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(54) Titre : MISE EN BANQUE DE CELLULES ENDOCRINES DE PANCREAS POUR LES TRANSPLANTATIONS
(54) Title: BANKING OF PANCREATIC ENDOCRINE CELLS FOR TRANSPLANTATION

(57) Abrégé/Abstract:
This invention relates to a novel process for banking pancreatic endocrine cells for transplantation. A method of freezing pancreatic endocrine cells for storage and thawing the pancreatic cells for use in transplantation for treatment of diabetes, which comprises: rapidly freezing the pancreatic endocrine cells in the presence of a suitable antifreeze, storing the frozen cells in a suitable sub-freezing environment, and then thawing the cells prior to transplantation.

# BANKING OF PANCREATIC ENDOCRINE CELLS FOR TRANSPLANTATION

## ABSTRACT OF THE INVENTION

This invention relates to a novel process for banking pancreatic endocrine cells for transplantation. A method of freezing pancreatic endocrine cells for storage and thawing the pancreatic cells for use in transplantation for treatment of diabetes, which comprises: rapidly freezing the pancreatic endocrine cells in the presence of a suitable antifreeze, storing the frozen cells in a suitable sub-freezing environment, and then thawing the cells prior to transplantation.

# BANKING OF PANCREATIC ENDOCRINE CELLS FOR TRANSPLANTATION

## FIELD OF THE INVENTION

This invention relates to a novel process for banking pancreatic endocrine cells for transplantation.

## BACKGROUND OF THE INVENTION

Successful pancreatic islet transplant in an insulin dependent diabetic person can potentially achieve physiological control of the metabolic abnormalities and prevention of long-term complication experienced in insulin-dependent diabetic subjects (Sutherland D.E.R., Chinn P.L., Morrow C.W.: Transplantation of pancreas islets, in Gupta S (ed): Immunology of Clinical Experimental Diabetes. New York, NY, Plenum, 1984, pp 147-246; Tze W.J., Sima A.A.F., Tai J.: Effect of endocrine pancreas allotransplantation on diabetic nerve dysfunction, Metabolism 34: 721-725, 1985). Currently, one major obstacle in the clinical application of islet transplantation is the difficulty in collecting enough donor pancreatic islets for transplantation.

Cryopreservation procedure is a potentially useful way of banking islet tissues until adequate quantities have been collected for transplantation. Cryopreserved whole islets and pancreatic fragments from adult and fetal sources have been shown to be functional in vitro and in vivo (Taylor M.J., Duffy T.J., Hunt C.J., et al.: Transplantation in vitro perfusion of rat Islets of Langerhans after slow cooling warming in the presence of either glycerol or dimethyl sulfoxide. Cryobiology 20:185-204, 1983). However, nearly all studies with cryopreserved whole islets reported some reduction of islet cell function following the freezing and thawing process (Toledo-Pereyra L.H., Gordon D.A., Mackenzie G.H.: Cryopreservation of islets of Langerhans. Cryobiology 18:2483-2488, 1981). Earlier, the inventors have

demonstrated that dispersed single pancreatic endocrine cells (PEC) grafts can normalize hyperglycemia in diabetic rats and monkeys, (Tze W.J., Tai J.: Successful intracerebral allotransplantation of purified pancreatic endocrine cells in diabet rat. Diabetes 32:1185-1187, 1983; Tze W.J., Tai J.: Intrathecal allotransplantation of pancreatic endocrine cells in diabetic rats. Transplantation 41:531-534, 1986; Tze W.J., Tai J.: Xenotransplantation of rat pancreatic endocrine cells in spontaneous and streptozotocin-induced diabetic monkeys. Transplant Proc 21:2736-2738, 1989) suggesting cryopreservation of PEC and islet fragments as an alternative approach to whole islet preservation. In addition, cryopreservation procedure has been suggested to reduce immunogenicity of islet tissue, thus making this approach even more attractive as a means of islet cell preservation.

## SUMMARY OF THE INVENTION

The invention is directed to a method of freezing pancreatic endocrine cells for storage and thawing the pancreatic cells for use in transplantation for treatment of diabetes, which comprises either rapidly or slowly freezing the pancreatic endocrine cells in the presence of a suitable antifreeze, storing the frozen cells in a suitable sub-freezing environment, and then thawing the cells prior to transplantation.

The antifreeze can be dimethyl sulphoxide or may be 10 to 20% dimethyl sulphoxide. The antifreeze can be selected from the group consisting of dimethyl sulphoxide, glycerol, propylene glycol and butane diol.

The cells can be frozen at an ultra rapid rate. They can be frozen in the presence of liquid nitrogen or using a vitrification process. The sub-freezing environment can be a freezer or liquid nitrogen.

The freezing can be conducted at a temperature drop rate of between 0.1°C to about 5°C per minute. The cells can be frozen at a rate of about 0.1°C to about 5°C per minute from ambient temperature to about -7°C, and at a freezing rate of about 0.1°C to about 3°C per minute from about -7°C to about -70°C, or they can be frozen at a rate of about 0.1°C to about 0.5°C per minute from ambient temperature to about -40°C, and at a freezing rate of about 0.5°C to about 5°C per minute from about -40°C to about -70°C.

The invention is also directed to a method of freezing pancreatic endocrine cells for storage and thawing the pancreatic endocrine cells for use in transplantation for treatment of diabetes, which comprises: cooling the pancreatic endocrine cells at a rate of between about 0.1°C to about 5°C per minute to about -70°C in the presence of between about 10% to about 20% dimethyl sulphoxide, storing the frozen cells in a sub-freezing environment, and then thawing the cells prior to transplantation.

