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ARTICLE

Long-distance transportation of primate embryos developing in culture: a preliminary study

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Abstract Non-human primate embryos are invaluable for conducting research relevant to human infertility and stem cells, but their availability is restricted. In this preliminary study, rhesus monkey embryos were produced by IVF at the Caribbean Primate Research Centre and shipped in tubes of gassed culture medium within a battery-powered transport incubator by overnight courier to Wayne State University in Michigan. Upon arrival, the embryos were incubated in fresh culture medium to evaluate further development. In 11 shipments comprising 98 cleavage-stage embryos developing from oocytes that were mature (MII) upon collection, 51 (52%) reached advanced preimplantation stages (morula to hatched blastocyst) during prolonged culture following transportation. However, most embryos produced from oocytes that were immature (MI) at collection arrested and only 5/51 (10%) reached advanced stages of development. This study demonstrates that non-cryopreserved primate embryos can be routinely transported between distant sites without loss of developmental ability. In this way, the processes of production and study of non-cryopreserved primate embryos need not be restricted to the same or nearby laboratories. This will expand the use of these embryos for research and facilitate generation of translationally relevant information.

KEYWORDS: blastocyst, embryo culture, embryo transportation, *in-vitro* fertilization, rhesus monkey

Introduction

Non-human primate embryos are increasingly regarded as valuable translational models for human health, including studies on infertility and embryonic stem cells. However, only a few centres have the capability to produce monkey embryos, both because of the need to maintain substantial numbers of expensive animals and the requirement for special expertise and facilities required to generate embryos by IVF. Numerous other research laboratories might benefit from studying monkey embryos but lack one or both of these essential requirements. During the past 4 years, the embryology laboratory at the Caribbean Primate Research Centre (CPRC) in Puerto Rico has routinely produced IVF rhesus monkey embryos. As part of a collaborative research programme between CPRC and Wayne State University (WSU), batches of these embryos have been periodically shipped to the Brenner research laboratory at WSU, but they were all fixed upon arrival for chromosomal analysis (Dupont et al., 2009a, b). Other studies, such as metabolic and gene expression analyses of blastocysts, as well as embryonic stem cell research, require live embryos, but WSU has no

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non-human primate facility for producing them. Monkey embryos can be cryopreserved successfully, as shown by the birth of offspring after embryo transfer (Wolf et al., 1989; Yeoman et al., 2001), so they could be transported between laboratories in liquid nitrogen dry-shipping containers. However, cryopreservation can damage embryos or affect their functional properties (Hasler, 1998; Lane et al., 2000; Society for Assisted Reproductive Technology [SART], 2009) so that results of studies, for example on gene expression or metabolism, could be compromised by inadvertently using defective embryos without overt signs of functional perturbations. Instead, this study investigated the possibility of shipping live monkey embryos in a way that would not compromise their cellular functions and would sustain their viability for extended research studies.

This study found that rhesus monkey cleavage-stage embryos could be successfully transported from Puerto Rico to Detroit using a portable incubator, and they subsequently developed to advanced stages (morula and blastocyst) at rates similar to those previously established for non-transported embryos (Schramm and Bavister, 1996). The objective of this study was to demonstrate that monkey embryos could be used for detailed studies at a laboratory geographically remote from the production site, thus expanding the usefulness of this material for clinically relevant research.

