Human Reproduction, Vol.29, No.11 pp. 2431–2438, 2014
Advanced Access publication on August 27, 2014 doi:10.1093/humrep/deu214

human reproduction

ORIGINAL ARTICLE ***Embryology***

Embryo vitrification using a novel semi-automated closed system yields *in vitro* outcomes equivalent to the manual Cryotop method

Tammie K. Roy[1,*], Susanna Brandi[1], Naomi M. Tappe[1], Cara K. Bradley[1], Eduardo Vom[2], Chester Henderson[2], Craig Lewis[2], Kristy Battista[2], Ben Hobbs[2], Simon Hobbs[2], John Syer[2], Sam R. Lanyon[1], Sacha M. Dopheide[2], Teija T. Peura[1], Steven J. McArthur[3], Mark C. Bowman[3], and Tomas Stojanov[3]

[1]Genea Biomedx, 321 Kent Street, Sydney, NSW 2000, Australia [2]Planet Innovation, 81-89 Cotham Road, Kew, VIC 3101, Australia [3]Genea, 321 Kent Street, Sydney, NSW 2000, Australia

*Correspondence address. E-mail: tammie.roy@geneabiomedx.com.au

Submitted on March 31, 2014; resubmitted on July 14, 2014; accepted on July 30, 2014

STUDY QUESTION: Can the equilibration steps prior to embryo vitrification be automated?

SUMMARY ANSWER: We have developed the 'Gavi' system which automatically performs equilibration steps before closed system vitrification on up to four embryos at a time and gives *in vitro* outcomes equivalent to the manual Cryotop method.

WHAT IS KNOWN ALREADY: Embryo cryopreservation is an essential component of a successful assisted reproduction clinic, with vitrification providing excellent embryo survival and pregnancy outcomes. However, vitrification is a manual, labour-intensive and highly skilled procedure, and results can vary between embryologists and clinics. A closed system whereby the embryo does not come in direct contact with liquid nitrogen is preferred by many clinics and is a regulatory requirement in some countries.

STUDY DESIGN, SIZE, DURATION: The Gavi system, an automation instrument with a novel closed system device, was used to equilibrate embryos prior to vitrification. Outcomes for embryos automatically processed with the Gavi system were compared with those processed with the manual Cryotop method and with fresh (non-vitrified) controls.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The efficacy of the Gavi system (Alpha model) was assessed for mouse (Quackenbush Swiss and F1 C57BL/6J x CBA) zygotes, cleavage stage embryos and blastocysts, and for donated human vitrified-warmed blastocysts. The main outcomes assessed included recovery, survival and *in vitro* embryo development after vitrification-warming. Cooling and warming rates were measured using a thermocouple probe.

MAIN RESULTS AND THE ROLE OF CHANCE: Mouse embryos vitrified after processing with the automated Gavi system achieved equivalent *in vitro* outcomes to that of Cryotop controls. For example, for mouse blastocysts both the Gavi system ($n = 176$) and manual Cryotop method ($n = 172$) gave a 99% recovery rate, of which 54 and 50%, respectively, progressed to fully hatched blastocysts 48 h after warming. The outcomes for human blastocysts processed with the Gavi system ($n = 23$) were also equivalent to Cryotop controls ($n = 13$) including 100% recovery for both groups, of which 17 and 15%, respectively, progressed to fully hatched blastocysts 48 h after warming. The cooling and warming rates achieved with the Gavi system were 14 136°C/min and 11 239°C/min, respectively.

LIMITATIONS, REASONS FOR CAUTION: Testing of the Gavi system described here was limited to *in vitro* development of embryos from two mouse strains and a limited number of human embryos. Validation of Gavi system advanced production models is now required to confirm the success of semi-automated vitrification, including clinical evaluation of pregnancy outcomes from the transfer of Gavi vitrified-warmed human embryos.

WIDER IMPLICATIONS OF THE FINDINGS: The Gavi system has the potential to revolutionize and standardize vitrification of embryos and oocytes. The success of the Gavi system shows that it is possible to semi-automate complicated labour-intensive ART methods and processes, and opens up the possibility for further improvements in clinical outcomes and efficiencies in the ART clinic.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by Genea Ltd. S.B., N.M.T., T.T.P., S.J.M., M.C.B. and T.S. are shareholders of Genea Ltd. E.V., C.H., C.L., S.R.L. and S.M.D. are shareholders of Planet Innovation Pty Ltd. The remaining authors are employees of either Genea Ltd. or Planet Innovation Pty Ltd.

Key words: automation / embryo / IVF / standardization / vitrification

Introduction

In the 35 years since the first human birth from *in vitro* fertilization (IVF), it is estimated that worldwide over 5 million babies have been conceived by assisted reproductive technologies (ART), half of which have been born in the last 6 years (Adamson *et al.*, 2013). During this time ART has seen substantial increases in success rates with skilled clinics achieving pregnancies in excess of 50% of embryo transfers (Cobo *et al.*, 2010; Roy *et al.*, 2014). These improvements can be attributed to multiple developments including low oxygen benchtop incubators designed specifically for embryo culture, the use of sequential embryo media and culture to the blastocyst stage, intracytoplasmic sperm injection, and vitrification for oocyte and embryo cryopreservation (Jansen, 2005; Wang and Sauer, 2006; AbdelHafez *et al.*, 2010; Bontekoe *et al.*, 2012). However, despite these advances IVF largely remains a manual time-consuming and operator-dependent process and so far only one embryology procedure has been semi-automated: time-lapse monitoring of embryo morphokinetics (Meseguer *et al.*, 2011, 2012). As a consequence there is limited standardization of ART procedures and clinical outcomes vary greatly between IVF clinics and between embryologists.