The sub-freezing environment can be liquid nitrogen. The cells can be thawed in a water bath of about 37°C temperature. The pancreatic endocrine cells can be single cells, cell aggregations, or islet cells.

The invention is also directed to a method of freezing pancreatic endocrine cells for storage and thawing the pancreatic endocrine cells for use in transplantation which comprises: cooling the pancreatic endocrine cells at a rate of about -0.3°C per minute to about -70°C in the presence of 10% dimethyl sulphoxide, storing the frozen cells in liquid nitrogen, and then thawing the cells in an about 37°C water bath prior to transplantation.

The invention is also directed to a method of cryopreserving pancreatic endocrine cells which comprises cooling the cells at a rate of about -5°C per minute to about 4°C, holding the cells for 3 minutes at about 4°C, subsequently cooling the

cells at a rate of about -0.3°C per minute to about -7°C, holding the cooled cells at about that temperature for about 3 minutes, then cooling the cells at a rate of about -0.3°C per minute to about -40°C, and then cooling the cells at a rate of about -5°C per minute from about -40°C to about -70°C in about 10% dimethyl sulphoxide, and finally, transferring the frozen cells to liquid nitrogen for storage.

The frozen cells can be thawed in a water bath maintained at about 37°C, and then transplanted into xenogeneic or allogeneic diabetic recipients to normalize the blood glucose level in the recipient. The cells can be insulinoma cells. The cells after being thawed in the water bath can be cultured overnight at about 26°C, prior to being transplanted into the diabetic recipient.

## DRAWINGS

In drawings which illustrate specific embodiments of the invention but which should not be construed as restricting the spirit or scope of the invention in any way:

Figure 1 illustrates the validity of rat insulinoma cells frozen with five alternative protocols;

Figure 2 illustrates the viability of rat PEC frozen with five alternative protocols;

Figure 3 illustrates the intrathetical transplantation of cryopreserved Wi PEC into allogeneic diabetic ACI rats; and

Figure 4 illustrates the functional period of cryopreserved Wi PEC in allogeneic diabetic ACI recipients.

DETAILED DESCRIPTION OF SPECIFIC
EMBODIMENTS OF THE INVENTION

To determine optimal freezing and thawing conditions for pancreatic endocrine cells (PEC) and insulinoma cells taken from rats, five different cryopreservation protocols were developed and compared. PEC and insulinoma cells were cooled at rates of between -0.3°C/min and -5°C/min to -70°C in the presence of 10%, 15%, or 20% dimethylsulfoxide (DMSO) with a programmable temperature controller and then transferred to liquid nitrogen for storage. Frozen cells were thawed by either a rapid (in 37°C water bath) or a slow (in air) thawing procedure. One hour after the thawing process, cellular visibility was determined by trypan blue dye exclusion. The visibility results for PEC and insulinoma cells were similar and showed that a slow cooling rate at -0.3°C/min in combination with a rapid thawing in 37°C water bath gave the best results, with up to 80% cellular visibility. Cryoprotectant DMSO used at 10% concentration was the most effective among the three concentrations tested. Later, transplantation studies were performed with PEC cryopreserved with the best protocol, which is -5°C/min to 4°C, held for 3 minutes, -0.3°C/min to -7°C, held for 3 minutes, -0.3°C/min to -40°C, and -5°C/min from -40°C to -70°C in 10% DMSO with a programmable temperature controller then transferred to liquid nitrogen for storage. Intraportal transplantation of cryopreserved Wistar (Wi) strain PEC into allogeneic ACI diabetic recipients was discovered to normalize their blood glucose (BG) for 8.3 ± 1.9 days (mean ± SD), which was not significantly different from that of a noncryopreserved preparation of 6.6 ± 1.5 days. Cytotoxic antibody titers in the ACI recipients of cryopreserved and noncryopreserved Wi PEC graft were found not to be significantly different. Intrathecal transplantation of frozen-thawed Wi PEC into allogeneic ACI diabetic recipients resulted in prolonged amelioration of diabetic state in 10 of 10 animals, which is similar to that seen with freshly prepared PEC. This study confirmed that cryopreservation is an effective procedure for the banking of PEC before a transplantation. The

in vivo survival period of PEC in allogeneic recipients was not significantly altered by the cryopreservation process used in this study.

In our investigations, a total of five cryopreservation protocols were assessed for the cryopreservation of PEC and small PEC aggregates and the PEC cryopreserved with the best protocol was then further assessed in vivo in allogeneic recipients. Yokogawa et al. (Yokogawa Y., Takaki R., Ouo J.: Cryopreservation of pancreatic islet cells. J Lab Clin Med 103:768-775, 1984) reported that consistently over 80% cellular viability was achieved with hamster PEC using a slow freeze and quick thaw protocol similar to ours. It has been suggested that cryopreservation can be used to decrease islet immunogenicity before transplantation (Bank H.L.: Cryobiology of isolated islets of Langerhans circa 1982. Cryobiology 20:119-128, 1983). Coulombe et al. (Coulombe M.G., Warnock G.L., Rajotte R.V.: Prolongtion of isleet xenograft survival by cryopreservation. Diabetes 36:1086-1088, 1987) recently reported slight prolongation in islet xenograft (rat-mouse) after freeze thawing process. Prolonged graft survival was achieved when additional immunosuppression was administered to the recipients. Taylor et al. (Taylor M.J., Bank H.L., Benton M.J.: Selective destruction of leucocytes by free freezing as a potential means of modulating tissue immunogeneity. Membrane integrity of lymphocytes and macrophages. Cryobiology 24:91-102, 1987) observed optimal survival of both lymphocytes and macrophages after freezing and thawing with cooling rates in the range of 0.3 to 5°C/min. Only after cooling at rates greater than 75°C/min was survival of these cells reduced to a negligible level. Further cryopreservation studies by vitrification would provide useful information on this issue.