Materials and methods

Embryo production

All animals were used with approval of the University of Puerto Rico's CPRC Institutional Animal Care and Use Committee. Rhesus monkey embryos were produced using modifications of published protocols (Schramm and Bavister, 1996). Briefly, females of reproductive age (7–14 years old) were stimulated with injections of 37.5 IU recombinant human FSH (Organon, Oss, The Netherlands; 81 IU/ampule) twice daily for 9 or 10 days, starting 1–3 days after observation of menses. On the last day of FSH, 1000 IU of recombinant human chorionic gonadotrophin (Ovidrel, Serono Laboratories, Rockland, MA) was also given and oocytes were collected by follicular aspiration at laparoscopy 32–34 h later. Oocytes were classified as mature (MII; metaphase II, with first polar body) or immature (MI; metaphase I, no polar body or germinal vesicle) at collection. Any oocytes that had failed to resume meiosis (germinal vesicle stage) were rejected. For each collection, all MII and MI oocytes were kept in two separate groups and inseminated with capacitated spermatozoa within 6–8 h after collection and co-incubated for 14–16 h in Tyrode's albumin lactate pyruvate medium. Many of the MI oocytes completed maturation before or during co-incubation with spermatozoa and were fertilized, as shown by the presence of two pronuclei and two polar bodies. Fertilized ova were cultured for several days in medium hamster embryo culture medium 6 (HECM-6) (McKiernan et al., 1995; Schramm and Bavister, 1996) and examined for cleavage development. Cleavage-stage embryos produced during two consecutive rhesus monkey breeding seasons (September to May 2007–2008 and 2008–2009) were transported in the manner described in this report.

Embryo transportation

The method used was a slight modification of a transportation system that is well established for shipping live bovine oocytes within the US mainland (Applied Reproductive Technologies, Madison, WI, USA). Embryos derived from oocytes that were MII or MI at collection were kept separate and transported to WSU on post-insemination days 2–4. Ideally, embryos were sent at the 4- to 8-cell stages but occasionally shipment had to be delayed because of inability to transport or receive embryos on weekends. On the morning of the shipment day, developing embryos were placed into 5 ml round-bottomed tubes (No. 352054; Falcon Plastics, Becton Dickinson, Franklin Lakes, NJ, USA) containing 2.5 ml of culture medium that had been pre-equilibrated with 5% CO_2 and 5% O_2 in air and maintained at 37.5°C. Embryos originating from fertilized oocytes that were MII or MI at collection were kept in separate tubes. Early shipments were sent using Connaught Medical Research Laboratories 1066 medium (mCMRL-1066) until mid-spring 2008, when the shipping medium was changed to HECM-6; both media support development of cleavage-stage monkey embryos. During the shipping day, the loosely capped transport tubes were kept in an atmosphere of 5% CO_2 and 5% O_2 in air in a 37°C incubator. Late that afternoon (at about 4 p.m.), embryos were transferred to these culture tubes, which were then tightly capped and sealed with parafilm to prevent gas leakage. These tubes were then immediately inserted into 15 ml round-bottomed tubes (No. 352001; Falcon Plastics) containing approximately 7 ml of equilibrated, warmed culture medium and these were also sealed with parafilm. This method of placing one tube inside another was designed to protect the small inner tube containing the embryos in case of any rough handling during shipment, which could cause tube cracking and potential medium/gas loss. The tubes were then placed into the transport incubator core that had been previously warmed to 37.5°C by turning on the heater during battery charging.

The shipping incubator was a CryoLogic BioTherm Transportable Incubator with fixed Type E10 core (CryoLogic, Victoria, Australia) (**Figure 1**). The E10 core is designed to snugly house 15 ml round-bottom or conical tubes. Tests in the laboratory showed that, with a fully charged battery, these incubators hold the set temperature for over 30 h. This portable incubator was placed into a padded box and prepared for shipment via Federal Express overnight (airmail). Packages were hand-delivered to a main Federal Express branch in Guaynabo, Puerto Rico between 5 and 6 p.m. on the day of shipment and tagged for overnight delivery to Wayne State University in Detroit, USA by 10.30 a.m. the following day, with an intermediate stop in Memphis, USA. The total distance flown was 2400 air miles. Upon arrival at the laboratory, embryos were removed from their shipping tubes and placed into equilibrated, fresh culture drops of culture medium (supplemented with fetal calf serum for blastocyst development) that had been set up the previous evening and culture was continued for several

Figure 1 The BioTherm transport incubator with lid open to show culture tubes in place in the core (left) and closed ready for shipment (right).

days. The total duration of embryo culture in the portable incubator was approximately 18 h.