The successful cryopreservation of supernumerary embryos is an essential practice during ART to maximize cumulative pregnancy rates, particularly for clinics performing single embryo transfers (Gerris, 2009). The last decade has seen a shift from conventional slow-freezing towards vitrification due to superior recovery rates and clinical outcomes (Loutradi *et al.*, 2008; AbdelHafez *et al.*, 2010). Vitrification is however one of the most time-consuming, manual and operator skill dependent procedures performed in an ART clinic (Gosden, 2011), with some centres employing scientists solely dedicated to this task. Embryo vitrification typically employs the use of high concentrations of cryoprotectants followed by cryopreservation with liquid nitrogen to produce extremely rapid cooling rates (Kuwayama, 2007; Gosden, 2011). This results in both the intracellular and extracellular solutions being rapidly solidified into a glasslike (vitreous) state which avoids detrimental ice crystal formation.

There are many vitrification devices and protocols available on the market today, which vary greatly in the constitution, exposure time and temperature of cryoprotectants, as well as in the volume of vitrification solution surrounding the embryo, the speed of cooling and warming rates, and whether or not the embryo comes in direct contact with liquid nitrogen (Kader *et al.*, 2009; Alpha Scientists In Reproductive Medicine, 2012). The gold standard of vitrification is arguably the Cryotop method, a room temperature vitrification system that has been used extensively and offers extremely high cooling and warming rates, reported to be ~23 000°C/min and 42 000°C/min respectively (Kuwayama *et al.*, 2005b; Kuwayama, 2007). This system has been shown to produce embryo survival rates following cryopreservation of >90% and implantation, live birth rates and neonatal outcomes equivalent to those of fresh transfers (Takahashi *et al.*, 2005; Kato *et al.*, 2012; Roy *et al.*, 2014). One of the limitations of the standard Cryotop system as well as many other available devices is that the embryo comes in direct contact with liquid nitrogen, potentially exposing it to pathogens, and for this reason closed aseptic systems are preferred by many ART clinics (Bielanski, 2012) and is a regulatory directive of the European Union (European Union, 2004).

To address the need for standardization of vitrification across users and clinics as well as a reduction in labour intensity, we have developed the Gavi system, which automates equilibration for vitrification using a novel closed system device.

Materials and Methods

Mouse embryos

Mouse F1 C57BL/6J x CBA embryos were supplied by Monash Animal Research Platform (Monash University, Melbourne, Australia), and were collected from superovulated females the morning after overnight matings and vitrified as zygotes (Day 1). Mouse Quackenbush Swiss embryos were supplied by CSIRO (Sydney, Australia) and were collected from superovulated females the morning after overnight matings then cultured *in vitro* in sequential embryo media until being vitrified as either 6–8 cell cleavage stage embryos (Day 3), blastocysts (Day 5) or fully hatching blastocysts (Day 6). Animal experiments were approved by the Monash University Animal Ethics Committee (application MMCA 2012/04) or CSIRO Animal Ethics Committee (application 2010/09).

Human embryos

Human embryos were generated by ART as described previously (Roy *et al.*, 2014). The embryos were donated for research via an informed consent process and were vitrified blastocysts that the patients had deemed as excess to their reproductive needs. All relevant clinical procedures and research protocols were approved by Genea's human research Ethics Committee, an independent committee constituted according to the requirements of Australia's National Health and Medical Research Council (NHMRC), and the Embryo Research Licensing Committee of the NHMRC (license 309 718).

Culture media and vitrification solutions

Human embryos, both prior and subsequent to donation to research, were cultured in in-house manufactured sequential media identical in formulation to Sydney IVF embryo culture medium suite (Cook Medical). Mouse embryos were cultured in either this formulation (experiments with vitrified zygotes) or a new in-house manufactured sequential media suite (anticipated as commercially available from Genea Biomedx in 2015). All embryos

were cultured under low (6%) oxygen in MINC benchtop incubators (Cook Medical).

Solutions for vitrification and warming were manufactured in-house in a HEPES buffered media containing 20 mg/ml human serum albumin and 0.01 mg/ml gentamicin identical in formulation to Cryobase (Cook Medical). Vitrification and warming solutions included: Vitrification Solution 1 containing 8% v/v ethylene glycol and 8% v/v DMSO; Vitrification Solution 2 containing 16% v/v ethylene glycol, 16% v/v DMSO and 0.68 mol/l trehalose; Warming Solution 1 containing 1 M trehalose; and Warming Solution 2 containing 0.5 M trehalose.

Description of the Gavi system

The Gavi system was developed at Genea (Sydney, Australia) and Planet Innovation (Melbourne, Australia) by teams of engineers, scientists and embryologists following a standard product development approach. The Gavi system performs automated equilibration for closed system vitrification of up to four embryos simultaneously. The system is comprised of: the instrument (Gavi) which performs fluid exchange using a robotic liquid handling unit with individual pipettes, has a heat sealing unit and includes the liquid nitrogen bucket; the single-use 'Pod' closed system device containing a novel microfluidic 'channel' that secures the embryo during fluid exchange (Supplementary data, Video S1) and vitrification; the 'Cassette' into which up to four Pods are loaded and which holds the Pods in liquid nitrogen storage; the 'Media Cartridge' containing Vitrification Solution 1 and Vitrification Solution 2; the 'Tip and Seal' consumable cartridge containing a pipette tip for fluid exchange and a lid to seal the Pod to prevent embryo or solution contact with liquid nitrogen; and the 'Tray' in which the Media Cartridge, Tip and Seal, and Cassette (containing Pods) are loaded (Supplementary data, Fig. S1).