## Materials and Methods

### Animals and Islet Cell Preparation

Rats of outbred Wistar (Wi) strain weighing 300 to 500 g were selected as donors of islets, and ACI (AgB 4/4) strain as streptozotocin (SZ) (65 mg/kg intravenously [IV]-induced diabetic recipients. Pancreatic tissue was digested with collagenase (type IV, Cooper Biomed, Freehold, NJ). The islets were hand picked under a dissection microscope and further purified by the single layer Hy-paque-Ficoll (H-F) separation technique. Each batch of 1,000 islets was digested with a combination of 0.04% EDTA in $Ca2^{+}$-$Mg2^{+}$ free Hanks' balanced salt solution (HBSS) and 0.02% purified trypsin. Dissociated cells were washed three times with cold HBSS and resuspended in 3 mL of H-F solution at a specific gravity of 1.080, and 2 mL of HBSS was layered on top of the cell suspension. Centrifugation was performed at room temperature for 8 minutes at 800 g, following which PEC at the interface were collected. A PEC preparation consisting mainly of single cells and some aggregates of fewer than 10 cells, viability over 90%, was resuspended in culture medium (medium 199 containing 10% fetal calf serum, 100 U/mL penicillin, 100 $\mu$g/mL streptomycin) and cultured overnight at 26°C in 5% $CO_2$-95% air. The PEC were purified with H-F again and cells at the interface were collected for freezing and transplantation studies.

### Insulinoma Cells

The rat insulinoma line (RIN) was a obtained from Dr. Oie (National Cancer Institute-New York Medical Oncology Branch, Bethseda, Md). Single cells were released from culture flasks with trypsin-EDTA solution. Cell preparation was regularly greater than 95% viable when assessed by trypan blue dye exclusion test.

Freeze-Thawing Procedure

Freezing of overnight cultured PEC and insulinoma cells was performed in a programmable temperature controller (Planer Dryo 10 Series; Planer Products, Sudbury-On-Thames, England). Fifty-milliliter sterile centrifuge tubes (Falcon 2070F, Falcon Plastics, Mississauga, Ontario) containing PEC preparation or insulinoma cells in culture medium (<3 mL) were placed on crushed ice. Equal volumes of precooled culture medium with 20%, 30%, and 40% dimethylsulfoxide (DMSO) (Sigma Chemicals, St. Louis, MO) were added to each tube dropwise over a 15 minute period to achieve the final DMSO concentrations of 10%, 15% and 20%. Aliquots of 1 mL each of the cell preparation were then distributed to cryovials (Cooke Laboratory Products, Alexandria, VA). The cells were then frozen with one of the following cooling schedules to -70°C and the vials were then plunged into liquid nitrogen (LN2) and stored for between 1 week to 6 months:

Schedule 1: -8°C/min to 0°C, hold 20 minutes, -5°C/min from 0°C to -70°C;

Schedule 2: -5°C/min to 10°C, hold 3 minutes, -1°C/min from 10°C to -70°C;

Schedule 3: -1°C/min to -70°C;

Schedule 4: -5°C/min to 10°C, -0.5°C/min from 10°C to -40°C, and -5°C/min from -40°C to -70°C;

Schedule 5: -5°C/min to 4°C, hold 3 minutes, -0.3°C/min from 4°C to -7°C, hold 3 minutes, -0.3°C/min from -7°C to -40°C and -5°C/min from -40° to -70°C.

Preliminary experiments showed that the storage duration in LN2 did not affect the cellular viability. The frozen cells were either thawed quickly with constant agitation

in a 37°C water bath or thawed slowly in air. Culture medium at room temperature was added dropwise over a 20 minute period to the thawed cell suspensions to dilute the DMSO concentration to less than 1% vol/vol. The cells were then transferred to Petri dishes and cultured at 37°C in a $CO_2$ incubator for 1 hour. Then, 200 cells from each preparation were counted and the percent of viable cells was determined by trypan blue dye exclusion. Preliminary experiments indicated that most of the cell death due to freezing and thawing occurred within the first hour of culture after thawing.

Transplantation Study

Rat PEC collected daily were frozen according to schedule 5 (above) and stored in LN2. They were accumulated until sufficient for transplantation study. Cells were quick-thawed in a 37°C water bath as described earlier. Frozen-thawed PEC in culture medium were dispensed into 100 x 20-mm plastic Petri dishes, and cultured overnight in a humidified 5% $CO_2$ incubator. They were centrifuged on H-F gradient for 8 minutes at 800 g. Viable cells collected at the interface were used for transplantation study. Two to three x $10^6$ viable PEC were suspended in a 50 $\mu$L volume in a U-100 insulin syringe (Sherwood Medical, St. Louis, MO) and injected intrathecally into the cisterna magna of diabetic ACI recipients. For intraportal transplantation, cryopreserved or noncryopreserved PEC were suspended in 200 $\mu$L volume in a monojet U-100 insulin syringe and injected over a 1-minute period intraportally into diabetic recipients. Random blood glucose (BG), body weight (BW), and 24-hour urine volume, were assessed before, and daily for 2 weeks following transplantation, and at regular intervals thereafter.

Antibody Study

Sera collected were stored at -20°C and heated at 56°C for 30 minutes before antibody determination. Cytotoxic antibody levels in the ACI recipients of intraportal transplant of fresh

or cryopreserved PEC were determined using pooled donor strain splenocytes as target cells, and rabbit serum (Low-Tox M rabbit complement cat. no. CL3111, Cedarlane Laboratory, Ontario, Canada) as complement source. Antibody titer is defined as the reciprocal of serum dilution that kills 50% of the target cells.