Fixation and examination of embryos

Rhesus IVF embryos form blastocysts usually by post-insemination days 6 or 7 and expand over the next 2 days; this schedule of in-vitro development is 1—2 days slower than for human IVF embryos (Boatman, 1987; Veeck, 1999). Some of the embryos were fixed at day 5 or day 7 for molecular analysis, while others were cultured until day 10 in order to examine gene expression at very late stages of preimplantation development (expanded and hatched blastocysts).

Results

For this study, during two breeding seasons, a total of 11 cohorts of oocytes were collected, each from a different female. After developing from oocytes that were MII at collection, 98 cleavage-stage IVF embryos were transported by air from CPRC to the laboratory at WSU (**Table 1**). These embryos yielded 51 advanced stages of development (morula and blastocyst) following culture for several days at WSU (52%). **Figure 2** shows a cohort of cleavage-stage embryos at the time of shipping from Puerto Rico on post-insemination day 3 and the resulting advanced embryos after culture for 4 more days (including the period of culture during air transportation). The overall yield of blastocysts would undoubtedly have been considerably higher than this; however, 13 morulae from cycles 2 and 4 (**Table 1**) were fixed on day 5 for molecular analysis (A Harvey et al., unpublished). In contrast, in some of the same stimulation cycles, oocytes that were MI at collection matured to MII *in vitro* and produced embryos, but their development was very poor. Out of 51 such embryos that were transported to WSU, only five (10%) developed into morulae or blastocysts. One entire batch of cleavage-stage embryos derived from oocytes that were MII or MI at collection all arrested during culture at the WSU laboratory (**Table 1**, Cycle No. 6) and, in another batch (No. 10), 11 of the 13 embryos from MI oocytes arrested. The reason for these failures is unknown; however, it was not an incubator problem because temperature and gas conditions were maintained during transportation.

Discussion

This study has shown that monkey embryos can be successfully transported over long distances while still actively developing. Ideally, some embryos from each cohort would have been kept in the originating laboratory for comparison of development with their transported siblings but this was not possible for logistical reasons. However, the results showed that transported embryos in most of the shipments continued developing in culture at rates similar to those reported for non-transported embryos (Schramm and Bavister, 1996). Of 11 shipments with embryos derived from oocytes that were MII at the time of collection, nine produced satisfactory outcomes (**Table 1**). Thus, 82% of the shipments can be considered very successful; however, the two failures do not appear to have been due to problems with the transportation method but rather to intrinsic faults with the embryos. In contrast to results with embryos derived from oocytes collected at MII, development of embryos derived from oocytes that were MI at collection was very poor beyond cleavage stages. This was also noted in previous studies (Zheng et al., 2002), suggesting that these oocytes/embryos are intrinsically defective, perhaps due to failure

Table 1 Development of embryos shipped from Caribbean Primate Research Centre, Puerto Rico to Wayne State University, Detroit in portable incubators.

Cycle No.[a]	*No. embryos shipped*[b]	*Embryo stage when shipped (cell no.)*	*Fixation day (post insemination)*	*Embryo development*
1	5	~12	7	3 exp. blast
2	11	~8	5	7 morulae
3[c]	13	~8	7	3 morulae
			10	7 exp. blast
4	14	8	5	6 morulae
5	7	4–8	7	1 early and 5 exp. blast
	(3)	4–8		2 morulae
6	9	4–8		All arrested
	(7)			All arrested
7	22	12–16	7	6 morulae 2 exp. blast, 3 hatched blast
	(6)	4–8	10	All arrested
8	(14)	4–6 with fragmentation		All arrested
9	9	4	7	4 exp. blast
	(5)	4		1 blast
10	1	2	7	Arrested
	(13)	2		2 exp. blast
11	7	>16	10	1 exp. blast, 3 hatched blast
	(3)	>16		All arrested
Totals	98			22 morulae, 1 early blast, 22 exp. blast, 6 hatched blast (52%)
	(51)			2 morulae, 1 blast, 2 exp. blast (10%)