Gavi system vitrification and warming

Vitrification using the 'Alpha' model Gavi system was performed on embryos using standardized procedures (Supplementary data, Video S2). Firstly, the instrument was turned on, the appropriate protocol was selected (zygote/oocyte, cleavage stage embryo or blastocyst) and liquid nitrogen added to the liquid nitrogen bucket. The Media Cartridge and Tip and Seal were then placed into the Tray and loaded into the instrument. Next, Pods were placed into the Cassette, individual embryos were loaded into the Pods and the Cassette was loaded into Gavi. Media Cartridge caps and the cover of the Tip and Seal were removed and the program was started. On completion of the 13- to 18-min program (depending on the protocol) which includes automatic sealing of Pods, the Cassette was manually removed, dunked in liquid nitrogen with vertical stirring and then transferred to a standard liquid nitrogen dewar for storage.

For warming of embryos vitrified using the Gavi system, the cassette was held in or just above liquid nitrogen and a single pod was removed and dunked into a 37°C water bath for ~2 s with oscillation. The Pod seal was manually removed and 10 µl of room temperature Warming Solution 1 immediately added to the Pod. After 1 min, the embryo was transferred to a standard Nunc 4-well dish (Nunclon) containing 0.5 ml of Warming Solution 2 for 3 min, and then into 0.5 ml Cryobase for 5 min followed by fresh 0.5 ml Cryobase for 1 min. Embryos were then transferred to Genea Cleavage or Blastocyst Medium (depending on developmental stage) for further culture.

Cryotop vitrification and warming

Manual Cryotop (Kitazato, Japan) vitrification and warming of mouse and human embryos was performed essentially as described by Roy *et al.* (2014). All solutions used were as per the Gavi system protocol and warmed to room temperature unless stated otherwise. In brief, embryos were incubated in Vitrification Solution 1 for up to 11 min for mouse embryos and up to 15 min for human embryos. After an initial shrinkage, embryos regained their original volume and were transferred into Vitrification Solution 2 for 1–1.5 min. Embryos were then transferred onto the Cryotop strip in an extremely small volume (<0.1 µl) of solution which was then plunged into liquid nitrogen. After addition of the protective cover, the Cryotop was transferred to liquid nitrogen storage.

Warming of Cryotop vitrified embryos was performed by removal of the protective cover under liquid nitrogen and immersion of the strip in 2.5 ml of 37°C Warming Solution 1 for 1 min. Embryos were then transferred to 0.5 ml of Warming Solution 2 for 3 min, and then into 0.5 ml Cryobase for 5 min followed by fresh 0.5 ml Cryobase for 1 min. Upon completion of the warming procedure, embryos were transferred to Genea Cleavage or Blastocyst Medium (depending on the developmental stage) for further culture.

Mouse embryo testing

Experiments with Quackenbush Swiss mouse embryos were performed at Genea while those with F1 C57BL/6J x CBA mouse embryos were performed at Planet Innovation on the same instrument model (Alpha prototype). On the day of vitrification, mouse embryos were divided equally based on embryo grade and developmental stage into three groups, those for Cryotop vitrification, those for Gavi system vitrification or a fresh group which did not undergo vitrification. Vitrification was subsequently performed in alternating groups of up to four embryos using Cryotop or Gavi system methodology. Vitrified embryos were warmed the following day in alternating Cryotop and Gavi groups, and then cultured in Cleavage Medium or Blastocyst Medium as appropriate, with the exception of vitrified-warmed fully hatched blastocysts which were cultured in dishes coated with collagen (Becton Dickinson; Cat # 354456) for assessment of embryo attachment and the outgrowth of embryonic cells. Experiments were repeated a minimum of four times with the exception of fully hatched blastocyst experiments which were repeated twice.

Human blastocyst testing

Experiments with human embryos were performed at Genea. The donated human embryos were received as vitrified blastocysts on Fibreplug (Cryo-Logic, Australia) or Cryotop carriers. These embryos were warmed with a protocol to match the vitrification procedure and then transferred to Blastocysts Medium. The human embryos were allowed to recover in culture for 4–24 h depending on the day of vitrification and the developmental stage of the embryo. They were then assessed for developmental stage and grade (only grade I and II embryos were utilized) and allocated into either Gavi or Cryotop groups keeping the proportion of higher quality and/or developmentally advanced embryos equal between the groups. Embryos were then re-vitrified using either the Cryotop or Gavi system methodology and upon warming were cultured in Blastocyst Medium.

Vitrification-warming outcomes assessment

The outcomes measured to assess vitrification varied depending on embryonic stage at vitrification and embryo species. Recovery of vitrified embryos was defined as the presence of an embryo immediately upon warming. Survival was evaluated at the completion of the warming procedure and was defined as 75% or more cells intact within a human blastocyst and 100% of cells intact for mouse zygotes and cleavage stage embryos. Re-expansion was assessed 2 h after warming for human blastocysts and was considered to be successful if an embryo's cells occupied 90% or more of its original volume. Embryo cleavage was assessed for experiments using vitrified-warmed zygotes on Day 2 and was defined as an embryo containing two or more cells. All vitrified-warmed mouse embryos were assessed on Day 6 and Day 7 of *in vitro* development for embryo grade and developmental stage, with the exception of fully hatched blastocysts which were assessed for their ability to attach and outgrow on collagen culture dishes at Day 7

and Day 8. Re-vitrified and warmed human blastocysts were assessed at 24 and 48 h after warming for embryo grade and developmental stage.