## Results

Figure 1 shows the viability of rat insulinoma cells frozen with the alternative five schedules and thawed at two rates with 10%, 15%, or 20% DMSO as cryoprotectant. It was found that a slow freezing rate was more effective than a quick freezing rate (Schedule 1) in preserving cellular viability. The best was Schedule 5, with the slowest freezing rate at -0.3°C/min, which achieved greater than 80% cellular viability after quick thawing. It was found that thawing rate also greatly affected the cellular viability of cryopreserved insulinoma cells. All samples thawed at room temperature in air (slow thawing) had consistently lower viability than similar preparations thawed in parallel in a 37°C water bath. The concentration of the cryoprotectant DMSO from 10% to 20% achieved similar protection of insulinoma cells with 10% and 15% achieving a slightly higher viability than 20% DMSO.

Figure 2 shows the viability of rat PEC frozen with five schedules and thawed at two different rates with 10% and 15% DMSO as cryoprotectant. The results were similar to that of insulinoma cells, with the highest cellular viability achieved with slow freezing at -0.3°C/min (Schedule 5) and quick thawing in a 37°C water bath. The cellular viability of more than 70% achieved for rat PEC was lower than that for insulinoma cells. For later transplantation studies, rat PEC were cryopreserved with schedule 5, which was shown to achieve the highest cellular viability for both insulinoma and rat PEC cells.

Figure 3 indicates that intrathecal transplantation of cryopreserved Wi PEC into allogeneic diabetic ACI rats resulted

in normalization of random BG in seven of 10 diabetic recipients within 7 days, while the remainder of the recipient rats achieved normalization more slowly. All these animals had normal weight gain and 24-hour urine volume, and became aglycosuric following transplantation. The metabolic patterns of these animals following transplantation were similar to those observed previously in diabetic ACI recipients of noncryopreserved Wi PEC.

Figure 4 shows that the functional period of cryopreserved Wi PEC (8.3 ± 1.9 days [mean ± SD]N = 7) in allogeneic diabetic ACI recipients though slightly longer, is not significantly different from that of noncryopreserved preparation (6.6 ± 1.5 days, N = 7).

Table 1 demonstrates that the intraportal recipients of both cryopreserved and noncryopreserved PEC preparations had cytotoxic antibody formation against donor alloantigens. Table 2 tabulates cytotoxic antibody titers in the ACI Recipients of Allogeneic Wi PEC Graft. The mean peak antibody titers on days 7 and 10 detected in the recipients of cryopreserved PEC were not significantly lower than in the recipients of noncryopreserved PEC.

TABLE 1

Functional Period of Fresh and Cryopreserved Wi PEC Transplanted in Allogeneic Diabetic ACI Recipients

| PEC Graft | Functional Days | Mean ± SD |
|---|---|---|
| Noncryopreserved | 4, 6, 6, 6, 8, 8, 8 | 6.6 ± 1.5° |
| Cryopreserved | 6, 7, 8, 8, 8, 9, 12 | 8.3 ± 1.9 |

°0.5 > P > .1, nonsignificant

TABLE 2

Cytotoxic Antibody Titers in the ACI Recipients of Allogeneic Wi PEC Graft

| PEC Graft | Individual AB Titer Following Transplantation (day) 7th | 10th |
|---|---|---|
| Noncryopreserved | 4, 8, 12, 16, 16, 16, 32 | 2, 4, 4, 8, 8, 16, 8 |
| Cryopreserved | 4, 4, 4, 4, 6, 8, 16 | 2, 4, 4, 4, 4, 6, 8 |

The major hindrances to islet transplantation in humans are lack of adequate donor tissue and graft rejection process. The former problem can partly be solved if isolated islets can be stored until sufficient quantity is available for transplantation. Several approaches for storage of islets, including tissue culture, low temperature storage, and cryopreservation, have been tried. For long-term storage, a cryopreservation procedure would be the most logical and practical approach. Since single and small aggregates of PEC can be used for transplantation, the inventors examined the feasibility of freezing single or small clumps of PEC instead of whole islets as a means of storage. The efficacy of several freezing protocols were compared in vitro in this study using PEC and rat insulinoma cells. Cellular death ranging from 20% to 90% was observed after the freeze-thaw process. The best protocol was discovered to be slow freezing at about -0.3°C in conjunction with a quick subsequent thawing phase.

With PEC and small aggregates of fewer than 10 cells, freer temperature and medium exchange can be achieved than is possible for whole islets. Another factor is that mammalian islets are not of uniform size. They can vary from 100 μm to 200 μm in diameter. A freeze thawing protocol that is ideal for one particular islet size may not be so for others in the same preparation. Therefore, it is a simpler task to formulate a freeze thawing protocol specially for PEC or clumps of relatively uniform size.

The concentration of dimethylsulfoxide DMSO used in this study ranged from 10% to 20%. Since the viability counts of freeze-thawed cells indicate that 10% DMSO was adequate for the protection of PEC during freezing and DMSO is known to have some toxic effects on cells, a 10% DMSO concentration would be preferable.