[a] Cycles from 11 different females over two breeding seasons.
[b] Values without parentheses are for embryos derived from MII oocytes; values in parentheses are for embryos derived from MI oocytes.
[c] **Figure 2** shows images of these embryos before and after shipping and subsequent culture. Early blast = blastocele cavity visible but not prominent; exp. blast = expanding/fully expanded blastocyst; hatched blast = blastocyst escaping/escaped from its zona pellucida.

to activate the embryonic genome (Schramm et al., 2003), a high incidence of chromosome defects in embryos from oocytes that were MI at collection (Dupont et al., 2009a) or insufficient coordination of cytoplasmic and nuclear maturation (Eppig, 1996). In addition, the oocyte culture medium may be inadequate for completion of normal maturation in oocytes collected at MI. Unless or until this developmental deficiency can be corrected, immature rhesus oocytes are not useful for producing competent embryos. For those few instances where embryos derived from oocytes that were MI at collection successfully developed into advanced preimplantation embryos, it is surmise that these oocytes were close to MII at collection and thus had a higher developmental capacity than most others in this category.

A similar method has been used to transport human IVF embryos between laboratories within the US mainland (Langley et al., 2001). In that report, human embryos were transported from the originating site to another laboratory for preimplantation genetic diagnosis (PGD) and then back again. The one-way transportation time was only 5 h and the embryos were hand-carried by a technician. Out of a total of 17 embryos in four shipments, most failed to develop past cleavage stages and only four developed to advanced stages (one morula and three blastocysts). It is unknown to what extent this was due to genetic defects in the embryos versus problems with the transportation method. Consequently, the reliability of the transportation method used in that study cannot be assessed. The results presented here comprise shipment of 11 cohorts of primate embryos and indicate an efficiency of more than 80%.

Transporting embryos during their development in culture medium avoids potential complications arising from cryopreservation. Monkey embryos have been successfully cryopreserved using slow cooling or vitrification (Wolf et al., 1989; Yeoman et al., 2001), but these procedures are not without problems. Subtle damage may not be immediately evident and could compromise results of molecular analyses such as gene expression or biochemical studies. Lane et al. (2000) showed that, immediately following warming, vitrified 2-cell hamster embryos lost their ability to control intracellular pH for up to 6 h after warming, resulting in the elevation of intracellular pH from a normal level of pH 7.2 to pH 7.35 and a significant reduction in oxidative metabolism. Although cryopreservation of in-vivo produced cattle embryos did not diminish their viability as judged by clinical pregnancy rates, cryopreserved in-vitro produced (IVP) embryos exhibited less than half the viability of fresh IVP embryos (Hasler, 1998). Cryopreserved human IVF embryos had significantly less viability than fresh embryos as judged by live birth rates, which were as much as 43% lower even when using donor oocytes (SART, 2009). Thus, in several species, the combination of IVP and cryopreservation appears to be traumatic to embryos. It is inferred that this would be a serious concern for transporting cryopreserved rhesus monkey embryos that are produced by IVF for use in any cellular and metabolic studies.

