

## Article

# Cryopreservation of biopsied cleavage stage human embryos



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James Stachecki has been the director of cryobiology at St Barnabas Medical Centre, New Jersey, USA since 1996, when he joined Drs Jacques Cohen and Steen Willadsen and shortly afterwards discovered a novel freezing medium that proved very effective for storing mouse and human oocytes. His work is focused on discovering new and improved methods of storing human oocytes, embryos, blastocysts, and spermatozoa. After obtaining a BSc in Biology from Alma College in Michigan, James went on to get an MSc from Central Michigan University in Genetics, and his PhD in Physiology from Wayne State University School of Medicine. For his work in oocyte cryopreservation, he received an award for the general prize paper in 1998 from the American Society of Reproductive Medicine. His ongoing research has far-reaching ramifications including the conservation of endangered species. His most recent work has focused on perfecting blastocyst vitrification. James is also an award-winning wildlife and nature photographer.

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## Abstract

The aim was to develop a method to optimize cryopreservation of biopsied multi-celled human embryos. Human day 3 embryos that were donated to research, along with those found to be chromosomally abnormal after blastomere biopsy and fluorescence in-situ hybridization (FISH), were cryopreserved using a slow-freezing protocol in either standard embryo cryopreservation solution [embryo transfer freezing medium (ETFM), a conventional sodium-based medium] or CJ3 (a choline-based, sodium-free medium). After thawing, the number of intact cells was recorded and the previously biopsied embryos were reanalysed using FISH. Biopsied embryos had a lower proportion of intact blastomeres after cryopreservation as compared with intact embryos. However, a significantly ( $P < 0.05$ ) higher proportion of blastomeres from intact and biopsied embryos cryopreserved in CJ3 (84.1 and 80.1% respectively) survived after thaw than those in ETFM (73.6 and 50.5% respectively). The proportion of aneuploid and mosaic embryos was not statistically different between the two groups. In addition, the frequency of lost cells by aneuploid and mosaic embryos was similar. This study describes a new method that improves the survival of cryopreserved biopsied embryos, and shows that it may also be beneficial for the storage of intact human multi-celled embryos.

**Keywords:** aneuploidy, choline, cryopreservation, embryo biopsy, PGD

## Introduction

Clinical embryo freezing is entering its 3rd decade (Trounson and Mohr, 1983; Zeilmaker *et al.*, 1984; Cohen *et al.*, 1985, 1988; Lassalle *et al.*, 1985). Well-defined protocols are routinely used and small modifications are introduced on a regular basis.

When examining the ability to successfully cryopreserve embryos, there are several ways to report the outcome, with the ultimate endpoint being replacement and development to term. Because of the widespread popularity of embryo freezing, the convenience it affords, and the fact that people achieve pregnancy after receiving frozen-thawed embryos, there is a tendency to believe that embryos are 'easy to freeze'. This is not the case, at least when trying to obtain 100% blastomere survival. There is general agreement that the loss of one or more

blastomeres leads to a substantial reduction in implantation. IVF clinics often report survival in terms of the embryo having 50% or more of its blastomeres intact (Van den Abbeel *et al.*, 1997; Mandelbaum and Menezo, 2001), but a more realistic measure of survival following cryopreservation is on a per blastomere basis. Data show that the more blastomeres that survive cryopreservation, the greater the chance to establish a pregnancy with that embryo (Van den Abbeel *et al.*, 1997; Burns *et al.*, 1999; Edgar *et al.*, 2000). Therefore, obtaining a maximum blastomere survival rate is a worthy aim and in the best interest of the patient. This is particularly evident in preimplantation genetic diagnosis (PGD), when embryos are already challenged by blastomere biopsy.

