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A modified cryopreservation method increases the survival of human biopsied cleavage stage embryos

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BACKGROUND: The relatively poor survival rate of human biopsied cleavage stage embryos following cryopreservation is a significant obstacle in the application of preimplantation genetic diagnosis (PGD). We have attempted to improve cryosurvival of biopsied embryos by modifying the standard embryo cryopreservation technique. METHODS: Biopsied embryos were cryopreserved in 1.5 mol/l 1,2-propanediol in the presence of an elevated concentration of sucrose (0.2 mol/l) and human serum albumin was replaced by maternal serum (20% vol:vol). An additional initial thawing step in the presence of 0.3 mol/l sucrose was also included. RESULTS: The proportion of biopsied embryos which survived cryopreservation with ⩾50% of their blastomeres intact was significantly higher using the modified method (138/185; 75%) than that observed using the standard propanediol method (20/46; 43%; $P = 0.022$). Total blastomere survival was also significantly increased as a result of the modifications (1010/1513; 67% versus 177/385; 46%; $P < 0.001$). Six fetal hearts have been detected to date following replacement of biopsied embryos cryopreserved with the modified method. CONCLUSIONS: Survival of human biopsied cleavage stage embryos can be restored to a level similar to that of non-biopsied controls by modification of the cryopreservation procedure. Embryos which have been cryopreserved using the modified method can implant following replacement *in utero*.

Key words: cryopreservation/embryo/implantation/PGD

Introduction

The development and introduction of more responsible approaches to the transfer of cleavage stage embryos in clinical IVF is partly due to the routine application of embryo cryopreservation (Mandelbaum *et al*., 1998), although it is also clear that cryopreservation results in a reduction in the developmental potential of a population of stored embryos (Edgar *et al*., 2000).

Preimplantation genetic diagnosis (PGD), in association with human IVF, offers the possibility of detection and circumvention of the clinical consequences of a variety of genetic disorders (Gianaroli *et al*., 1997; Sermon, 2002), but the prerequisite for zona pellucida (ZP) opening and removal of one or more blastomeres (embryo biopsy) has been reported to result in a further reduction in the capacity of human embryos to survive subsequent cryopreservation (Joris *et al*., 1999; Magli *et al*., 1999; Ciotti *et al*., 2000). This phenomenon is discouraging in view of the relative 'normality' of biopsied embryos which require cryopreservation and is of particular concern in the application of more advanced PGD techniques such as comparative genomic hybridization (CGH) where the period required for completion of diagnosis currently necessitates cryopreservation of all biopsied embryos with replacement of the 'normal' embryos in a subsequent menstrual cycle (Wilton *et al*., 2001).

Modification of standard cryopreservation methodology incorporating exposure to an increased sucrose concentration prior to freezing has been reported to result in improved survival of human oocytes (Fabbri *et al*., 2001) and has also been used in protocols for cryopreservation of embryos (Kaufman *et al*., 1995; Chi *et al*., 2002). Our own observations (unpublished data) have also suggested that the macromolecular component of cryopreservation solutions may play a role in the protection of human oocytes during freezing, with higher survival observed in the presence of human serum (which contains a significant globulin content) when compared with purified human serum albumin (HSA).

In the present study we confirm the observation that survival of biopsied embryos is compromised relative to non-biopsied controls and report an increase in the survival of biopsied embryos when a modified cryopreservation technique is employed.

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Materials and methods

Embryos

Embryos were obtained from patients undergoing IVF in conjunction with PGD, and were generated by both ICSI and conventional IVF. Embryos were biopsied ~64–66 h post-insemination. Biopsied embryos were cryopreserved between 72–88 h post-insemination. Frozen biopsied embryos diagnosed as euploid were thawed for subsequent transfer. Frozen biopsied embryos classified as aneuploid were also thawed in order to confirm the original diagnosis. A small number ($n = 19$) of non-biopsied embryos, of similar age to the frozen biopsied embryos, were also frozen to assess the impact of embryo age alone on cryosurvival.

