Clinical Studies & Development and Use of global® Medium

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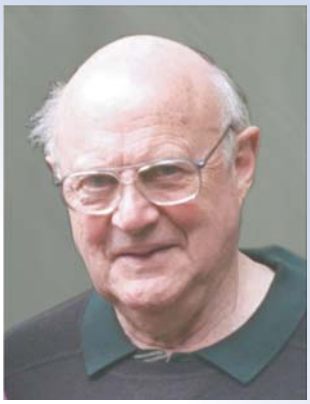
Vision, Science, Research, Standards, and Quality

global® embryo culture medium was developed through innovative scientific research, has been proven in centers worldwide, and is manufactured to the highest Quality Control standards and its on-going quality is assured by independent testing of every batch.



John Biggers, PhD

global® medium is used in Fertility centers worldwide, to help increase the number and quality of human embryos for transfer embryos on either Day 3 culture or at the blastocyst stage, yielding more embryos available for transfers and increased pregnancy rates.

Jacques Cohen, PhD

Fertility clinics who have adopted the use of **global®** have reported improved embryonic development, blastocyst development and an increase in the number of embryos available for fresh transfer or for cryopreservation.

global® has proven to be stable in its use; it maintains its pH and osmolality. It is formulated to produce the lowest levels of ammonium build-up over time. Superior stability, quality, and performance.

Klaus Wiemer, PhD

LifeGlobal®, 'The Art Media Company' was established to meet the industry needs for high quality, specially designed media products. All products are designed with the nutritional needs of the human embryo in mind, with the strictest quality controls, tested ingredients and are FDA compliant. Each batch is independently tested. Our research team of John Biggers, Jacques Cohen, Klaus Wiemer, and Don Rieger have years of research and clinical experience. Together, they have developed these products to offer you the best quality and performance. LifeGlobal has a well established worldwide distribution network to assure you quality customer service, and fresh delivery of the products.

Don Rieger, PhD

The LifeGlobal® scientific team now includes John Biggers, Jacques Cohen, Klaus Wiemer, Don Rieger, and many outside expert consultants in the industry. Together, they are working on exciting projects; using breakthrough technologies to develop and improve products for embryo culture, stem cell culture, gamete and embryo freezing, and many other aspects of human ART.

What makes our team outstanding is not only their commitment and dedication to scientific research, but also their openness to communication in the industry by encouraging multiple center testing and research collaboration, and their willingness to help with clinical applications and testing. Our team is your team, and open to work with you and answer any question you might have on the scientific background or clinical use of global or any other of the family of LifeGlobal ART media.

Please feel free to contact our team at: experts@LifeGlobal.com

Recent Clinical Studies of the Development of Human Embryos in global® Medium

Since the original publication of this monograph, the following four studies of the development of human embryos in global® medium have been presented at major international scientific congresses in 2005.

Study I. **Racowsky C (2005)** QC/QA/QI in the IVF Lab: Using your data to improve clinical protocols. 13th World Congress on In-vitro Fertilization, Assisted Reproduction and Genetics, Istanbul, Turkey, May 26-29.

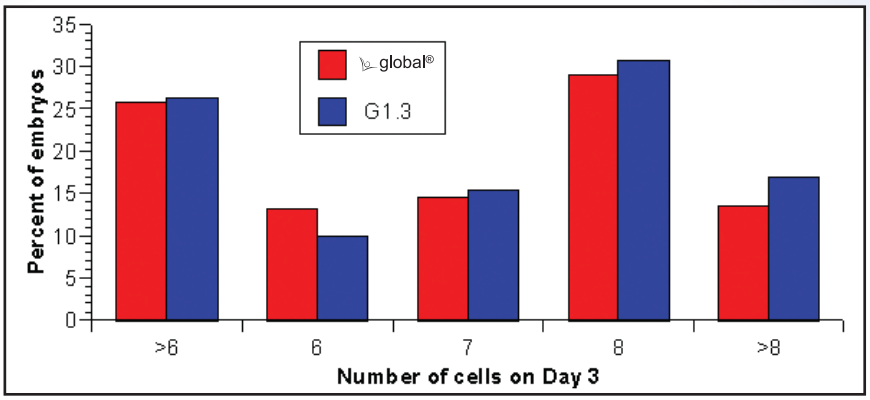


Figure A. The number of cells in human embryos following culture from Day 1 to Day 3 in either global® medium or G1.3. There was no statistically significant difference in cleavage rates between the two media treatments (P = 0.84).