The percentage of embryos recovered was calculated as the number of embryos recovered per the total number of embryos vitrified. All other percentage calculations were based upon the number of embryos recovered, for example, the percentage of embryos survived was calculated as the number of survived embryos per the number of embryos recovered. The exception to this was the fresh group in which all percentage calculations were based upon the total number of embryos cultured in that group.

Measurement of cooling and warming rates

The Gavi Pod cooling rate was measured using a fine gauge type K thermocouple (RS 397-1589; RS Components, Australia) with a calibrated portable data acquisition module (DT 9806; Data Translation, Massachusetts, USA). The thermocouple probe was placed as close as possible to where the embryo would normally be loaded, 1 μL of Vitrification Solution 2 dispensed to cover the probe and the Pod heat sealed. Pods were then carefully loaded into cassettes and immersed in liquid nitrogen with vertical stirring. The Pod warming rate was measured as described for cooling with the exception that vitrified Pods were removed from cassettes under liquid nitrogen and individually submerged in a 37°C water bath with oscillation. The cooling and warming rates were calculated as the rate (°C/minute) of cooling from 0°C to −133°C and rate of warming from −133°C to 0°C respectively. As a control for testing procedures, the cooling rate for the Rapid-i closed system vitrification device (Vitrolife) was also measured, the result being on average 1215°C/min, a deviation of only 175°C/min from the reported claim by the manufacturer of 1400°C/min.

Statistical analyses

Statistical analyses comparing mouse embryo vitrification outcomes between Gavi, Cryotop and fresh controls were conducted using Minitab 16 (Minitab, Inc.), with a *P*-value of <0.05 considered statistically significant. Comparison of contingency tables was performed by Pearson's chi-squared test with the exception of analyses where an expected cell count was <5, in which case Fisher's exact (two-tailed) test was performed.

Results

Mouse zygotes

The recovery of mouse zygotes vitrified after automated processing with the Gavi system was 97%, which was comparable to Cryotop controls ($P = 0.22$) (Table I). *In vivo* embryo development was also equivalent, including identical development to fully hatched blastocysts with both Gavi and Cryotop groups being 43% by Day 6 and 57% by Day 7. However, further Gavi protocol optimization is required to improve zygote survival after vitrification-warming.

Mouse cleavage stage embryos

Cleavage stage mouse embryos (6–8 cell embryos) processed with the automated Gavi system achieved equivalent *in vitro* outcomes to that of the Cryotop method (Table II). Recovery was 100% for both Gavi and Cryotop vitrified-warmed embryos, development to the blastocyst stage by Day 5 was 85% for Gavi and 92% for Cryotop controls ($P = 0.26$), and development to fully hatched blastocysts by Day 6 was 38% for Gavi and 30% for Cryotop controls ($P = 0.33$).

Mouse blastocysts

Mouse blastocysts processed with the automated Gavi system had an exceptionally high embryo recovery rate after vitrification-warming of 99%, which was identical to the Cryotop controls (Table III). Development to fully hatched blastocysts by Day 6 and Day 7 was comparable between Gavi and Cryotop vitrified-warmed groups, being 41 and 45% ($P = 0.4$), respectively, for Day 6, and 54 and 50% ($P = 0.46$), respectively, for Day 7. Using this same protocol to process fully hatched blastocysts using the Gavi system resulted in equivalent embryo attachment to collagen coated dishes after vitrification-warming to that of Cryotop controls (Table IV). Furthermore, the outgrowth of embryonic cells to collagen coated dishes at Day 8 was significantly higher for embryos vitrified-warmed using the Gavi system, being 70% as compared with 41% for Cryotop controls ($P = 0.02$).

Human blastocysts

The recovery of human embryos after re-vitrification and warming was 100% for blastocysts processed with either the Gavi system or manual Cryotop method (Table V). The proportion of cells which were considered to have survived after warming was 91% for Gavi vitrified-warmed embryos when compared with 77% for Cryotop controls. In line with this, there were more grade 1 and grade 2 fully hatched blastocysts 24 h after warming for Gavi processed embryos, being 17% for Gavi compared with 8% for Cryotop controls. This difference likely reflects low embryo numbers and was no longer apparent at 48 h post-warming.

Cooling and warming rates

The rate of cooling of the Gavi Pod (Fig. 1), as calculated from 0°C to −133°C, was determined to be 14 136 °C/min (Table VI). The rate of

Table I Evaluation of the Gavi system for mouse zygotes.

				Cleaved	≥ Expanding blastocyst	Fully hatched blastocyst	
Treatment	#	Recovered	Survived	Day 2	Day 5	Day 6	Day 7
Fresh	63	n/a	n/a	57 (90%)	45 (71%)	24 (38%)	33 (52%)
Cryotop	68	68 (100%)	67 (99%)*	62 (91%)	54 (79%)	29 (43%)	39 (57%)
Gavi	60	58 (97%)	51 (88%)*	49 (84%)	44 (76%)	25 (43%)	33 (57%)

Outcomes for F1 C57BL/6J x CBA mouse zygotes vitrified after automated processing with the Gavi system, as compared with the manual Cryotop method and fresh controls.
*Significant difference ($P < 0.05$) between test groups.