Embryo biopsy

ZP drilling and embryo biopsy were performed under mineral oil in HEPES-buffered human tubal fluid (HTF) medium supplemented with 10% (vol:vol) maternal serum. Embryos were incubated for ~5 min in Ca^{2+}–Mg^{2+} free medium before acidified HEPES-buffered HTF medium (pH 2.4) was used to breach the ZP. Following blastomere removal, embryos were incubated in fresh equilibrated culture medium. All the above media were obtained from SAGE BioPharma (Bedminster, NJ, USA).

Embryo cryopreservation

Standard method

Initially, embryos were frozen using 1,2-propanediol (PROH) and sucrose as the cryoprotectants (Lassalle *et al*., 1985) in HEPES-buffered HTF medium as previously described (Edgar *et al*., 2000). HSA (SAGE Biopharma) was used to supplement both freezing (10 mg/ml) and thawing (4 mg/ml) solutions. Embryos were equilibrated in 1.5 mol/l PROH for 10 min at room temperature before being transferred to 1.5 mol/l PROH with 0.1 mol/l sucrose and loaded individually into plastic straws. Cooling was conducted in programmable freezers (Kryo 10 Series; Planar Products, Sunbury-on-Thames, UK) at a rate of –2°C/min to –7°C, at which point seeding was induced manually. Cooling was then continued at rates of –0.3°C/min to –30°C and –50°C/min to –150°C before plunging and storage in liquid nitrogen. Embryos were thawed rapidly by removing straws from storage, exposure to air for 30 s and immersion in a water bath at 30°C for 45 s. PROH was removed by serial transfer into 0.75 mol/l PROH in the presence of 0.2 mol/l sucrose at room temperature for 5 min followed by 0.2 mol/l sucrose for a further 5 min. Rehydration was completed by transfer to sucrose-free HEPES-buffered HTF medium for 10 min. Embryos were transferred to culture medium at 37°C before being assessed for blastomere survival.

Modified method

Adjustments were made to both the freezing and thawing solutions but not to the freezing or thawing rates. The concentration of PROH in the freezing solutions remained at 1.5 mol/l, but the sucrose concentration was doubled to 0.2 mol/l. The HSA protein supplement was replaced by 20% (vol:vol) heat inactivated maternal serum. In order to maintain the osmotic buffering effect of increased sucrose during rehydration the concentration was also increased to 0.3 mol/l during the first thawing steps. Thawed embryos were rehydrated by sequential transfer to 0.75 mol/l PROH + 0.3 mol/l sucrose (5 min), 0.3 mol/l sucrose (5 min), and 0.2 mol/l sucrose (10 min). The concentration of HSA during thawing was 20 mg/ml. Rehydration was completed by transfer to sucrose-free HEPES buffered HTF medium (10 min) and embryos were transferred to culture medium at 37°C prior to assessment. Thawed embryos were transferred in cycles as previously described (Bourne *et al*., 1995).

Analysis of results

Statistical analysis was performed using the χ^2-test. Although not applicable in the very strictest theoretical sense, this method was considered to be reasonable given that the embryos came from sources which would not be expected to differ in any significant way and given that the differences in survival were so marked.

Results

Survival of cryopreserved biopsied embryos using standard method

Our early results from cryopreservation of biopsied embryos using our standard protocol are shown in Table I. Only 43% of biopsied embryos had ⩾50% of the original blastomeres intact after thawing and only 22% remained fully intact. In addition, only 46% of all cryopreserved blastomeres survived after thawing.

Survival of cryopreserved non-biopsied embryos of similar age to biopsied embryos using standard method

As described above, biopsied embryos were cryopreserved at least 72 h post-insemination, whereas our previously published data was based on cryopreserved non-biopsied day 2 embryos. To confirm that reduced cryosurvival is a consequence of the biopsy procedure(s) rather than embryo age, a small number of non-biopsied embryos of equivalent age were cryopreserved and thawed. These embryos had a slightly reduced mean cell number compared with the biopsied embryos (7.2 versus 8.4) which reflects the fact that they were not considered suitable for biopsy. They did, however, represent the only embryo age-matched control material available. In addition, no differences in survival related to developmental stage were observed in biopsied or non-biopsied embryos. The results are shown in Table I. The proportion of embryos with ⩾50% of the original blastomeres intact (79%) and the overall blastomere survival