Our investigations tested the in vivo function of cryopreserved PEC by transplantation into diabetic recipients. Rat PEC frozen with Schedule 5 and quick thawed in a 37° water bath followed by overnight culture at 26°C were transplanted intrathecally into diabetic recipients. The rates of BG decline in seven of 10 diabetic recipients were similar to that seen in the recipients of intrathecal fresh PEC grafts as observed in a previous study. The remaining three had a slower decline in BG, but once their BG were normalized, the metabolic parameters and BW gain were comparable to the other seven animals and similar to those recipients of fresh PEC and normal controls. The slower response in the last three rats would likely have resulted from a transient subnormal functional state of cryopreserved PEC or a fewer number of PEC being transplanted. The results suggest to the inventors that cryopreserved PEC were effective in the amelioration of the diabetic state in the recipients.

The prolonged survival of intrathecally implanted cryopreserved allogeneic PEC that we have observed was likely due to the protection by the immunoprivileged nature of subarachnoid space, rather than to the decreased immunogenieity of PEC after the freeze thawing process. To further assess the possibility of reduction of graft immunogenieity by cryopreservation, PEC were transplanted intraportally into allogeneic diabetic recipients. The similar rejection period of the cryopreserved and noncryopreserved PEC allograft following intraportal transplantation and comparable levels of antidonor antibody attained in both groups of recipients observed would suggest that cryopreservation with the present protocol has an insignificant effect on the immunogenieity of PEC graft.

The cryopreservation protocol with slow freezing and rapid thawing to achieve high PEC viability used in this study was similar to that used for the cryopreservation of lymphoid cells. Therefore, it is not surprising that no significant reduction of islet cell immunogeneity was achieved. The marginal prolongation of the survival of cryopreserved PEC and the

marginal reduction in antidonor antibody titers in the allogeneic recipient compared with noncryopreserved PEC observed could be due to the additional washing steps with the PEC following the freeze thawing process. Although the results of this study did not detect any significant reduction of immunogenieity of PEC following cryopreservation, it is still possible that reduction of islet cell immunogenieity can be achieved by cryopreservation process if optimal differential cooling and thawing rates can be found for the PEC and the contaminating immunogenic cells.

The results of our investigations show that cryopreservation procedure with slow freezing and quick thawing is an effective procedure for the banking of single or small aggregates of PEC. Cryopreserved PEC were functional in vivo in diabetic rat recipients. However, the cryopreservation protocol used in our investigations that resulted with high cellular viability following the freeze thawing process did not achieve significant reduction of PEC immunogenieity when assessed by in vivo allotransplantation.

As will be apparent to those skilled in the art in the light of the foregoing disclosure, many alterations and modifications are possible in the practice of this invention without departing from the spirit or scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the following claims.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A method of freezing pancreatic endocrine cells for storage and thawing the pancreatic cells for use in transplantation for treatment of diabetes, which comprises: rapidly freezing the pancreatic endocrine cells in the presence of a suitable antifreeze, storing the frozen cells in a suitable subfreezing environment, and then thawing the cells prior to transplantation.

2. A method of freezing pancreatic endocrine cells for storage and thawing the pancreatic cells for use in transplantation for treatment of diabetes, which comprises: slowly freezing the pancreatic endocrine cells in the presence of a suitable antifreeze, storing the frozen cells in a suitable sub-freezing environment, and then thawing the cells prior to transplantation.

3. A method according to claim 1 or claim 2 wherein the antifreeze is dimethyl sulphoxide.

4. A method according to claim 1 or 2 wherein the antifreeze is 10 to 20% dimethyl sulphoxide.

5. A method according to claim 1 or 2 wherein the antifreeze is selected from the group consisting of dimethyl sulphoxide, glycerol, propylene glycol and butane diol.

6. A method according to claim 1 wherein the cells are frozen at an ultra rapid rate.

7. A method according to claim 1 wherein the cells are frozen in the presence of liquid nitrogen.

8. A method according to claim 1 wherein the cells are frozen using a vitrification process.

9. A method according to claim 1 or claim 2 wherein the sub-freezing environment is a freezer.

10. A method according to claim 1 or claim 2 wherein the sub-freezing environment is liquid nitrogen.

11. A method according to claim 2 wherein the freezing is conducted at a temperature drop rate of between 0.1°C to 5°C per minute.

12. A method according to claim 2 wherein the cells are frozen at a rate of 0.1°C to 5°C per minute from ambient temperature to about -7°C, and thereafter at a freezing rate of 0.1°C to 3°C per minute to about -70°C.

13. A method according to claim 2 wherein the cells are frozen at a rate of 0.1°C to 0.5°C per minute from ambient temperature to about -40°C, and thereafter at a freezing rate of 0.5°C to 5°C per minute to about -70°C.

14. A method of freezing pancreatic endocrine cells for storage and thawing the pancreatic endocrine cells for use in transplantation for treatment of diabetes, which comprises: cooling the pancreatic endocrine cells at a rate of between 0.1°C to 5°C per minute to about -70°C in the presence of between 10% to 20% dimethyl sulphoxide, storing the frozen cells in a sub-freezing environment, and then thawing the cells prior to transplantation.

15. A method according to claim 14 wherein the subfreezing environment is liquid nitrogen.

16. A method according to claim 15 wherein the cells are thawed in a water bath of about 37°C temperature.

17. A method according to claim 1, 2 or 14 wherein the pancreatic endocrine cells are single cells.

18. A method according to claim 1, 2 or 14 wherein the pancreatic endocrine cells are cell aggregations.

19. A method according to claim 1, 2 or 14 wherein the pancreatic endocrine cells are islet cells.

20. A method of freezing pancreatic endocrine cells for storage and thawing the pancreatic endocrine cells for use in transplantation which comprises: cooling the pancreatic endocrine cells at a rate of -0.3°C per minute to -70°C in the presence of 10% dimethyl sulphoxide, storing the frozen cells in liquid nitrogen, and then thawing the cells in an about 37°C water bath prior to transplantation.

