## The latest product from the Sydney IVF multi-stage media development program.

Tomas Stojanov, PhD; Julia Schaft, PhD; and Robert Jansen, MD Sydney IVF Limited, 321 Kent Street, Sydney, Australia



### Background: Culture Medium Development at Sydney IVF

The development of embryo culture medium has been a vital part of Sydney IVF research from the inception of the company over 20 years ago. From the late 1990s, IVF clinics began implementing processes that decrease the health risks associated with multiple pregnancies without reducing pregnancy rates. This was made possible by advances in embryo viability in culture and increasingly allowing the transfer of a single, fresh embryo, so-called "elective single embryo transfer," or eSET [1,2]. Commonly, these improved methods involve the culture of embryos outside of the human body through the embryonic process of blastulation, which puts an increased reliance on the IVF culture system and the media used to sustain the human embryo in vitro. The last 11 years have seen significant improvements in the ability of Sydney IVF embryo culture media to closely match the embryo's physiological requirements. During this time we have also advanced our testing processes to make sure that Sydney IVF medium readily fulfills the highest regulatory standards.

More and more, our research base for culture medium development incorporates in vivo and in vitro tests using, respectively, excess embryos donated for ethically approved, licensed research and human embryonic stem cells developed from Sydney IVF blastocysts, as well as the traditional mouse embryo model systems where these still remain appropriate.

Initial evaluations are performed in a mouse embryo culture system. In vivo fertilized mouse oocytes are subjected to the changed media and embryonic development is assessed by blastocyst development rate, inner cell mass (ICM) cell counts and various gene expression studies. In addition, monitoring of the epigenetic status of the embryo's DNA (DNA "imprinting") is of particular interest to address the repeatedly raised but not substantiated concern that extended in vitro culturing of embryos could systematically or consistently increase the risk of DNA methylation aberrations. Genomic imprinting defects are behind disorders such as Beckwith-Wiedemann syndrome, Prader-Willi syndrome and Angelman syndrome [3,4,5]. The imprinting defects are mediated through impaired DNA methylation. We monitor a panel of imprinted genes in an in vitro assay system based on hES cells. These imprinted genes are analyzed using methylation-specific PCR before any new culture medium formulation is cleared for the next step in the testing regimen.

Pluripotent stem cells are derived from the early human embryo and exhibit two unique features: (1) a karyotypically and epigenetically stable capacity for continued self-renewal and (2) an ability to differentiate into any of the more than 200 somatic cell types present in the human body. Our stem cells display a similar surface antigen profile to the inner cell mass (ICM) of the human blastocyst, and the gene expression profile of these stem cells is similar to that of early embryos. These features have led us to consider these stem cells to be a consistent, practical and useful tool for modeling early preimplantation human embryonic development in vitro. Our stem-based media testing regimen utilizes proliferation assays to determine whether a new formulation of medium changes regular growth patterns, DNA-methylation patterns or the expression of genes of interest under the test culture conditions.

Sydney IVF has now introduced a third level of testing prior to the clinical trial phase for a medium: trials with human research embryos. These embryos are excess IVF embryos, no longer needed by the donating couples (or family) for reproduction and consented by them for research. Under the Australian National Health and Medical Research Council licensing regime, Sydney IVF is able to conduct research on human embryos to analyze for any genetic reprogramming and alterations in embryo metabolism. Sydney IVF holds five of the ten licenses issued to date and has applied for a further five. The granted licenses 309701, 309702A and 309702B enable us to research across a spectrum of minimal physiological variations, unaccompanied by invasive testing, to more major innovations checked by thorough analyses of embryo gene expression, structure and function.

Increasingly, therefore, we use our human stem cell-based in vitro bioassays to screen for both major and minor variations in culture medium formulation to develop and refine media innovations before we test the new conditions using licensed embryos. This eventually leads to controlled clinical trials in which retrieved eggs are randomly divided into standard and nonstandard formulations to detect clinically important advances. At the clinical practice level, we at Sydney IVF are beginning to use our human stem cell-based in vitro bioassays for batch toxicity testing, aiming to at least partially replace mouse embryo testing [6].