Table I. Survival of embryos cryopreserved using standard and modified PROH methods

Cryopreservation method	Embryos	*n*	% Surviving blastomeres				Embryos with ⩾50% surviving blastomeres	Total blastomere survival
			0	1–49	50–99	100		
Standard	Biopsied	46	18 (39%)[a]	8 (17%)	10 (22%)	10 (22%)	20 (43%)[b]	177/385 (46%)[c]
Standard	Non biopsied	19	0 (0%)	4 (21%)	8 (42%)	7 (37%)	15 (79%)	104/137 (76%)
Modified	Biopsied	185	20 (11%)[a]	27 (15%)	80 (43%)	58 (31%)	138 (75%)[b]	1010/1513 (67%)[c]

[a]$P < 0.001$.
[b]$P = 0.022$.
[c]$P < 0.001$.

(76%) in this group were similar to our previous results with non-biopsied day 2 embryos.

Survival of cryopreserved biopsied embryos using the modified method

The results obtained after freezing biopsied embryos with the modified cryopreservation method are shown in Table I. The proportion of embryos with ⩾50% of the original blastomeres intact was significantly ($P = 0.022$) higher with the modified method (75%) compared with the standard method (43%). The proportion of embryos undergoing complete lysis was significantly reduced using the modified method (11 versus 39%; $P < 0.001$). Total blastomere survival was also significantly higher using the modified method (67 versus 46%; $P < 0.001$). The differences observed were all significant when analysed using the χ^2-test. Although this analysis is not strictly applicable since the embryos were derived from different patient cycles (only one patient had embryos frozen by both methods) in different time periods, the differences were such that a randomized controlled trial to compare the methods would be unethical. The embryos in both the standard and modified groups were derived from patients with similar mean ages (35.9 and 36.3 years respectively) and the survival distributions were uniformly distributed. Comparison of the blastomere survivals in the modified versus the standard groups (1010/1513 versus 177/385) generated a ratio of 1.45 with 95% confidence limits of 1.3–1.6.

Implantation of biopsied embryos cryopreserved using the modified method

To date, 41 thaw cycles have resulted in 36 transfer procedures in which a total of 50 embryos (cryopreserved using the modified method) have been transferred. Eight of the 36 transfers resulted in a positive (>100 IU/l) β-hCG test (22%). Six of the eight progressed to the detection of a fetal heart-beat with the remaining two classified as biochemical pregnancies only. A total of six fetal hearts have been detected (12% implantation rate).

Discussion

Cryopreservation of human embryos generated by IVF allows maximum utilisation of the clinical material available from a single cycle of oocyte collection (Jones *et al*., 1997) while removing the temptation to replace large numbers of embryos in any single transfer procedure (Cohen and Jones, 2001). However, it has become clear from a number of studies that blastomere loss in frozen–thawed early cleavage stage embryos is associated with a reduction in implantation potential (Van den Abbeel *et al*., 1997; Burns *et al*., 1999; Edgar *et al*., 2000; Guerif *et al*., 2002). Given that it is important to minimise the impact of cryopreservation on the blastomere constitution of embryos, recent reports (Joris *et al*., 1999; Magli *et al*., 1999; Ciotti *et al*., 2000) that blastomere and embryo survival are further reduced in embryos which have been subjected to embryo biopsy have raised concerns relating to the potential efficiency of PGD.

Embryos which are cryopreserved following characterization by PGD may, in general, be considered to be more 'normal' than embryos that are not selected. In some cases they will have been screened for the presence of specific aneuploidies (Gianaroli *et al*., 1997; Munné *et al*., 1999) or specific monogenic disorders (Sermon, 2002) and be surplus to those which can be replaced safely in a fresh transfer procedure. More recently, the application of CGH in PGD has offered the possibility that the whole genome can be screened for numerical chromosome errors (Voullaire *et al*., 2000; Wells and Delhanty, 2000). Although this technology has been used to screen embryos prior to replacement *in utero* and resultant live birth (Wilton *et al*., 2001), the time currently required for completion of diagnosis dictates that all embryos must be frozen pending a definitive result. The consequences of suboptimal cryopreservation will, therefore, be more significant in this case since the 'normal' embryos which would routinely be transferred fresh after PGD are also exposed to the risk of cryodamage. The above considerations emphasise the need for improved survival of biopsied embryos.