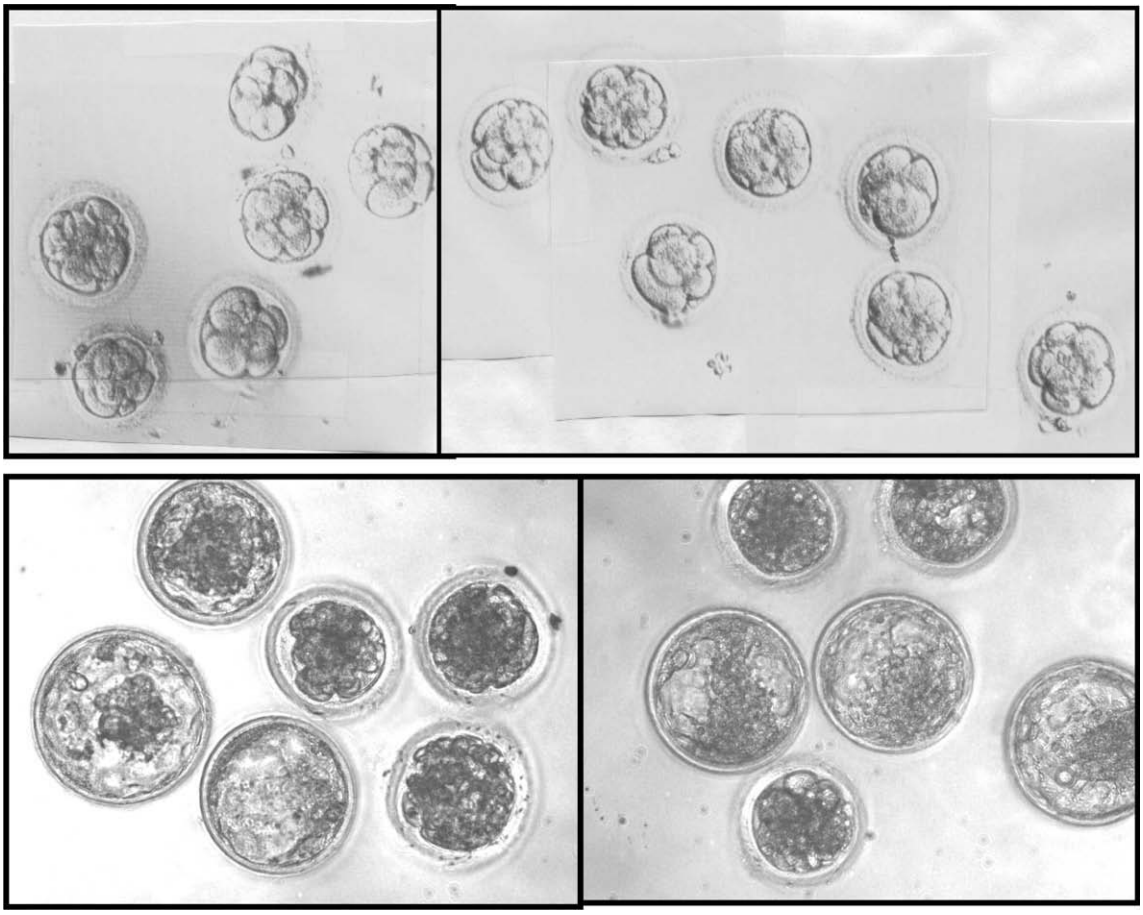

Figure 2 Cohort of 13 IVF rhesus embryos (Cohort No. 3 in Table 1). (**A**) Embryos as shipped to Wayne State University on post-insemination day 3, magnification ×10; (**B**) same embryos shown after culture at Wayne State University, which by post-insemination day 7 had developed into three morulae and seven blastocysts; magnification ×20.

In summary, using the method described here for transporting monkey embryos from production laboratories to distant sites for detailed analysis could substantially expand the usefulness of this scarce, valuable material for translational studies including research into human infertility and the production of embryonic stem cells. Certainly, this approach could be used for embryos of any species that are amenable to development in culture. Because the transport incubator used could maintain temperature for considerably longer than the 18 h needed in this study, it should be possible to transport embryos between continents. This demonstration of the reliability of this method could also make it more attractive for transporting human preimplantation embryos between laboratories. In addition, it would be possible to transport IVF embryos produced with gametes of scarce or endangered non-human primates to distant facilities for implantation into surrogate females; in this way, these animals could be propagated and their genetic diversity expanded without the logistical problems involved in transporting the animals themselves.

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