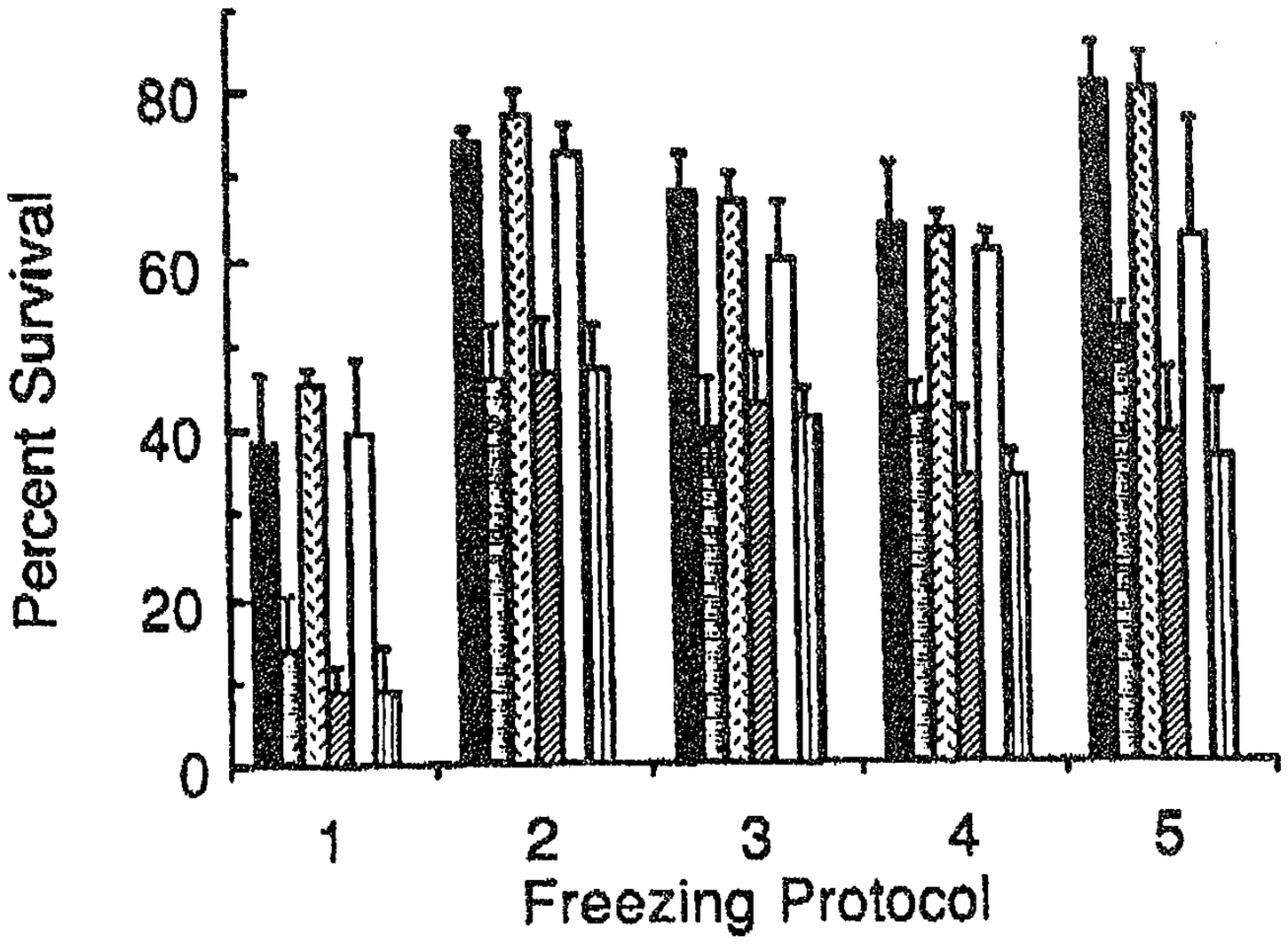
21. A method of cryopreserving pancreatic endocrine cells which comprises cooling the cells at a rate of about -5°C per minute to about 4°C, holding the cells for 3 minutes at about 4°C, subsequently cooling the cells at a rate of about -0.3°C per minute to about -7°C, holding the cooled cells at that temperature for 3 minutes, then cooling the cells at a rate of about -0.3°C per minute to about -40°C, and then thereafter cooling the cells at a rate of -5°C per minute to about -70°C in 10% dimethyl sulphoxide, and finally, transferring the frozen cells to liquid nitrogen for storage.

22. A method according to claims 20 or 21 wherein the frozen cells are thawed in a water bath maintained at about 37°C, and then transplanting the thawed cells into xenogeneic or allogeneic diabetic recipients to normalize the blood glucose level in the recipient.

23. A method according to claim 20 or 21 wherein the cells are insulinoma cells.

24. A method according to claim 20, 21 or 22 wherein the cells after being thawed in the water bath are cultured overnight at about 26°C, prior to being transplanted into the

diabetic recipient.

