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Cryopreservation of Mouse Pancreatic Islets: Effects of Human Serum on Islet Survival

Stellan Sandler, Bo Nilsson and Arne Andersson

Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden

ABSTRACT

The aim of this study was to compare the survival of cryopreserved mouse pancreatic islets frozen in the presence of either a simple salt solution (Hanks' balanced salt solution) or a complete tissue culture medium (RPMI 1640). Moreover, the addition of 10% human serum to the freezing solutions was evaluated. Collagenase isolated islets were kept in culture for three days, before being cooled at a rate of 5°C/min or 25°C/min to -70°C, at which temperature the islets were transferred to liquid nitrogen. All freezing media were supplemented with 2 M dimethylsulphoxide as cryoprotectant. The islets were rapidly thawed at 37°C and subsequently cultured for another three days. The recovery of islets was higher when the more rapid cooling rate was used and the addition of serum further improved the recovery. Compared to non-frozen cultured islets there was a loss of cells in all groups of cryopreserved islets, as measured by their DNA content, and this was accompanied by a lowered insulin content. All groups of frozen-thawed islets responded to a high glucose stimulus in vitro with a 5-9 fold increase in insulin secretion. There was no obvious advantage of using a complete tissue culture medium for islet cryopreservation, but the addition of serum had some beneficial effects. Data obtained from non-frozen control islets suggest that human serum slightly impairs the function of mouse pancreatic B-cells.

INTRODUCTION

We have recently demonstrated that cryopreserved mouse pancreatic islets cooled at a rapid rate (25°C/min) are able to synthesize (pro)insulin and release insulin, in response to glucose in vitro, at rates not different from those of non-frozen cultured control islets (15). Such cryopreserved islets significantly reduced alloxan-induced hyperglycemia when transplanted into syngeneic mice. It was also found that the function of more rapidly cooled islets was better preserved than that of islets cooled at a rate of 5°C/min. In two previous studies we observed that a prerequisite for a successful

cryopreservation of isolated islets is that the islets are maintained for a period in culture before freezing (12,16).

The aim of the present study was to investigate whether the viability of cryopreserved pancreatic islets could be improved by using a complete tissue culture medium (RPMI 1640)(10) instead of a simpler salt solution (Hanks' balanced salt solution)(2) during the freezing procedure. We have recently found that supplementation of the culture medium with human serum promotes the development of islet-like structures in vitro (14) when using a recently described method for the culture of human fetal pancreas (13). Future attempts to cryopreserve human islets intended for transplantation to human insulin-dependent diabetics may involve the addition of human serum to media. Therefore, we examined the effects of adding human serum not only to the freezing medium but also to the medium used during culture of the islets before and after freezing.

MATERIALS AND METHODS

Islet isolation, culture and cryopreservation. Pancreatic islets were isolated by a collagenase digestion method (5) from male, adult NMRI mice (Anticimex AB, Sollentuna, Sweden). Prior to freezing the islets were kept for three days free-floating in tissue culture medium RPMI 1640 (Flow Laboratories Ltd., Irvine, Scotland) supplemented with 100 U/ml benzylpenicillin (Astra Läkemedel, Södertälje, Sweden), 0.1 mg/ml streptomycin (Glaxo Laboratories, Greenford, England) and 10% pooled heat-inactivated human serum (v/v) (Blood Center, University Hospital, Uppsala, Sweden). The cultures were maintained at 37°C in an atmosphere of humidified air + 5% CO_2. The culture medium was changed after two days.

Cooling was performed using a programmable temperature controller (Planer Minifreezer, Model R 202/200R, Planer Products Ltd., Sunbury-on-Thames, England). Groups of about 75 islets were transferred at room temperature to sterilized glass ampoules containing 0.4 ml freezing medium. The medium was either Hanks' solution or medium RPMI 1640 with or without 10% (v/v) human serum. In all freezing media 2 M dimethylsulphoxide (Me_2SO; Sigma Chemicals, St Louis, MO, USA) was added as cryoprotectant. First, the islets were cooled at a rate of 8°C/min from room temperature to 0°C where the temperature was maintained for 20 min to allow permeation of the cryoprotectant. Cooling was then resumed at either 5°C/min or 25°C/min down to -70°C, after which the ampoules were immersed in liquid nitrogen.

