lecithin-glycerol-WPE mixture compared to the lecithin-glycerin-WPE mixture for cryopreservation of INS832/13 cells. This discrepancy was puzzling, given that glycerin and glycerol have the same molecular structure. However, glycerin is produced from plants and its synthesis involves a trans-esterification step, while glycerol is of animal origin and its synthesis requires a saponification step. We verified whether a by-product generated during synthesis of either of these two compounds could be responsible for the difference in metabolic activity. A GC/MS analysis revealed that...
glycerin contains similar proportions (approx. 50:50) of glycerol and the by-product N-methylpyrrolidone (NMP). We next determined whether NMP could be beneficial during cryopreservation. When cells were preincubated with 1% NMP alone, post-thaw viability was low (<5%) (Fig. 4). However, high viability (>90%) was obtained when cells were preincubated for 1 h with a combination of lecithin-glycerol-NMP (1% each), and was equivalent to that of the lecithin-glycerol mixture and of fresh cells (Fig. 4). This suggests that the NMP contaminant was contributing to the cryoprotective effect of glycerin. Furthermore, when cells were preincubated with the lecithin-glycerol-NMP mixture prior to cryopreservation (Fig. 2C), there was no protein coating after 1 and 14 days in culture. The morphology of these thawed cells more closely resembled fresh cells (Fig. 2D), compared to cells that were cryopreserved with the WPE alone (Fig. 2A) or with Me2SO (Fig. 2B). Given that glycerol is more readily available than natural glycerin, further experiments were carried out using glycerol and NMP.

Metabolic functions are maintained in INS832/13 cells cryopreserved with the optimized WPE formulation

For pancreatic cells, key metabolic functions include glucose-induced secretion of insulin and C-peptide, and glucose uptake. Therefore, stimulation ratios for glucose-induced insulin and C-peptide secretion were determined in thawed INS832/13 cells after 5 and/or 14 days in culture. Stimulation ratios for glucose-induced insulin release on day 14 were significantly lower ($P < 0.001$) in cells cryopreserved with the WPE following preincubation with lecithin alone or combined with glycerol or NMP, compared to fresh cells (Fig. 5A). However, the highest stimulation ratios for insulin release were obtained in cells that were preincubated with the combinations lecithin-glycerol-NMP or lecithin-glycerin, prior to cryopreservation with the WPE (Fig. 5A). These stimulation ratios were not significantly different from the ratio in fresh cells, and were significantly higher ($P < 0.001$) than that in Me2SO-cryopreserved cells. For release of C-peptide, the optimal stimulation ratio occurred in cells that were preincubated with lecithin-glycerol-NMP, prior to cryopreservation with the WPE, and there was no significant difference from fresh cells (Fig. 5B). These higher stimulation ratios for insulin and C-peptide were due to an increase in the glucose-induced rates, whereas the basal rates did not change (data not shown). Glucose uptake was functional in cells that were cryopreserved either with the WPE following preincubation with lecithin-glycerin, or with 10% Me2SO-50% FBS (Fig S2). Levels of glucose uptake were not significantly different when compared to fresh cells.

Expression of key genes associated with pancreatic functions is comparable between fresh cells and those cryopreserved with the WPE formulation

We also determined whether cryopreservation with the WPE could alter the expression of key genes associated with β-cell metabolism and function: Glut1, Glut2, Glut3, Ins, Pdx1 and Reg1α. Glut genes encode for glucose transporters expressed in β cells. These include glucose transporter 1 (GLUT1), responsible for basal glucose uptake, GLUT2, a low affinity glucose transporter, and GLUT3, a high affinity transporter [23]. Preproinsulin, the primary translation product of the insulin gene (Ins), is a precursor that is processed to proinsulin, which undergoes proteolytic cleavage to produce insulin and C-peptide [42]. In response to insulin, increased expression of the gene encoding the transcription factor pancreas and duodenum homeobox-1 (Pdx1) occurs. This transcription factor is important for survival and differentiation of β cells [37] and primarily acts by up-regulating the transcription of several β cell-specific genes, including Ins and Glut2 [1]. The regenerating islet-derived 1 alpha (Reg1α) gene encodes a protein that is associated with the regeneration of pancreatic islets [32]. Data from semi-quantitative RT-PCR show that at day 14 post-adhesion, there were no significant differences in the expression levels of all six genes between fresh cells and those that were cryopreserved with the WPE (following preincubation with lecithin-glycerol-NMP) or with 10% Me2SO-50% FBS (Fig. 6).