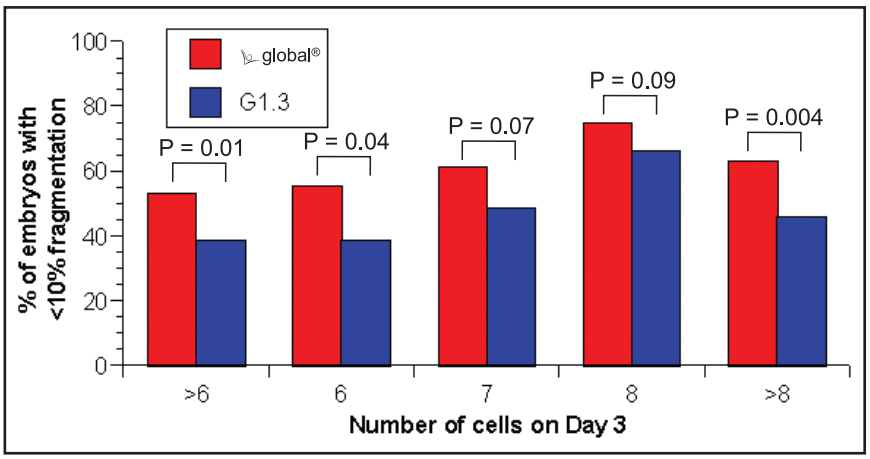


Figure B. Fragmentation in human embryos following culture from Day 1 to Day 3 in either global® medium or G1.3.

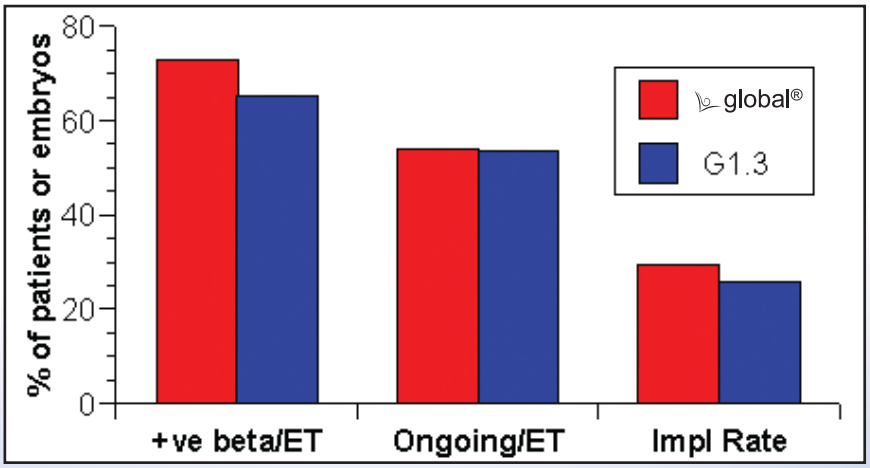


Figure C. Clinical outcomes following transfer of human embryos that had been cultured from Day 1 to Day 3 in either global® medium or G1.3. There were no statistically significant differences in clinical outcomes between the two media treatments.

Study II. **Greenblatt E, Di Berardino D, Chronis-Brown P, Holt D, Lains A (2005)** Comparison of global® medium and G1/G2 cleavage/blastocyst sequential media for culture of human embryos after IVF. *Hum. Reprod.* **20, Suppl. 1,** i21 (Abstract).