After storage for 60 min at -196°C the islets were rapidly thawed by stirring the glass ampoules in a 37°C water bath. The resulting warming rate was about 240°C/min. When the ice of the freezing medium had just melted the islets, with the freezing medium were poured into culture dishes containing 10 ml of the same culture medium as that used before the freeze-thawing experiments. This lead to a rapid dilution of the Me_2SO to less than 0.08M. Finally, 5 ml of culture medium was removed from the dishes and

the islets were cultured for another 3 days before testing. In a previous study we have shown that this Me_2SO concentration does not impair the B-cell function of islets maintained in culture in the presence of Me_2SO (11).

The non-frozen control islets were obtained from the same islet isolations as the frozen-thawed islets, but maintained in tissue culture in medium RPMI 1640 + 10% human serum for 6 days, with medium exchanges every second day.

Islet recovery. The islet recovery after cryopreservation was calculated by counting the number of islets in a stereomicroscope just before transfer to the freezing ampoules and subsequently on day three after thawing. The recovery of the non-frozen control islets was calculated by counting the islets present in the culture dishes on day 3 and day 6 of culture.

Islet DNA content. On day 3 after thawing i.e. on experimental day 6, frozen-thawed and control islets in groups of 30 were ultrasonically disrupted in 0.2 ml redistilled water. The DNA content of the resulting aqueous homogenates was measured by fluorophotometry (4,7)

Islet insulin content. A 50 µl fraction of the islet homogenates was mixed with 125 µl of acid ethanol (0.18 M HCl in 96% (v/v) ethanol), extracted overnight at +4°C and then stored at -20°C. The insulin concentration in the samples was determined by radioimmunoassay (3), using mouse crystalline insulin as standard (Novo, Copenhagen, Denmark) and ^{125}I-labelled insulin as tracer (Novo).

Islet insulin release. Triplicate groups of 10 islets were incubated for 2 consecutive hours at 37°C (O_2:CO_2; 95:5) in a slowly shaking water bath in sealed glass vials (6) containing 0.25 ml of a Krebs-Ringer bicarbonate buffer (8), supplemented with 2 mg/ml bovine albumin (Miles Laboratories, Slough, England) and 10 mM N-2-hydroxyethylpiperazine-N'-2ethanesulphonic acid (Hepes; Sigma), hereafter referred to as KRBH. During the first hour the incubation medium contained 1.67 mM glucose. After this incubation the medium was carefully removed and replaced by 0.25 ml of KRBH supplemented with 16.7 mM glucose. The insulin concentration in the incubation media was determined by radioimmunoassay as described above.

Statistical analysis. Results are expressed as means ± SEM. Groups of data were compared using Student's unpaired t-test.

RESULTS

In general the recovery of cryopreserved islets was higher after cooling at a rate of 25°C/min as compared to that after cooling at 5°C/min (Table 1). The highest values

TABLE 1. Effects of human serum (HS) addition and cooling rate on islet recovery, islet DNA content and islet insulin content of mouse pancreatic islets cryopreserved in the presence of 2 M Me_2SO.

Freezing condition (Cooling rate; medium)	Islet recovery (%)		Islet DNA content (μg DNA/10 islets)		Islet insulin content (ng insulin/10 islets)	
5°C/min; Hanks'	62.5 ± 8.8	(7)**	0.33 ± 0.041	(7)**	71.5 ± 11.7	(7)***
5°C/min; Hanks' + 10% HS	71.5 ± 9.0	(7)	0.30 ± 0.028	(7)***	72.8 ± 11.7	(7)***
5°C/min; RPMI 1640	41.2 ± 10.7	(7)***	0.41 ± 0.037	(6)	50.7 ± 9.0	(7)***
5°C/min; RPMI 1640 + 10% HS	68.6 ± 9.5	(7)	0.33 ± 0.029	(7)**	67.4 ± 13	(7)***
25°C/min; Hanks'	78.5 ± 4.2	$(7)^{+}$	0.30 ± 0.022	(7)***	80.2 ± 15	(7)***
25°C/min; Hanks + 10% HS	89.0 ± 2.0	(8)	0.35 ± 0.022	(8)**	108 ± 7.8	(8)***
25°C/min; RPMI 1640	74.7 ± 2.0	$(8)^{**++}$	0.37 ± 0.023	(8)*	85.8 ± 5.6	(8)***
25°C/min; RPMI 1640 + 10% HS	85.6 ± 2.0	(8)	0.38 ± 0.025	(8)*	93.0 ± 10	(8)***
Non-frozen cultured islets	87.2 ± 2.6	(9)	0.48 ± 0.033	(9)	223 ± 15	(9)