Discussion

Overall, our cellular, biochemical and molecular analyses show that the plant-based cryopreservation formulation maintains cell viability, adhesion and metabolic functions following ultra-low temperature storage of insulin-secreting cells. Our optimized
cryopreservation protocol for INS832/13 cells involves a two-step procedure that is based on the preincubation of $7.5 \times 10^5$ cells with a mixture of lecithin-glycerol-NMP (1% each) or lecithin-glycerin for 1 h at 37 °C followed by the addition of 5 mg of WPE and immediate cryopreservation. The preincubation with the mixture has the added benefit that the quantity of wheat proteins required for cryopreservation could be decreased from 20 to 5 mg per $7.5 \times 10^5$ cells.

During initial cryopreservation experiments, the plant proteins coated the cell membrane, which interfered with cell adhesion and glucose-induced insulin secretion in thawed cells. The decrease in glucose sensitivity due to cell membrane coating is likely explained by blocked access of glucose to glucose transporters. Glucose-induced insulin secretion was functional when cells were incubated with the WPE at 37 °C, indicating that this membrane coating only occurred during freezing of the proteins. During the freezing of a protein in solution, there are rapid increases in concentrations of solutes due to ice formation, and changes in pH and protein dehydration, which can cause a decrease in protein stability due to denaturation or changes in folding [35]. Therefore, it appears that freezing may modify the plant proteins, causing them to become gel-like and to adsorb strongly to membranes of INS832/13 cells. Another explanation could be changes in membrane structure and fluidity that arise during freezing [2], which could render the membrane more susceptible to adhesion by the wheat proteins.

The protein membrane-coating phenomenon observed for INS832/13 cells did not occur during cryopreservation of hepatocytes with the WPE. However, these are two very different cellular systems. The hepatocytes are primary cultures of cells isolated from liver, whereas INS832/13 is a continuous cell line that can be maintained in culture after adherence in dishes. Hepatocytes are differentiated cells that have a polarized nature [3]. Compared to cell lines, the hepatocyte membrane is less uniform and composed of several domains: apical (or canicular) and basolateral (lateral and sinusoidal). These domains have different biochemical compositions including different membrane receptors and enzymes. Hepatocytes are cubic, have numerous microvilli, and specialized junctions and bile canaliculi form within the membranes of adjacent cells. The reason for this difference in protein membrane coating between hepatocytes and INS832/13 cells remains unknown.

The WPE was previously reported to successfully cryopreserve primary cultures of hepatocytes, which exhibited high viability, good adhesion, and retained hepato-specific functions such as albumin secretion and induction of cytochrome P450 (CYP) isoenzymes [11–13]. Due to membrane coating, the cryopreservation protocol developed for hepatocytes was modified for INS832/13 cells to avoid interference with adhesion and metabolic functions. This modified protocol with the lipid mixture improved the cryoprotective effects of the WPE by preventing adsorption of wheat proteins to the membrane, thus allowing cells to adhere to culture plates and maintain their metabolism and gene expression post-thaw. This plant-based protocol was more effective than Me$_2$SO for cryopreservation of insulin-secreting cells.
Cryopreservation-induced damage to cells appears to involve multiple mechanisms [6,22]. The formation of intracellular ice is known to cause damage during freezing of many different cell types [21]. Membrane damage and physical cell rupture that occur during cryopreservation have been attributed to intracellular ice crystal formation and increased solute levels. Moreover, extracellular ice can cause mechanical stress as well as cellular deformation [22].

The fundamental mechanisms involved in the cryoprotective effect of the wheat protein formulation are unknown. This is particularly difficult to establish since the principle component of the WPE, following partial acetone purification, is a mixture of numerous proteins. It is likely that multiple mechanisms are involved, conferred mainly by the different plant proteins, but lecithin, glycerc ol and NMP also contribute. In addition, we do not know if these proteins can enter cells. Proteins are relatively large macromolecules and it seems unlikely that the wheat proteins would cross the cytoplasmic membrane; therefore, they are likely to exert their cryoprotective effects at the extracellular level. In general, soluble proteins are able to associate with membrane phospholipids through hydrophobic interactions [25], although these could be somewhat diminished by association of low (1%) levels of lecithin with the membrane. It is possible that some of the plant proteins in the WPE possess properties that confer cryoprotective effects by stabilizing the cell membrane against freeze-induced injury, thus lessening physical damage to the membrane due to the accumulation of extracellular ice. This mechanism has been described for cells of plants subjected to freezing [48]. The WPE made from winter wheat contains many different proteins including basal levels of different freezing tolerance associated proteins such as dehydrins (e.g. WCS120, WCS19, WCRD40) and antifreeze proteins (AFPs; e.g. TaIRI-2) (unpublished observations). Dehydrins are thought to coat vital cellular proteins and protect them against conformational changes or agglomeration under conditions of stress such as freezing [18]. AFPs such as TaIRI-2 can inhibit growth of ice crystals at the extracellular level, thus protecting the plasma membrane during freezing [49]. Other proteins in the WPE are very likely involved in the cryoprotective effect, but their identity remains to be determined. The advantages of using a natural plant-based technology for cell cryopreservation, as an alternate to Me2SO, is that the protein extracts are non-toxic and contain no gluten or animal material.