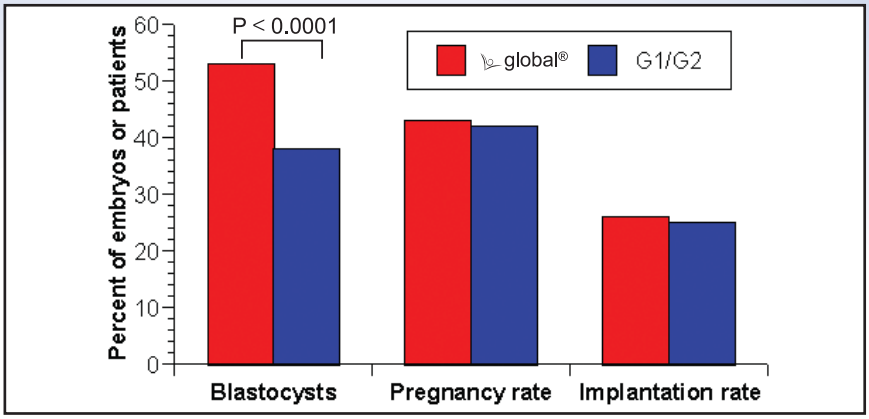


Figure D. Development to blastocyst and subsequent pregnancy and implantation rates for human embryos that had been cultured in global® medium from Day 1 to Day 3 and then Day 3 to Day 5, or in G1 from Day 1 to 3 and then in G2 from Day 3 to 5. There were no statistically significant differences in pregnancy or implantation rates between the two media treatments.

Study III. **Mellon J, Salvador C, Fluker M, Yuzpe A, Kelk D (2005)** A randomized prospective comparison of a sequential versus single media culture system. *Fertil. Steril.* **84, Suppl. 1,** S449-S450 (Abstract).

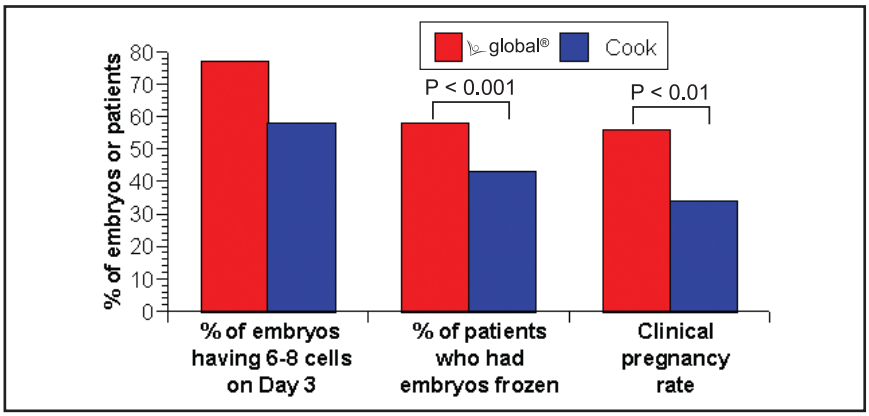


Figure E. Cell numbers on Day 3, % of embryos subsequently frozen, and clinical pregnancy rates for human embryos that were cultured in global® medium from Day 1 to Day 3 and then Day 3 to Day 5, or in Cook sequential media. The authors report that more zygotes developed to the 6-8 cell stage by Day 3, but no significance level was given.

Study IV. **Desai N, Goldfarb J (2005)** Comparison of Day 5 vs. Day 3 transfer outcomes and examination of embryo development in global® Blastocyst medium. *Fertil. Steril.* **84, Suppl. 1,** S251 (Abstract).

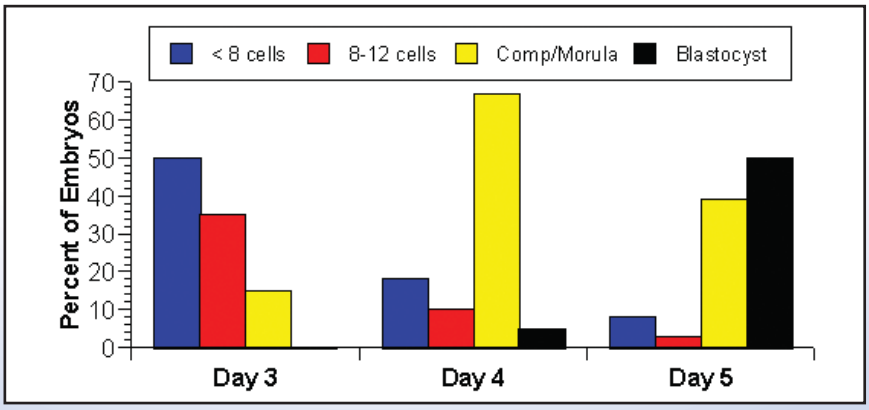


Figure F. The development of human embryos that were cultured in HTF from Day 1 to Day 3 and then in global® medium from Day 3 to Day 5.