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AUTHOR: TZE FIG #: 1
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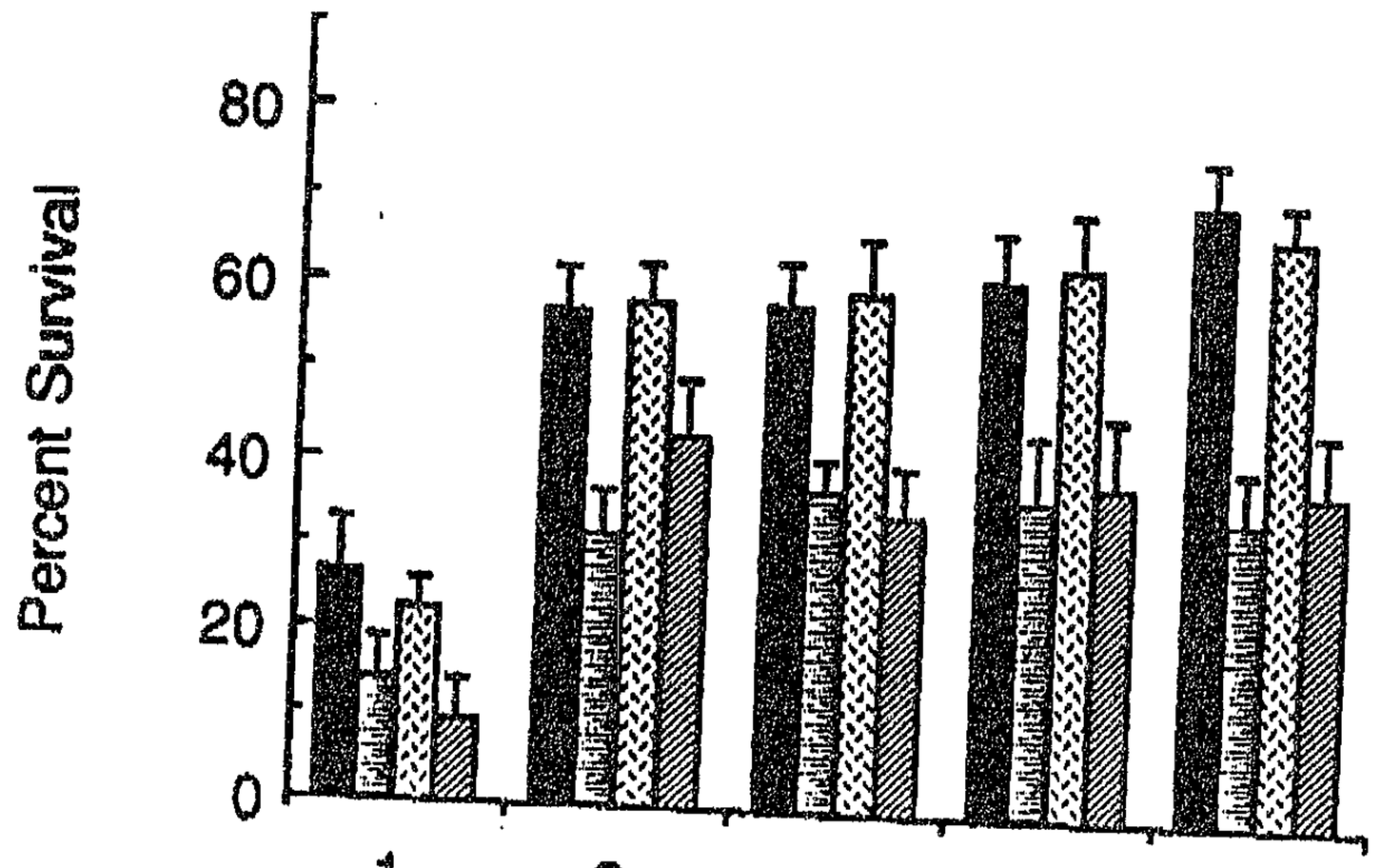