Pancreatic islets were isolated from NMRI mice and cultured for three days in medium RPMI 1640 + 10% HS prior to freezing. The islets were frozen at the cooling rate and in the medium given in the first column. After storage for 1-2 hours at -196°C the frozen samples were rapidly thawed at 37°C and the islets cultured for another three days. Non-frozen islets from the same isolations served as controls. The number of islets were counted in a stereomicroscope just before transfer to the freezing ampoules, and subsequently on day three after thawing, and the islet recovery was expressed as the percentage of islets remaining. The recovery of the control islets was calculated by comparing the number of islets present on day 3 and day 6 of culture. Islet DNA content was measured fluoro-photometrically in water homogenates of the islets, and the insulin contents assessed by radioimmunoassay of acid ethanol extracts of the islets. Data are given as means ± SEM, with number of observations within parenthesis. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ respectively, compared to non-frozen islets using unpaired t-test. $^{+}$ and $^{++}$ denote $P<0.05$ and $P<0.01$ when comparing islets frozen at the same cooling rate and in the same salt solution but with or without HS, using unpaired t-test.

TABLE 2. Effects of human serum (HS) addition and cooling rate on glucose-stimulated insulin release of mouse pancreatic islets cryopreserved in the presence of 2 M Me_2SO.

Freezing condition (Cooling rate; medium)	Insulin release		
	1.67 mM glucose (ng insulin/10 islets x 60 min)	16.7 mM glucose (ng insulin/10 islets x 60 min)	Stimulation factor (16.7 vs 1.67 mM glucose)
5°C/min; Hanks'	1.9 ± 0.2 (7)**	12.5 ± 4.1 (7)**	6.3 ± 2.1 (7)
5°C/min; Hanks' + 10% HS	2.0 ± 0.5 (7)*	9.8 ± 2.1 (7)***$^{++}$	5.0 ± 0.8 (7)
5°C/min; RPMI 1640	1.6 ± 0.4 (7)**	11.2 ± 4.5 (7)***	5.9 ± 1.3 (7)
5°C/min; RPMI 1640 + 10% HS	2.9 ± 0.6 (7)*	12.5 ± 3.1 (7)***$^{+}$	4.3 ± 0.7 (7)
25°C/min; Hanks'	3.4 ± 0.8 (7)*	20.5 ± 5.5 (7)*$^{++}$	6.3 ± 1.2 (7)
25°C/min; Hanks' + 10% HS	3.3 ± 0.4 (8)*	19.9 ± 3.3 (8)**$^{+++}$	7.5 ± 1.1 (8)
25°C/min; RPMI 1640	2.9 ± 0.5 (8)*	22.0 ± 4.0 (8)**$^{+++}$	8.4 ± 1.2 (8)
25°C/min; RPMI 1640 + 10% HS	3.7 ± 0.7 (8)*	27.8 ± 7.8 (8)$^{++}$	7.4 ± 1.8 (8)
Non-frozen cultured islets	12.2 ± 3.0 (9)	41.7 ± 4.9 (9)$^{+++}$	5.0 ± 1.2 (9)

The groups of islets were treated as described in Table 1. Islet insulin release experiments were performed by incubating the islets in groups of ten in KRBH buffer containing 1.67 mM glucose and albumin (2 mg/ml) at 37°C (O_2:CO_2, 95:5). After 60 min the medium was removed and the islets incubated for another 60 min in medium containing 16.7 mM glucose. The stimulation factor was determined in each individual experiment by calculating the ratio of insulin release between the second and first incubation hour. Data are given as means ± SEM, with number of observations within parenthesis. *, ** and *** denote $P<0.05$, $P<0.01$ and $P<0.001$ respectively, compared to non-frozen islets using unpaired t-test. $^{+}$, $^{++}$ and $^{+++}$ denote $P<0.05$, $P<0.01$ and $P<0.001$ when comparing the same group of islets incubated at 1.67 mM and 16.7 mM glucose, using unpaired t-test.

were obtained when human serum was added to either Hanks' solution or RPMI 1640 used for freezing islets at a rate of 25°C/min. In these two cases the recovery figures were very close to that of the non-frozen control islets. The number of islets decreased by approximately 13% during culture between day 3 and day 6. In all test situations the addition of human serum yielded a higher recoveries when compared to the same group of islets frozen without serum.