I. Historical Background

A. Ringer's Saline Solution and its Derivatives

The history of defined tissue culture media begins with Ringer (1882) who developed a simple salt solution based on the constituents of blood serum for the study of the beating frog heart, *in vitro*. It was composed of sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$) and a low concentration of sodium bicarbonate ($NaHCO_3$, 2.7 mM). This was modified by Locke and Rosenheim (1907), notably by the addition of 11.1 mM glucose, for the study of rabbit heart. Tyrode (1910) added sodium phosphate (NaH_2PO_4) and magnesium chloride ($MgCl_2$) and increased the concentration of $NaHCO_3$ to 11.9 mM for use in the study of rabbit intestine. Krebs and Henseleit (1932) used no glucose, but increased the concentration of $NaHCO_3$ to 25 mM, for the study of nitrogen metabolism by rat tissues. The 25 mM concentration of $NaHCO_3$ in this Krebs-Ringers bicarbonate solution (KRB) is what is conventionally used in conjunction with 5% CO2 in many tissue culture media to produce a physiological pH. (see The Biological Bulletin, Compendium of Physiological Solutions, http://www.mbl.edu/BiologicalBulletin/COMPENDIUM/Comp-TabCont.html)

B. Embryo Culture Media based on KRB

Whitten (1956) showed that 8-cell mouse embryos would develop to the blastocyst stage when cultured in KRB supplemented with 5.55 mM glucose, and McLaren and Biggers (1958) showed that such blastocysts would produce live young when transferred to recipient mothers. Whitten (1957) added lactate to his 1956 formulation and was able to culture out-bred 2-cell mouse embryos, but not zygotes, to the blastocyst stage. This observation led to the concept of the "two-cell block" to mouse embryo culture. In a series of papers, Brinster showed that phosphoenolpyruvate, pyruvate, lactate and oxaloacetate, but not glucose, could support the development of the 2-cell mouse embryo to the 8-cell stage, while glucose could support the development of the 8-cell embryo to the blastocyst stage (see Brinster 1973). Based on these findings, Brinster modified Whitten's medium by reducing the calcium concentration and adding 0.27 mM pyruvate to produce BMOC2, which would support the development of the mouse 2-cell embryo to the blastocyst stage, at high rates. Whittingham (1971) modified Brinster's medium by decreasing the concentration of lactate and increasing the concentration of pyruvate to produce M16 medium.

Brinster's medium and M16 were significant advances for embryo culture and were widely used. However, except for inbred strains of mice, they could not overcome the 2-cell block. Blocks to development *in vitro* were similarly found for hamster (2-cell), cattle (8-16 cell), pig (4-8 cell) and human (4-8 cell) embryos, all approximately coincident with the major onset of expression of the embryonic genome (see Rieger 1992). The 2-cell block to in-vitro development of the mouse embryo was finally overcome by Chatot *et al*. (1989) with CZB medium, a modified version of BMOC2 with no glucose, 1.0 mM glutamine, and 0.1 mM EDTA. This led to the concept that glucose is inhibitory to early embryo development.

C. Embryo Culture Media based on the Composition of Oviduct and Uterine Fluids

As noted above, much of the development of embryo culture media was based on simple salt solutions. An alternative approach was the formulation of media based on the measured concentrations of the components of oviduct and uterine fluids. These include SOF (synthetic oviduct fluid), based on ovine oviduct fluid (Tervit *et al*. 1972); B2, based on bovine oviduct and uterine fluids (Ménézo 1976); HTF (human tubal fluid), based on human oviduct fluid (Quinn *et al*. 1985); and MTF (mouse tubal fluid), based on mouse oviduct fluid (Gardner and Leese 1990).