Following blastomere biopsy, the embryo usually remains in an incubator until the analysis is completed, anywhere from 3 to

50 h. Although PGD has proven beneficial, many clinics do not yet have access to this technology. The ability to freeze biopsied embryos would allow more clinics to offer PGD to their patients because they would have time to ship the fixed biopsied cells to distant laboratories for specific analysis and then be able to thaw and replace only those embryos found to be chromosomally normal. This would permit newer technologies that require longer analysis times to be used more widely, such as comparative genome hybridization (CGH; Wells *et al.*, 1999). Currently, CGH has been applied to biopsied cells from embryos frozen after biopsy, or to polar bodies (Voullaire *et al.*, 2000; Wilton *et al.*, 2001; Wells *et al.*, 2002), but the high rate of embryo loss after thawing with current methods precludes the use of CGH for clinical PGD.

Many embryos analysed with PGD are found to be abnormal, and are discarded; however, sometimes the number of chromosomally normal embryos obtained is greater than would be acceptable for transfer, and these become candidates for cryopreservation. Several recent studies have shown that human embryo survival after biopsy and cryopreservation is extremely poor and not considered worth doing (Joris *et al.*, 1999; Magli *et al.*, 1999; Ciotti *et al.*, 2000; Lee and Munné, 2000). The reasons for the reduced survival of these embryos are unknown.

This study examines the effectiveness of CJ3, a choline-based medium, used in conjunction with a modified protocol for the storage of intact and blastomere-biopsied human embryos (Stachecki and Willadsen, 2000).

## Materials and methods

### Biopsy of embryos

Human day 3 embryos (day 0 = day of collection) from a total of 16 patients were biopsied (Grifo *et al.*, 1990), and blastomeres processed for chromosome testing following previously published protocols (Munné *et al.*, 1999). The embryos were cultured for an additional day *in vitro* at 37°C. Embryos were obtained from cycles of PGD for infertility, in which fluorescence in-situ hybridization (FISH) for chromosomes X, Y, 13, 15, 16, 17, 21 and 22 was performed. Embryos that were chromosomally abnormal following biopsy, or were intact (from a total of 16 patients) and considered unsuitable for replacement based on morphology and development, were used for this study. For all embryos used, consent for research was obtained in accordance with internal review board protocols.

### Cryopreservation of embryos

Cryopreservation protocols were applied to non-biopsied day 3 embryos that were considered unusable for embryo transfer or clinical cryopreservation due to anomalies of development after consent was obtained from the patient (group A). Day 4 embryos that had undergone blastomere biopsy on day 3 for PGD analysis and were found to be chromosomally abnormal were also selected for experimental cryopreservation (group B). Depending on the number of embryos available per patient, they were split equally into two protocol groups: (i) control and (ii) experimental. The control group was frozen in ETFM (embryo transfer freezing medium; Gibco BRL, Gaithersburg, MD, USA) using a standard clinical protocol for freezing cleaved human embryos, and the

experimental group was frozen in CJ3 (modified form of the choline-based freezing medium CJ2: Stachecki and Willadsen, 2000). For cryopreservation, embryos were equilibrated at 23°C with medium containing 1.5 mol/l PrOH for 10 min, followed by exposure to the same medium containing 0.2 mol/l sucrose for 5–10 min, during which period the oocytes were transferred to 0.25 ml French straws (IMV International, Minneapolis, MN, USA). The straws were heat-sealed at both ends and placed in a BioCool III programmable freezer (FTS Systems, Stone Ridge, NY, USA). The straws were cooled from 20°C at –2°C/min to –7°C, seeded and held for 10 min at –7°C, followed by slow cooling at –0.3°C/min. The straws were then plunged directly into liquid nitrogen (LN<sub>2</sub>) after cooling to –33°C in a BioCool freezer or cooled beyond –33 to –150°C at a rate of –50°C/min in a Planer Kryo 640 freezer before plunging into LN<sub>2</sub> (Planer Products Ltd, Middlesex, UK). After storage for at least 7 days, the straws containing embryos frozen in ETFM were thawed by holding in room temperature air for 30 s before immersion in a 30°C water bath for an additional 10 s. Embryos frozen in CJ3 were thawed by holding in room temperature air for 60 s before immersion in a 30°C water bath for an additional 10 s (Stachecki and Willadsen, 2000). After thawing, the cryoprotectants were removed in five steps at 5-min intervals as previously described (Stachecki *et al.*, 1998a). These steps were: CJ3 or ETFM supplemented with (i) 0.2 mol/l sucrose and 1.0 mol/l PrOH, (ii) 0.2 mol/l sucrose and 0.5 mol/l PrOH, (iii) 0.2 mol/l sucrose, (iv) 0.1 mol/l sucrose, and (v) CJ3 or ETFM respectively. The embryos frozen in CJ3 were held for an additional 5 min in mCZB, a sodium-based medium (Chatot *et al.*, 1989; Kimura and Yanagimachi, 1995). All embryos were then incubated on a slide warmer in mCZB at 37°C for 5 min. After cryoprotectant removal, the embryos were visually examined for blastomere survival. Biopsied embryos were then transferred to human tubal fluid supplemented with 10% human serum albumin (Sage, Trumbull, CT, USA) and placed into a 37°C incubator before being fixed for analysis using FISH.