Sydney IVF has an AusIndustry Commercial Ready grant to develop these human stem cell-based bioassays by incorporating high-content analysis (HCA) technology to gain more comprehensive, faster and more accurate hES cell-based media evaluation. Our assays will investigate parameters such as nuclear fragmentation, cytoskeleton organization, cell size, selected cell surface marker expression, DNA content, cell cycle distribution and mitochondrial integrity, and will enable us to gain insights into the cells' viability, apoptosis fragility, proliferation and differentiation capabilities upon exposure to new culture medium conditions. Our new HCA technology will also be applied to our embryo development analysis for more accurate and faster analysis of cell numbers and apoptosis under the test conditions.

Media formulations that show improvement in embryo development with no negative effects on embryo quality enter the final stage of our multi-stage testing process. Sixteen laboratories throughout Australia and three countries are available for human clinical trials of the medium. This multi-center facility ensures that our media are first tested under a variety of different laboratory conditions and processes during their formulation. They are then (whenever innovative conditions require it) proven with licensed research embryos in vitro and entered into efficient clinical comparisons. In these comparisons IVF couples in up to three countries have their cohort of eggs and embryos for that treatment cycle exposed both to the standard medium and to the modified medium under study.

#### Sydney IVF Blastocyst and Oocyte Vitrification Media

Sydney IVF Vitrification Medium is the latest culture medium product developed by Sydney IVF.

There are currently two common methods for cryopreserving excess embryos. In order to succeed, any embryo cryopreservation strategy must minimize the impact of ice crystal formation, solution effects and osmotic shock. The traditional method is to slowly cool the embryos and the surrounding solution to the storage temperature with the deliberate initiation of the formation of ice crystals remote from the embryo. A more recent method, known as vitrification, is different and, by utilizing extremely rapid cooling, transforms the solution into a glass-like, amorphous solid that is free from any crystalline ice structure.

Both methods use special compounds known as cryoprotectants to avoid cell damage. These can be divided into permeating and nonpermeating agents. Permeating cryoprotectants include ethylene glycol (EG), dimethyl sulphoxide (DMSO) and glycerol, all of which are small molecules that readily permeate the membranes of cells; they form hydrogen bonds with water molecules to inhibit ice crystallization. At low concentrations in water, permeating cryoprotectants lower the freezing temperature of the resulting mixture. At higher concentrations, the formation of ice crystals is inhibited and the solution is able to form a solid, glass-like or vitrified state. The potential toxicity of these chemicals at these concentrations is significant, and the cell must therefore be exposed to this solution either for a very short period of time or at very low temperatures, in which condition the metabolic rate of the cell is very low.

# Figure 1: The effectiveness of permeable cryprotectants in Sydney IVF vitrification media (comparison of ethylene glycol and propylene glycol).



Nonpermeating cryoprotectants include the disaccharides sucrose (glucose + fructose) and trehalose (glucose + glucose), which remain extracellular. Nonpermeating cryoprotectants act by drawing free water from within the cell, thus dehydrating the intracellular space. As a result, when used in combination with a permeating cryoprotectant, the net concentration of the permeating cryoprotectant is increased in the intracellular space. This further assists the permeating cryoprotectant in the prevention of ice crystal formation.

# Figure 2: The effectiveness of permeable cryprotectants in Sydney IVF vitrification media (comparison of trehalose and sucrose).



During vitrification, permeating cryoprotectants are typically added at a high concentration while the cell's temperature is controlled at a predetermined level above the freezing point. Because of the potential toxicity of permeating cryoprotectants at these concentrations, the embryo cannot be kept at this temperature for long. After a very short time for equilibration the embryos are plunged directly into liquid nitrogen temperatures.

A typical vitrification process involves exposing the cells to three or more cryoprotectant solutions in a multi-well dish. The cells are transferred to the first solution, where they are washed carefully by moving the cells through the solution with a pipetting device. The washing process is repeated in the second, third and fourth wells for predetermined periods of time until the cells are ready for cryopreservation.