Despite an early report (Wilton *et al*., 1989) that mouse biopsied embryos were able to survive cryopreservation and form fetuses *in utero*, lower levels of survival have been reported in human biopsied (relative to non-biopsied) embryos cryopreserved using both propanediol (Magli *et al*., 1999; Ciotti *et al*., 2000) and dimethylsulphoxide (Joris *et al*., 1999) as cryoprotectants. The latter study also suggested that the increased susceptibility of biopsied embryos could be a consequence of both zona drilling and blastomere removal. Micromanipulation, however, does not necessarily appear to reduce the cryosurvival of embryos in all cases since it has been shown that embryos derived from ICSI survive cryopreservation at similar rates to those derived from conventional IVF (Van Steirteghem *et al*., 1994; Kowalik *et al*., 1998).

Our own observations on cryopreserved biopsied embryos using a standard propanediol method (Table I) reinforce previous reports that only 9% (Magli *et al*., 1999) and 14.8% (Joris *et al*., 1999) of cryopreserved biopsied embryos survived fully intact after cryopreservation. Similarly, our finding that only 46% of all frozen blastomeres in biopsied embryos survived was in agreement with a reported survival rate of 29.5% (Joris *et al*., 1999), a survival index of 38% (Magli *et al*., 1999) and the reduced cell survival (58 versus 85%) reported by Ciotti *et al*. (2000). The results also confirmed the reduced survival following cryopreservation when compared with our historical results with non-biopsied embryos (Edgar *et al*., 2000). Previous work with non-biopsied embryos has suggested that cell number at freezing may impact on survival (Hartshorne *et al*., 1990) but our results with non-biopsied embryos of similar stage to the biopsied group (Table I) suggest that cleavage stage is not a significant factor in the reduced survival of biopsied embryos.

The potential toxicity of permeating cryoprotectants, such as propanediol or dimethyl sulphoxide, mitigates against the use of elevated concentrations for embryo cryopreservation but the use of an elevated level (0.2 mol/l) of the non-permeating sucrose may allow more complete dehydration and has been used in protocols for freezing human oocytes in combination

with propanediol (Fabbri *et al*., 2001), multicellular embryos in combination with ethylene glycol (Chi *et al*., 2002) and blastocysts in combination with glycerol (Kaufman *et al*., 1995). Although little has been published on the role of macromolecular components in cryopreservation media, it has been suggested that protein preparations containing significant levels of globulins (similar to serum concentrations) may provide a more appropriate milieu for human embryos (Pool and Martin, 1994; Weathersbee *et al*., 1995). The polyhydroxy domains in the globulin fraction and their interaction with water may also be of significance during dehydration prior to cryopreservation. Protective effects of polymers such as dextran and Ficoll (Dumoulin *et al*., 1994) in the cryopreservation of ZP and embryos has also been demonstrated. Our modified cryopreservation method was based on the above considerations in an attempt to provide a greater degree of cryoprotection to the more vulnerable biopsied embryos.

The proportion of embryos which survived cryopreservation with ⩾50% of the initial blastomeres intact (75%, Table I) was restored to a level consistent with our expectation of non-biopsied embryos as was the overall blastomere survival. The proportion of embryos which survived with all blastomeres intact was still lower than reported by us for day 2 embryos (Edgar *et al*., 2000) but this may simply be a reflection of the greater number of cells present in the more advanced (⩾72 h post-insemination) biopsied embryos. The implantation rate of biopsied embryos cryopreserved using the modified method to date is consistent with reported results with fresh biopsied embryos from an equivalent (recurrent IVF failure) patient population (ESHRE PGD Consortium Steering Committee, 2002).

The method described results in improved survival of biopsied embryos and should impact on the efficiency of PGD treatment. The ability of biopsied embryos, frozen using the modified method, to implant following transfer confirms the applicability of the technique.

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