### Chromosome abnormality criteria for embryo analysis

The following classification scheme was followed when reanalysing thawed biopsied embryos: (i) when the cell(s) had two copies of each chromosomes analysed, the embryo was classified as normal, (ii) when the cell(s) had three or more copies of each chromosome the embryo was classified as polyploid, (iii) when the cell(s) had one or fewer copies of each chromosome the embryo was classified as haploid, (iv) when the cell(s) had one or two chromosomes with an abnormal number of copies the embryo was classified as aneuploid, and (v) when the cell(s) had three or more chromosomes with an abnormal number of copies but the cell was not haploid or polyploid, the embryo was classified as mosaic (Silber *et al.*, 2003). While criteria (i)–(iii) may seem obvious, criteria (iv) and (v) may seem arbitrary, but they are based on the observation that triple and higher multiple aneuploidies are extremely rare even in cleavage-stage embryos, and that after full analysis of embryos with three or more abnormal chromosomes, these are almost always mosaic (Munné *et al.*, 1995; Márquez *et al.*, 2000).

### Statistical methods

Contingency tables were used to evaluate statistical differences between experimental groups. Yates' correction was applied due

to small sample sizes.  $P < 0.05$  was considered significant.

## Results

It was found that compared with controls (intact), embryos that had undergone blastomere removal were compromised in their ability to survive cryopreservation, which was reflected in the reduced blastomere survival rates regardless of the method used to freeze the cells (ETFM: 50.5 versus 73.6%; CJ3: 80.1 versus 84.1% respectively; **Table 1**). A significantly higher proportion of embryos and individual blastomeres from biopsied embryos cryopreserved in CJ3 (94.3 and 80.1% respectively) were intact after thawing and cryoprotectant removal than those cryopreserved in ETFM (55.6 and 50.5% respectively,  $P < 0.05$ ; **Table 1**). Additionally, a greater proportion of control non-biopsied embryos and their respective blastomeres survived cryopreservation when frozen in CJ3 (BioCool: 95.2, 84.1% respectively or Planer: 90.5, 83.1% respectively) than in ETFM (77.8, 73.6% respectively; **Table 1**).

**Table 2** shows the chromosome abnormalities detected by PGD in the embryo groups. Every embryo was analysed unless all the blastomeres were lysed after thawing. Some embryos gave unclear or no results and/or blastomeres were lost during analysis and thus they were not included in the results presented. There were only nine out of 72 embryos with no FISH results. Of the embryos that could be reanalysed, 3/33 (9%) were misdiagnosed and were in fact normal. The proportion of aneuploid (aneuploid, **Table 2**) and mosaic (other, **Table 2**) embryos was not statistically different between freezing groups. Likewise, the frequency of lysed cells by aneuploid and other (mosaic) embryos was similar, with 28.6 and 25.9% of embryos with 0 or 1 cell lysed, 35.7 and 37.0% with 2 or 3 cells lysed, and 35.7 and 37.0% with 4 or more cells lysed respectively. Thus, the difference in survival rate was not caused by differences in chromosome abnormalities among the groups studied, but was a result of the freezing protocol.