The cells are then drawn up in vitrification solution with a pipettor. A droplet containing the cell to be vitrified is wiped onto the hook of a fiber plug. The fiber hook may be transferred directly into liquid nitrogen or onto the surface of a precooled vitrification block. The fiber hook is placed directly onto the surface of the block, upon which the cell and fluid are instantly vitrified. The fiber hook is then inserted into a prechilled straw located in a slot in the vitrification block before being transferred to long-term cold storage in liquid nitrogen or liquid nitrogen vapor.

Since January 2006, our blastocysts have been vitrified in Sydney IVF Vitrification Media [7,8] using our refined nonimmersion vitrification protocol, where embryos have no direct exposure to liquid nitrogen (thus minimizing any risk of cross-contamination within the storage system). **Table 1** (see next page) compares the clinical vitrification results with historical data for our former, standard slow-freezing procedure.

Using our slow-freezing protocol, 55 percent of the pregnancies obtained were from embryos that had 90-100% cell survival after thawing. Our 2005 data indicate that 41% (432/1,047) of slow-frozen embryos were in this survival band. Since 2006, data from our 16 clinics show a higher embryo rate of 90% or greater cell survival for vitrified embryos of 63% (421/685) (P < 0.005). To date, we have obtained 100 clinical pregnancies from 260 embryo transfers (39%, 1.2 embryos per transfer). In comparison, slow-frozen embryos in 2005 gave a clinical pregnancy rate of 24% (77/327), also transferring on average 1.2 embryos (P = 0.095, which is significant at a one-tailed testing level). We are continuing to monitor these results.

Our clinical results indicate that our innovative, (nonimmersion) vitrification of human blastocysts can provide a safe and viable alternative to current slowfreezing protocols. The high post-thaw survival and pregnancy rates also enable Sydney IVF to encourage an increased proportion of patients to elective single embryo transfer (eSET), the modern way to avoid the complications associated with multiple gestations without sacrificing pregnancy rates.

	Slow-Frozen Pre-2006			Vitrified 2006		
Thaw cycles	< 38 yrs	≥ 38 yrs	all	< 38 yrs	≥ 38 yrs	all
Embryo transfers	299	58	357	217	59	276
Embryos transferred	277	50	327	204	56	260
Embryos transferred	337	65	402	249	77	326
Mean	1.2	1.3	1.2	1.2	1.3	1.2
BhCG-positive	32%	26%	31%	46%	39%	44%
pregnancies per transfer	(90/277)	(13/50)	(102/327)	(93/204)	(22/56)	(115/260)
Clinical	24%	24%	24%	41%	30%	39%
pregnancies per transfer	(66/277)	(12/50)	(77/327)	(84/204)	(17/56)	(100/260)

Table 1: Comparison of slow-freezing and vitrification cryopreservation protocols.

In summary, using our mouse and stem cell models and our preliminary clinical data, we have developed an excellent protocol for the vitrification of blastocysts. We have also developed an in-house device for particularly efficient laboratory vitrification that largely removes the risk of operator error.

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Extending the technology, a protocol developed for the vitrification of cleavage stage embryos is now in its final stages of clinical evaluation. Vitrification medium and protocols for cryopreservation of human oocytes have also progressed, and we are proud to announce, to our knowledge, the first pregnancy in Australia achieved with a vitrified human oocyte (2007).

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COOK MEDICAL INCORPORATED P.O. Box 4195, Bloomington, IN 47402-4195 USA Phone: 812.339.2235, Toll Free: 800.457.4500, Toll Free Fax: 800.554.8335

O'Halloran Road, National Technology Park Limerick, IRELAND, Phone: +353 61 334440, Fax: +353 61 334441

www.cookmedical.com

COOK (CANADA) INC. 111 Sandiford Drive, Stouffville, Ontario, L4A 7X5 CANADA Phone: 905.640.7110, Toll Free: 800.668.0300, Fax: 905.640.6804

WILLIAM A. COOK AUSTRALIA PTY. LTD. 95 Brandl Street, Brisbane Technology Park, Eight Mile Plains Brisbane, QLD 4113 AUSTRALIA, Phone: +61 7 3841 1188

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