Table II Evaluation of the Gavi system for mouse cleavage stage embryos.

Treatment	#	Recovered	Survived	Blastocyst Day 5	Fully hatched blastocyst Day 6	Fully hatched blastocyst Day 7
Fresh	72	n/a	n/a	36/40 (90%)[a]	34 (47%)*	44 (61%)*,[#]
Cryotop	60	60 (100%)	60 (100%)	55 (92%)	18 (30%)*	23 (38%)*
Gavi	68	68 (100%)	67 (99%)	58 (85%)	26 (38%)	29 (43%)[#]

Outcomes for Quackenbush Swiss mouse 6–8 cell embryos vitrified after automated processing with the Gavi system, as compared with the manual Cryotop method and fresh controls.
[a]Not all embryos were assessed on Day 5.
*,[#]Significant difference ($P < 0.05$) between test groups.

Table III Evaluation of the Gavi system for mouse blastocysts.

Treatment	#	Recovered	Fully hatched blastocyst Day 6	Fully hatched blastocyst Day 7
Fresh	180	n/a	86 (48%)	115 (64%)*
Cryotop	172	171 (99%)	77 (45%)	86 (50%)*
Gavi	176	175 (99%)	71 (41%)	95 (54%)

Outcomes for Quackenbush Swiss mouse blastocysts vitrified after automated processing with the Gavi system, as compared with the manual Cryotop method and fresh controls.
*Significant difference ($P < 0.05$) between test groups.

warming, as calculated from −133°C to 0°C, was determined to be 11 239°C/min.

Discussion

Cryopreservation of embryos is an essential component of a successful ART clinic, with vitrification the method-of-choice due to excellent embryo survival and clinical outcomes (Loutradi *et al.*, 2008; AbdelHafez *et al.*, 2010; Roy *et al.*, 2014). However, vitrification is a labour-intensive and time-consuming task that involves embryologists physically moving the embryo from one solution to another. This makes it impossible to control and standardize the many vitrification variables such as actual temperatures, the exact duration of incubations and the diffusion gradients of solutions at the embryo level. As a result, the success of vitrification can be highly variable between embryologists and ART clinics (Gosden, 2011). Demanding labour requirements and the recognized need for standardization inspired us to attempt to automate the vitrification process. Through extensive voice-of-customer studies, we determined that ART clinics had several key requirements for an automated vitrification instrument. Firstly, vitrification outcomes had to be comparable to an approved closed vitrification device or ideally to the gold standard Cryotop open manual system. Secondly, a closed system whereby the embryo was protected from contact with liquid nitrogen was required to prevent cross-contamination and to comply with increasingly stringent regulatory directives, for example in the European Union (European Union, 2004). Additionally, the instrument had to be capable of vitrifying multiple embryos simultaneously. Furthermore, the process had to be 'low skill' and 'user friendly', allowing the embryologist to simply add the embryos to the instrument, press start and then return at the end of automatic equilibration for manual immersion of the carrier device into liquid nitrogen. Finally, the instrument had to be capable of successfully vitrifying embryos at different developmental stages including cleavage stage embryos and blastocysts.

Here we report the development of a vitrification instrument, Gavi, and its associated vitrification device, the Pod, which fulfils these voice-of-customer requirements. The Gavi system allows automated exchange of vitrification solutions for up to four embryos simultaneously, occurring in the Pod via a robotic liquid handling unit with individual pipettes and single-use pipette tips. Subsequently, the instrument seals each Pod with a lid to create a closed system to prevent embryo or solution contact with liquid nitrogen. At all stages of development, we benchmarked Gavi system vitrification outcomes to that of the manual Cryotop open system, which Genea ART clinics have used extensively to achieve excellent clinical results (Roy *et al.*, 2014). This study shows that the automated Gavi system can successfully equilibrate embryos at different developmental stages for vitrification, including mouse zygotes (Table I), cleavage stage embryos (Table II) and blastocysts (including fully hatched blastocysts) (Tables III and IV), as well as human blastocysts (Table V). This includes an excellent embryo recovery rate of 99% (354/358) with the Gavi system.

The development of the Gavi system to its current configuration involved 4 years of intensive research with close collaboration between teams of embryologists and engineers, two successive, improved main instrument prototypes (the Alpha model reported here and its predecessor concept demonstrator prototype) and 12 Pod revisions, over 30 protocol variations and 1250 vitrification runs. The Gavi system allows precise and reproducible control of all vitrification variables including temperature, time, volume, media concentration, fluidics speed and cooling rate, an impossible feat if performed manually. Embryo equilibration in the Pod by automated fluidics also results in reduced embryo handling therefore minimizing embryo stress and the potential for adverse events including embryo loss. Unlike manual vitrification, the only skill necessary to successfully operate the Gavi system is basic embryo handling, required for embryo loading into the Pod, and we have not observed operator-dependent outcomes even with 'junior' technicians. Furthermore, valuable time for other work is gained by embryologists when embryos are vitrified using the Gavi system. We estimate 21 min of 'hands-on' time is required to vitrify four human blastocysts using Gavi, including paperwork and preparation for automated equilibration. This is compared with 57 min using Cryotop, including paperwork, preparation time, manual

Table IV Evaluation of the Gavi system for mouse fully hatched blastocysts.