**Table 1.** Embryo and blastomere survival following cryopreservation in CJ3 or embryo transfer freezing medium (ETFM).

Embryos	Medium	Freezer	Experiments (n)	Embryos (n)	Embryos intact (%) <sup>1</sup>	No. with 100% survival (%) <sup>3</sup>	Blastomeres (n)	No. with blastomeres intact (%)
Intact	ETFM	Planer	10	27	21 (77.8) <sup>a</sup>	10 (37.0)	182	134 (73.6) <sup>a</sup>
Intact	CJ3	Planer	4	21	19 (90.5) <sup>a,b</sup>	10 (47.6)	124	103 (83.1) <sup>a,b</sup>
Intact	CJ3	BioCool	16	63	60 (95.2) <sup>b</sup>	31 (49.2)	340	286 (84.1) <sup>b</sup>
Biopsied	CJ3	BioCool	12	35	33 (94.3) <sup>b</sup>	11 (31.4)	231	185 (80.1) <sup>a,b</sup>
Biopsied	ETFM	BioCool	11	27	15 (55.6) <sup>c</sup>	1 (3.7)	182	92 (50.5) <sup>c</sup>
Biopsied	ETFM <sup>2</sup>	BioCool	4	10	4 (40.0) <sup>c</sup>	2 (20)	83	40 (48.2) <sup>c</sup>

Biopsied embryos from each patient were split equally between groups. The number of experiments is also the number of patients in each group. Embryos were frozen in separate straws and intact blastomeres counted before and after cryopreservation.

<sup>1</sup>An intact embryo is an embryo with  $\geq 50\%$  of its blastomeres intact after thawing and cryoprotectant removal.

<sup>2</sup>Embryos i□

30°C water for 10 s, instead of 30 s at room temperature before immersion in a 30°C water bath for 10 s).

<sup>3</sup>Embryos with all of their blastomeres intact after thawing.

<sup>a,b,c</sup>Within columns, values with different superscripts are significantly different ( $P < 0.05$ ).

ETFM = embryo transfer freezing medium.

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**Table 2.** Chromosome abnormalities detected by preimplantation genetic diagnosis (PGD) and reanalysis.

Medium	Thaw rate <sup>c</sup>	PGD/reanalysis outcome		
		Normal Intact cells/total cells (embryos) <sup>d</sup>	Aneuploid <sup>a</sup> Intact cells/total cells (embryos)	Other <sup>b</sup> Intact cells/total cells (embryos)
CJ3	60/10	31/32 (5)	65/80 (11)	73/98 (15)
ETFM	30/10	15/24 (3)	35/70 (10)	14/52 (9)
ETFM	60/10	8/8 (1)	26/52 (6)	6/23 (3)

PGD was performed on day 3 and cryopreservation on day 4 in a BioCool III programmable freezer. Embryos were re-analysed by fluorescence in-situ hybridization following thawing.

<sup>a</sup>Aneuploid refers to monosomic or polysomic embryos.

<sup>b</sup>Other refers to mosaic, polyploidy, or haploid embryos.

<sup>c</sup>Embryos were thawed by holding in room temperature air for either 30 or 60 s and then submerged into 30°C water for 10 s (30/10, 60/10 respectively).

<sup>d</sup>The number of intact blastomeres after and before cryopreservation, and the number of embryos analysed.

ETFM = embryo transfer freezing medium.

## Discussion

The results of this study show that using conventional embryo freezing, biopsied human embryos are significantly more sensitive to cryopreservation damage compared with intact embryos. Embryos stored in the choline-based medium, CJ3, have a significantly higher rate of blastomere survival ( $P < 0.05$ ).