The DNA content in all groups of cryopreserved islets was reduced by 20-30% (Table 1). The addition of human serum to the freezing medium did not affect the islet DNA content. The insulin content of the cryopreserved islets was lowered to 50% or less of the value found in the non-frozen cultured islets (Table 1). The addition of human serum to the freezing media slightly increased the insulin content of the islets in the various groups of frozen islets, however the differences did not attain statistical significance.

The basal insulin release of the non-frozen control islets at 1.67 mM glucose, was elevated compared to all groups of frozen-thawed islets (Table 2). When the islets were challenged with high glucose (16.7 mM) the insulin secretion was increased in all groups of islets by 5-9 times. However, in the two groups of islets cooled at a rate of 5°C/min, in the absence of human serum, the high-glucose stimulated insulin release was not significantly different from the basal secretion. The rate of insulin release at the high glucose concentration was lower in the cryopreserved islets than in the controls, except for the islets cooled at a rate of 25°C/min in RPMI 1640 plus human serum. It should be noted that the stimulation factor of insulin release in response to the high glucose challenge in the control islets was fairly low due to the high basal insulin release.

DISCUSSION

Cryopreservation appears to be the only suitable method for prolonged storage of isolated pancreatic islets in vitro intended for transplantation to insulin-dependent diabetics. During the last 5-10 years a number of different cryopreservation protocols have been presented employing various cooling rates, modes of addition and removal of the cryoprotectant and warming rates (for a review see Bank, (1)). Although theoretical calculations can be made on how a particular cell preparation should be cryopreserved (9), when designing the optimal freeze-thawing conditions for a given cell population the different steps in the cryopreservation procedure must often be tested separately. This communication is such a step in the efforts to find the best cryopreservation procedure for isolated mouse pancreatic islets. In this context it was also of interest to study the effect of human serum addition to the freezing medium, since this biological supplement will most probably be used in conjunction with human islet cryopreservation.

Overall, the use of RPMI 1640 compared to Hanks' solution as the freezing medium afforded of no clear advantage for the viability of the frozen-thawed islets. The addition

of human serum increased the islet recovery after cryopreservation, which indicates that the presence of serum has a beneficial effect for the survival of the islet cells during the freeze-thawing manoeuvres. The reason for this is obscure. It could be speculated that serum has a supportive role for islet viability as during tissue culture, but it cannot be excluded that factors in the serum have cryoprotective properties.

The islet insulin content was markedly reduced after cryopreservation, which confirms our previous findings (12,15). However , when comparing the insulin content of the control islets cultured with 10% human serum with that of islets cultured in the presence of 10% calf serum (12,15), it is obvious that human serum reduces the insulin contents of mouse pancreatic islets. It may be that human serum is slightly toxic to mouse islets, leading to a passive insulin secretion or alternatively an impaired insulin biosynthesis. The finding of an increased insulin secretion by the control islets at the low glucose concentration speaks in favour of an effect on the secretory apparatus. If the addition of 10% human serum to islets in culture slightly impairs their viability, it can be expected that their functional capacity will be further reduced by cryopreservation, since the prefreezing status of the islets is crucial for their ability to resist the stresses of the freeze-thawing process.

In conclusion the data of the present study suggest that the use of a complete tissue culture medium (RPMI 1640) as freezing medium does not increase the viability of cryopreserved pancreatic islets. On the other hand, the addition of 10% human serum to the freezing medium supported the survival of the cryopreserved islets, but human serum may, to some degree, impair the viability of mouse pancreatic islets in culture. We have, however, found that this supplement may stimulate the generation of pancreatic B-cells from human fetal pancreatic glands (14).

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Address for correspondence:
Dr Stellan Sandler
Department of Medical Cell Biology
Biomedicum, P.O.Box 571
S-751 23 Uppsala, Sweden