Treatment	#	Recovered	Attached[a]		Outgrown[b]
			Day 7	Day 8	Day 8
Fresh	33	n/a	28 (85%)	32 (97%)*	20 (61%)
Cryotop	32	32 (100%)	26 (81%)	25 (78%)*	13 (41%)*
Gavi	31	30 (97%)	28 (93%)	25 (83%)	21 (70%)*

Outcomes for Quackenbush Swiss mouse fully hatched blastocysts vitrified after automated processing with the Gavi system, as compared with the manual Cryotop method and fresh controls.
[a]Embryo attachment to collagen coated dishes.
[b]Outgrowth of embryonic cells (after attachment) in collagen coated dishes.
*Significant difference ($P < 0.05$) between test groups.

Table V Evaluation of the Gavi system for human blastocysts.

Treatment	#	Recovered	Survived	Re-expanded	24 h		48 h	
					Fully hatched blastocyst	Hatching/expanded blastocyst	Fully hatched blastocyst	Hatching blastocyst
Cryotop	13	13 (100%)	10 (77%)	7/10 (70%)[a]	1 (8%)	9 (69%)	2 (15%)	3 (23%)
Gavi	23	23 (100%)	21 (91%)	14/18 (78%)[a]	4 (17%)	12 (52%)	4 (17%)	6 (26%)

Outcomes for human blastocysts vitrified after automated processing with the Gavi system, as compared with the manual Cryotop method. Embryo classifications at 24 and 48 h were restricted to grade I and grade II embryos.
[a]Not all embryos were assessed for re-expansion.

equilibration (each embryo processed 5 min apart with 15 min in Vitrification Solution 1 and 1.5 min in Vitrification Solution 2) and vitrification.

One of the greatest challenges in the development of the Gavi system was how to hold the embryo in place during fluid exchange. The microfluidic dynamics of the Pod channel secures the embryo and prevents it from floating during automated fluid exchange (Supplementary data, Video S1) and as such may have applications beyond vitrification for automation of IVF procedures including general culture media changes. This patented approach (Henderson *et al.*, 2011) is completely novel compared with those of other early stage projects which have attempted to automate vitrification using an enclosed microfluidics method (Swain *et al.*, 2013). Furthermore, the Gavi Pod has been specifically developed for heat sealing to allow for closed system vitrification, with its design limiting dissipation of heat from the seal location down to the bottom of the Pod where the embryo is located. As a result the sealing process does not affect vitrification outcomes as the temperature does not increase beyond physiological levels for the embryo if transferred to liquid nitrogen within the recommended time (unpublished data).

The success of the Gavi system in vitrifying-warming embryos, on par with that of the manual Cryotop method, challenges many of the preconceived fundamentals of vitrification. This includes the assumption that the ultra-fast speed of cooling and warming achieved with open vitrification devices is critical to cryopreservation success. We determined the Gavi Pod to have cooling rates in the order of 14 100°C/min and warming rates in the order of 11 200°C/min (Table VI), which is much slower than those reported for Cryotop (Kuwayama *et al.*, 2005b; Kuwayama, 2007) and is an unavoidable consequence of a closed system. Despite this, our data indicate that it is possible to achieve comparable *in vitro* cryopreservation outcomes to the Cryotop, which is supported by other studies which found equivalent clinical outcomes when comparing open and closed vitrification devices (Kuwayama *et al.*, 2005a; Isachenko *et al.*, 2007; Desai *et al.*, 2013). In fact, our cooling rate far exceeds that of other closed vitrification devices such as the Rapid-i and Vitrisafe, estimated to be ~1300°C/min (Vanderzwalmen *et al.*, 2009; Desai *et al.*, 2013). This is because heat transfer to the embryo in the Pod occurs rapidly through plastic (with the embryo residing on the inner Pod surface with the outer surface in contact with liquid nitrogen) when compared with the Rapid-i and Vitrisafe where heat transfer occurs much more slowly through air (with the embryo residing on a plastic rod surrounded by air inside a separate straw with its outer surface in contact with liquid nitrogen).

Encouraged by the extremely promising vitrification results reported here for mouse and human embryos, we are now undertaking further research and development to ready the Gavi system for use in ART clinics. This includes verification and validation with successive models to the Alpha prototype including further extensive *in vitro* testing with mouse embryos from multiple strains as well as donated human blastocysts. Additionally, to satisfy regulatory requirements for medical devices, stability and toxicity testing of Gavi system consumables including Pods, premixed media and tips will be performed. If successful, clinical evaluation of pregnancy outcomes following transfer of human embryos vitrified-warmed using Gavi production units will commence in 2015. It is anticipated that the Gavi system will be initially released with protocols for human blastocysts, with future software updates to include oocyte, zygote and cleavage stage protocols.

In summary, we have developed an automated fluids exchange instrument and novel closed system device to allow rapid, efficient and reproducible vitrification of embryos for ARTs. Our *in vitro* results