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AUTHOR: TZE FIG #: 2
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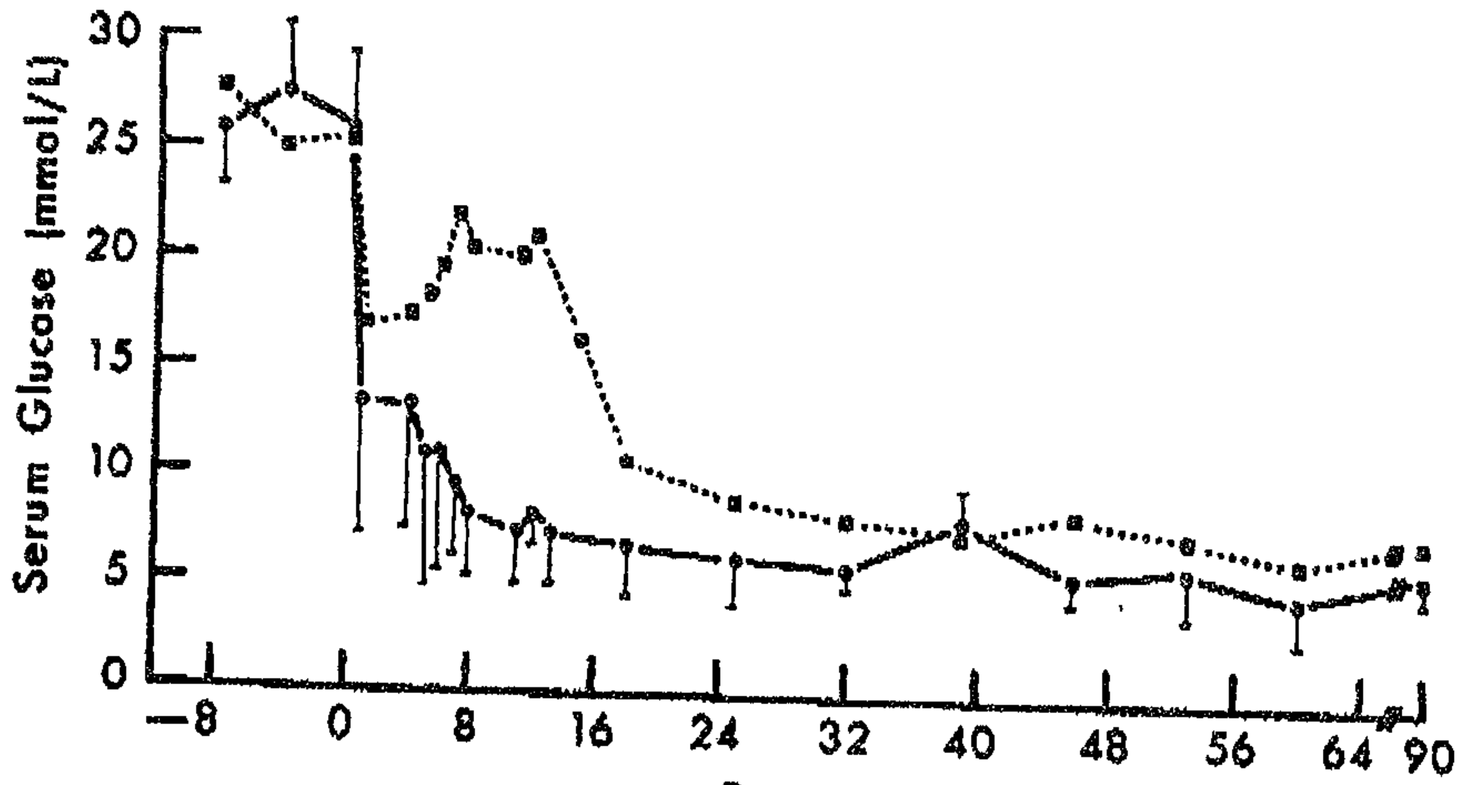
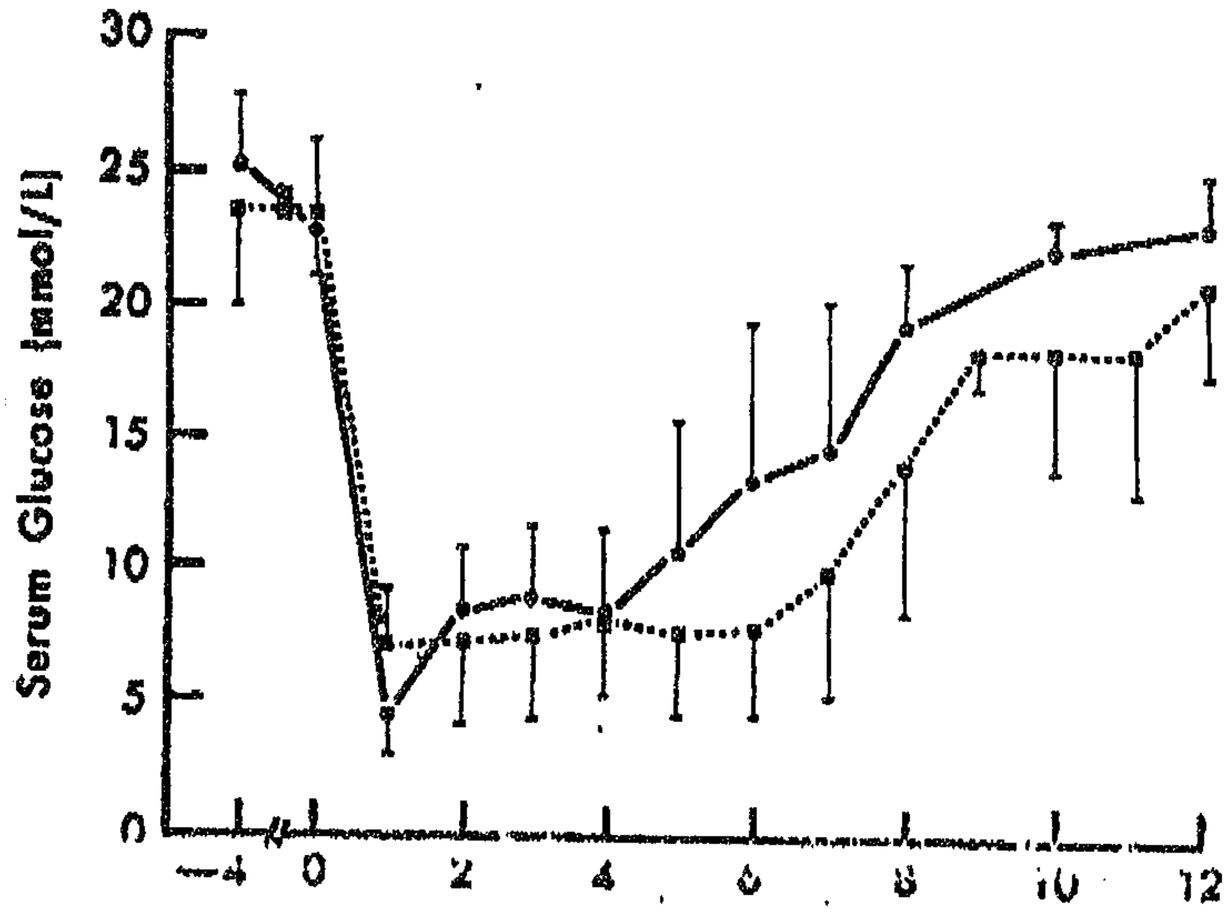



JOB NAME: META
JOB #: 7360-311-0
AUTHOR: TZE FIG #: 3
80 % FINAL SIZE: 29 , 0 X 15 , 10
FOLDER #: H1/12




JOB NAME: META
JOB #: 7360-311-0
AUTHOR: TZE FIG #: 4
79 % FINAL SIZE: 25 , 0 X 18 , 12
FOLDER #: H1/12