It is difficult to understand fully why embryos and blastomeres frozen in CJ3 survived at a significantly higher rate, but the difference is clear. Both groups of biopsied embryos had similar types of chromosome abnormalities, similar average cell numbers prior to freezing (6.6 CJ3 and 7.1 ETFM), they were split equally between both freezing regimes (35 CJ3 and 37 ETFM), and they were treated similarly up to the point of cryopreservation (Table 1). In addition, the frequency of cells lost was equally spread among all abnormalities. The only difference, besides the medium used for cryopreservation, was in the thawing time (60 versus 30 s in air, for those embryos frozen in CJ3 versus ETFM respectively). However, a small group of ETFM frozen biopsied embryos were thawed similarly to the CJ3 embryos (60 s air; Table 1, last row), and this had no observable effect on their survival. It appears that the differences between freezing media caused the change in blastomere survival after thawing. CJ3 is, in fact, very different than ETFM or other cryopreservation or culture media currently used, mainly because it is devoid of sodium ions, except for those in the serum supplement. Also, CJ3 contains choline chloride as its major ion, rather than sodium. In the past, several papers have been published regarding the ability of the low-sodium, choline-based media to obtain very high survival, fertilization, development and pregnancy rates for unfertilized mouse oocytes (Stachecki and Willadsen, 2000; Stachecki et al., 1998a,b, 2002).

Naturally, freezing and thawing protocols are as important as the freezing media for optimal results. The existing protocols used by many laboratories for freezing human embryos are adequate to obtain blastomere survival rates of at least 50%. The basic slow-cooling protocols used for embryo storage during the past 20 years have worked fairly well, and there have been no major revisions reported, save for alternative methods such as vitrification. Numerous protocols were previously tested using choline-based medium to cryopreserve mouse oocytes and embryos and discarded human embryos, in order to reduce observable stress and damage to the cells. These pilot studies helped determine the freezing and thawing regimes that gave the most consistent and optimal results.

A breach of the zona pellucida can impact embryo cryo-survival, especially if blastomeres near the breach are altered by the technique used. Methods for opening the zona include partial zona dissection, acidified Tyrode's solution, or laser ablation. All of these techniques, if used improperly, can damage blastomeres, and this may in turn affect the blastomeres' ability to survive cryopreservation. One study using mouse embryos (Thompson et al., 1995) reported a significant detrimental effect of biopsy and cryopreservation on in-vitro development, but this could have been due to the particular method of biopsy or the cryopreservation protocol used. By contrast, numerous studies that have analysed the effects of blastomere biopsy

and cryopreservation of mouse (Wilton et al., 1989; Liu et al., 1993; Snabes et al., 1993; Ludwig et al., 1998) and bovine embryos (Agca et al., 1998) conclude that cryopreservation had no significant negative impact on either the in-vitro or in-vivo developmental potential of the biopsied embryos. A similar study in ovine embryos (Leoni et al., 2000) showed that while biopsy and cryopreservation had no negative effect on in-vitro development, it negatively affected in-vivo production of lambs. Although there is evidence in other species that blastomere biopsy does not significantly affect embryo cryopreservation outcome, human embryos seem to be more sensitive to this combination of stresses. This study confirms other reports that describe detrimental effects of blastomere biopsy in conjunction with cryopreservation of human embryos (Joris et al., 1999; Magli et al., 1999; Ciotti et al., 2000; Lee and Munné, 2000). Although Lee and Munné (2000) achieved a pregnancy after polar body biopsy and embryo freezing, it was probably anecdotal and does not necessarily show that blastomere biopsy in conjunction with embryo cryopreservation is not detrimental in some way. Additionally, in that study, the hole made during polar body biopsy was smaller, no acid was used, and no extra perivitelline space was created by removing one cell. The reduction in the survival rate of biopsied human embryos following cryopreservation could be due to the combination of stresses including IVF, in-vitro culture, biopsy, freezing, and thawing, all of which the embryo must endure. Further study in all of these areas may be required before biopsied embryos can be successfully and reproducibly frozen, but the results here show that a significant improvement can be obtained with CJ3 as the cryopreservation medium when used in conjunction with appropriate freezing and thawing protocols.

## Acknowledgements

Our thanks to Giles Tomkin for his critical review of the manuscript and for EggCyte™ database analysis of figures on blastomere survival.

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Received 4 August 2005; refereed 31 August 2005; accepted 23 August 2005.