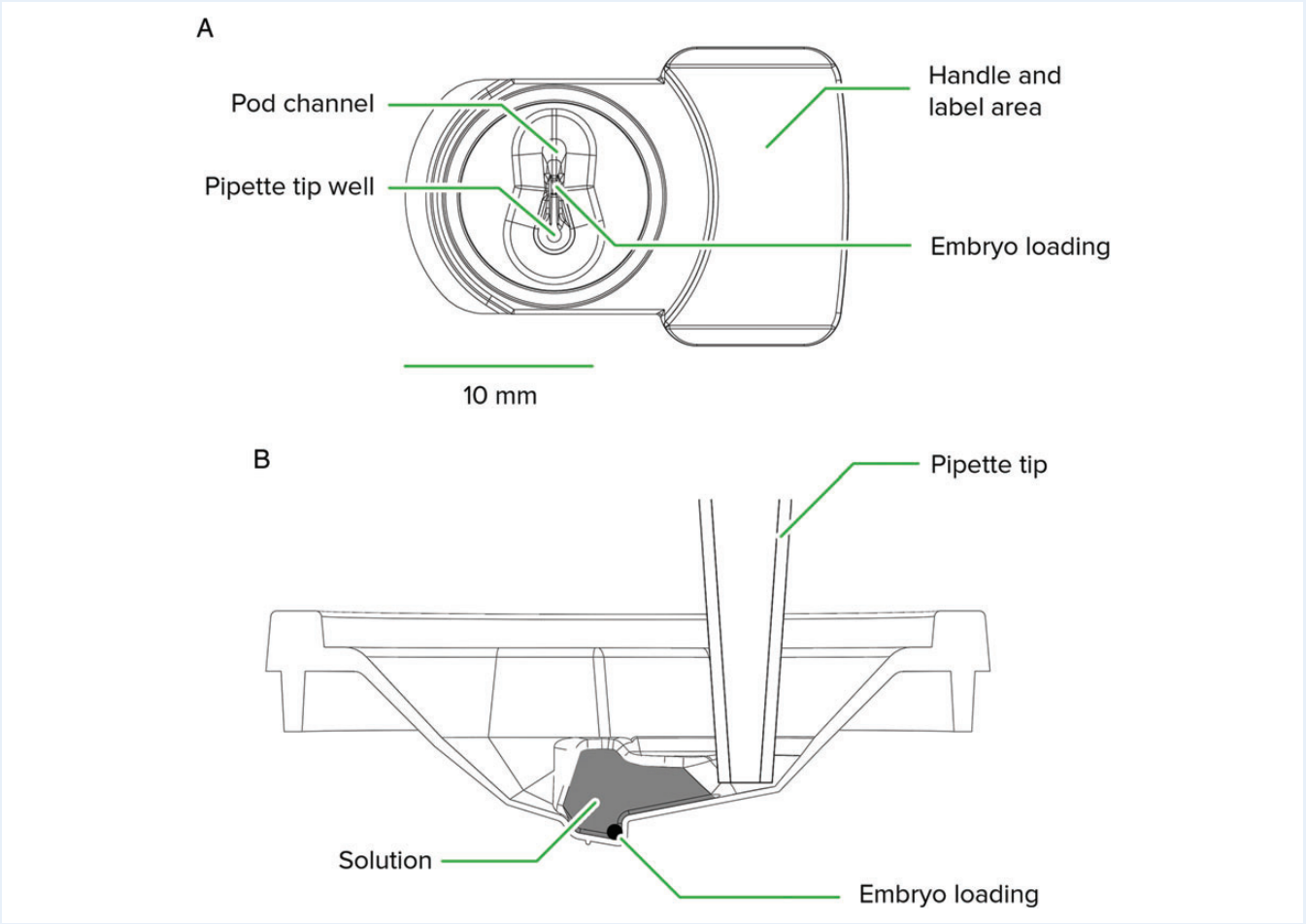

Figure 1 Design of Gavi Pod. (**A**) Schematic top view of Pod (Alpha prototype). (**B**) Schematic view of microfluidics 'channel' of Pod (vertical cross-section through Pod channel shown in (A)).

Table VI Gavi Pod cooling and warming rates.

	Cooling	Warming
ave ± std	14 136 ± 1528	11 239 ± 2480
min – max	12 667–15 709	7176–13 951

Cooling and warming rates (°C/min) of Pod (Alpha prototype). Data are average (ave) ± standard deviation (std) and minimum (min) – maximum (max) value obtained from at least of four repeats.

using the Gavi system Alpha model are extremely promising, being comparable to that of manual Cryotop, and testing of advanced instrument models is currently underway. The success of the Gavi system demonstrates that it is possible to automate highly skilled and labour-intensive ART procedures and opens up the door for further innovations to standardize methodologies and improve patient outcomes.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

T.K.R. participated in the instrument design, study design, execution and analysis, manuscript drafting and critical discussion. S.B. participated in the instrument design and study design, execution and analysis. N.M.T. participated in the instrument design and study execution and analysis. C.K.B. participated in the study analysis and manuscript drafting. E.V. participated in the instrument design, study design and analysis and critical discussion. C.H. participated in the instrument design. C.L. participated in the instrument design and critical discussion. K.B. participated in the instrument design. B.H. participated in the instrument design. S.H. participated in the instrument design, study design and critical discussion. J.S. participated in the instrument design. S.R.L. participated in the instrument design. S.M.D. participated in the instrument design, study design and critical discussion. T.T.P. participated in the study design and critical discussion. S.J.M. participated in the instrument design, study design and critical discussion. M.C.B. participated in the study design and critical discussion. T.S. participated in the instrument design, study design and critical discussion.

Funding

The development and testing of the Gavi system was funded by Genea Ltd.

Conflict of interest

S.B., N.M.T., T.T.P., S.J.M., M.C.B. and T.S. are shareholders of Genea Ltd, a public company with over 180 shareholders. Genea Ltd is the sole owner of the Gavi system. E.V., C.H., C.L., S.R.L. and S.M.D. are shareholders of Planet Innovation Pty Ltd. Planet Innovation Pty Ltd has been contracted by Genea Ltd to develop engineering aspects of the Gavi system and to initially manufacture the instrument upon commercialization. The remaining authors are employees of either Genea Ltd or Planet Innovation Pty Ltd.