Interestingly, antifreeze proteins from fish have been tested as cryoprotectants. Synthetic analogues of C-linked antifreeze glycoprotein (C-AFGP), which has antifreeze function in deep sea teleost fish, were shown to be an alternative to Me2SO for the cryopreservation of human embryonic liver cells [27]. A synthetic galactose-lacking fish AFGP analogue (synAFGP) improved pancreatic islet recovery post-cryopreservation as well as glucose-stimulated insulin secretion, when combined with Me2SO and 10% fetal calf serum (FCS) [28]. The fish antifreeze protein type III (AFP III) was reported to inhibit the ice nucleation process by adsorbing onto the surfaces of both ice and foreign (e.g. dust) particles [8]. The adsorption of AFPS onto the surface of ice caused a similar effect on both the formation (nucleation) and growth kinetics of ice crystals.

The major factor that improved the cryoprotective effect of the WPE was preincubation with low levels of lecithin. Lecithin is a phospholipid that is composed of glycerol, two fatty acids, choline and a phosphate group. The fatty acid components in lecithin can vary, in terms of the number of carbon atoms they contain and their saturation level. Lecithin will associate with phospholipids in the cell membrane through non-covalent hydrophobic interactions and will likely form a protective layer around cells. These lipid-lipid interactions appear to diminish the association between plant proteins and the cell membrane, thus preventing the membrane coating effect, and at the same time allow the proteins to provide cryoprotection, probably by decreasing extracellular ice formation. Several other studies have reported a role for lipids in cryoprotection. Liposomes were reported to stabilize membranes during freezing and thawing of bull sperm [9] and red blood cells [17]. In particular, unsaturated lipids such as 1,2-dioleoyl-sn-glycerol-3-phosphocholine (DOPC) were protective. It appears that liposomes can alter membranes by promoting cholesterol-lipid exchange [43]. Cholesterol depletion causes several alterations in physical properties of membranes including increased fluidity and permeability for hydrophilic solutes [24].

Interestingly, there was no difference in cell viability between 1% lecithin alone or combined with the WPE. However, although the WPE did not provide any added benefit with regard to viability following cryopreservation, the WPE did provide significant improvement to glucose-induced secretory function relative to 1% lecithin alone. It should be noted that viability was measured with SYTOX Green, which detects cytoplasmic membrane integrity but does not indicate the metabolic state of cells. Cells can suffer metabolic and functional damage even though the membrane is intact. Our data suggest that lecithin is protecting the cytoplasmic membrane against damage during cryopreservation, whereas the presence of plant proteins is also required to protect the metabolic function of cells, likely through multiple mechanisms as discussed above.

Glycerol is a highly viscous endogenous cryoprotective agent found in plants and insects that endure cold environments [7,34]. It decreases the amount of body water that freezes at a given temperature, thus preventing excessive cellular dehydration [5,45]. Glycerol has been commonly used as a colligative cryoprotectant that penetrates cells [36], generally at high concentrations of 5–30%. It binds to water molecules by forming strong hydrogen bonds and cryoprotects cells mainly by preventing intracellular ice formation [39,44]. For INS832/13 cells, glycerol was used at low concentrations that did not cryoprotect cells when used alone, but good cryoprotection was obtained when combined with lecithin and NMP as a preincubation mix before the addition of plant proteins.

NMP, which was found to be a contaminant in plant-derived glyc erin, is a biodegradable polar aprotic solvent that has strong solubilisation properties. It has been used as a cryoprotectant in microbiology and its cryoprotective activity was similar to that of glycerol and Me2SO in E. aerogenes [30]. The structural analogue poly(ethylene)glycol (PEG) has been used commonly in both cryobiology and general cryobiology [19]. PVP is cell-impermeable and was shown to reduce damaging effects of intracellular ice formation during cryopreservation of adipose tissue derived adult stem cells [14]. The cryoprotective mechanism of NMP is not known.

Current cryopreservation protocols for Islets of Langerhans are suboptimal and survival is only about 50% [46]. Pancreatic islets are patches of endocrine tissue that consist of clusters of cells. They contain several different cell types, the most common being the insulin-secreting β cell. Important differences exist between individual insulin-secreting cells and pancreatic islets. Islets are separated from other pancreatic tissue by a thin connective tissue, and have intrinsic mass transport limitations. They are highly vascularized organs and require a steady supply of nutrients and oxygen, as well as the removal of metabolites and secreted hormones. Should the coating of islets by the plant proteins occur during cryopreservation, there may be less interference with metabolic functions compared to individual insulin-secreting cells. Our future work will evaluate whether this non-toxic, natural plant-based formulation could provide an alternative technology for improving the cryopreservation of pancreatic islets, albeit a more complex system than insulin-secreting cells.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cryobiol.2012.02.008.

References