References

AbdelHafez FF, Desai N, Abou-Setta AM, Falcone T, Goldfarb J. Slow freezing, vitrification and ultra-rapid freezing of human embryos: a systematic review and meta-analysis. *Reprod Biomed Online* 2010;**20**:209–222.

Adamson GD, Tabangin M, Macaluso M, de Mouzon J. The number of babies born globally after treatment with the assisted reproductive technologies (ART). *Fertil Steril* 2013;**100**:S42.

Alpha Scientists In Reproductive Medicine. The Alpha consensus meeting on cryopreservation key performance indicators and benchmarks: proceedings of an expert meeting. *Reprod Biomed Online* 2012;**25**:146–167.

Bielanski A. A review of the risk of contamination of semen and embryos during cryopreservation and measures to limit cross-contamination during banking to prevent disease transmission in ET practices. *Theriogenology* 2012;**77**:467–482.

Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. *Cochrane Database Syst Rev* 2012;**7**:CD008950.

Cobo A, Meseguer M, Remohi J, Pellicer A. Use of cryo-banked oocytes in an ovum donation programme: a prospective, randomized, controlled, clinical trial. *Hum Reprod* 2010;**25**:2239–2246.

Desai NN, Goldberg JM, Austin C, Falcone T. The new Rapid-i carrier is an effective system for human embryo vitrification at both the blastocyst and cleavage stage. *Reprod Biol Endocrinol* 2013;**11**:41.

European Union. Directive 2004/23/EC of the European Parliament and of the Council of 31 March 2004 on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. *Offic J Eur Union* 2004;**102**:48–58.

Gerris J. Single-embryo transfer versus multiple-embryo transfer. *Reprod Biomed Online* 2009;**18**:63–70.

Gosden R. Cryopreservation: a cold look at technology for fertility preservation. *Fertil Steril* 2011;**96**:264–268.

Henderson CJ, Lewis CM, Roy TK, Vom E. Improved micromanipulation and storage apparatus and methods. *World Intellectual Property Organization* 2011, WO2011146998A1.

Isachenko V, Katkov II, Yakovenko S, Lulat AG, Ulug M, Arvas A, Isachenko E. Vitrification of human laser treated blastocysts within cut standard straws (CSS): novel aseptic packaging and reduced concentrations of cryoprotectants. *Cryobiology* 2007;**54**:305–309.

Jansen RP. Benefits and challenges brought by improved results from *in vitro* fertilization. *Intern Med J* 2005;**35**:108–117.

Kader AA, Choi A, Orief Y, Agarwal A. Factors affecting the outcome of human blastocyst vitrification. *Reprod Biol Endocrinol* 2009;**7**:99.

Kato O, Kawasaki N, Bodri D, Kuroda T, Kawachiya S, Kato K, Takehara Y. Neonatal outcome and birth defects in 6623 singletons born following minimal ovarian stimulation and vitrified versus fresh single embryo transfer. *Eur J Obstet Gynecol Reprod Biol* 2012;**161**:46–50.

Kuwayama M. Highly efficient vitrification for cryopreservation of human oocytes and embryos: the Cryotop method. *Theriogenology* 2007;**67**:73–80.

Kuwayama M, Vajta G, Ieda S, Kato O. Comparison of open and closed methods for vitrification of human embryos and the elimination of potential contamination. *Reprod Biomed Online* 2005a;**11**:608–614.

Kuwayama M, Vajta G, Kato O, Leibo SP. Highly efficient vitrification method for cryopreservation of human oocytes. *Reprod Biomed Online* 2005b;**11**:300–308.

Loutradi KE, Kolibianakis EM, Venetis CA, Papanikolaou EG, Pados G, Bontis I, Tarlatzis BC. Cryopreservation of human embryos by vitrification or slow freezing: a systematic review and meta-analysis. *Fertil Steril* 2008;**90**:186–193.

Meseguer M, Herrero J, Tejera A, Hilligsoe KM, Ramsing NB, Remohi J. The use of morphokinetics as a predictor of embryo implantation. *Hum Reprod* 2011;**26**:2658–2671.

Meseguer M, Rubio I, Cruz M, Basile N, Marcos J, Requena A. Embryo incubation and selection in a time-lapse monitoring system improves pregnancy outcome compared with a standard incubator: a retrospective cohort study. *Fertil Steril* 2012;**98**:1481–1489.

Roy TK, Bradley CK, Bowman MC, McArthur SJ. Single-embryo transfer of vitrified-warmed blastocysts yields equivalent live-birth rates and improved neonatal outcomes compared with fresh transfers. *Fertil Steril* 2014;**101**:1294–1301.

Swain JE, Lai D, Takayama S, Smith GD. Thinking big by thinking small: application of microfluidic technology to improve ART. *Lab Chip* 2013;**13**:1213–1224.

Takahashi K, Mukaida T, Goto T, Oka C. Perinatal outcome of blastocyst transfer with vitrification using cryoloop: a 4-year follow-up study. *Fertil Steril* 2005;**84**:88–92.

Vanderzwalmen P, Ectors F, Grobet L, Prapas Y, Panagiotidis Y, Vanderzwalmen S, Stecher A, Frias P, Liebermann J, Zech NH. Aseptic vitrification of blastocysts from infertile patients, egg donors and after IVM. *Reprod Biomed Online* 2009;**19**:700–707.

Wang J, Sauer MV. *In vitro* fertilization (IVF): a review of 3 decades of clinical innovation and technological advancement. *Ther Clin Risk Manag* 2006;**2**:355–364.