Based on measured differences in the composition of oviduct and uterine fluids, and on measured changes in the metabolic activity of embryos during early development, Gardner and Lane (2002) have suggested that "in order to optimize mammalian embryo development in culture, sequential media are required, each designed to meet the changing requirements of the developing embryo." Although this seems logical, this "back to nature" approach relies on several questionable assumptions.

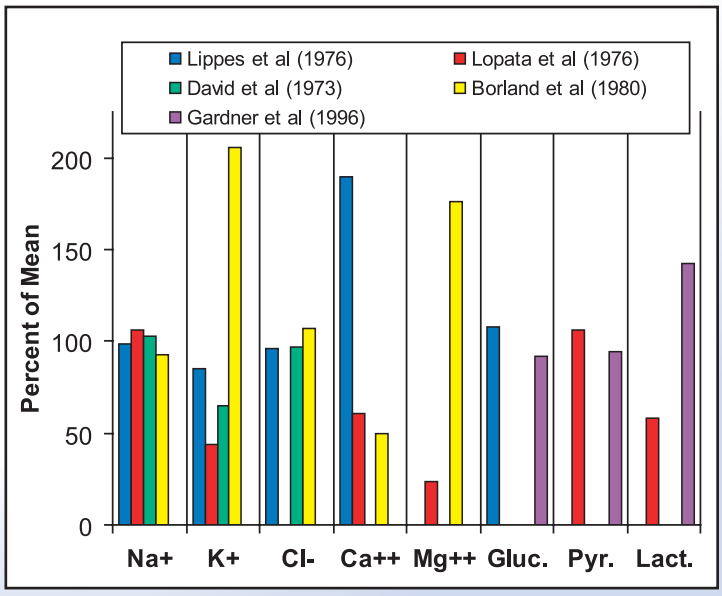


Figure 1. Measurements of the components of human oviduct fluid. (Adapted from Summers and Biggers 2003)

First, as noted by Summers and Biggers (2003) and shown in Figure 1, the measurements of the components of oviduct and uterine fluids are highly variable, and almost certainly subject to physiological inductance. Second, such measurements only reflect the overall composition of the tract fluids and not the micro-environment around the embryo. Third, as shown in Figure 2, the physical and chemical environment of the embryo *in vivo* is completely different from its environment *in vitro*. Except in pathological conditions, there is no pool of fluid in the reproductive tract. The embryo is surrounded by a thin layer of fluid and is in close apposition to the maternal tissues, allowing rapid exchange of nutrients, gases, wastes, and effectors between the embryo and the mother. In contrast, *in vitro*, the embryo is bathed in a relatively large pool of fluid in which the nutrients are continually decreasing and the waste products are continually increasing during culture. Clearly, the stresses on the embryo *in vitro* are very different from those *in vivo*, and culture media must be designed to optimize embryo development under in-vitro conditions.

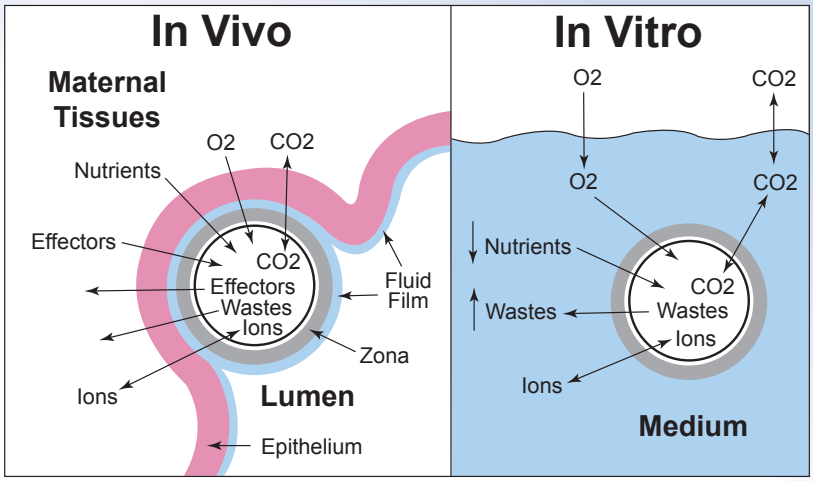


Figure 2. A comparison of the interaction of an embryo with its environment, *in vivo* and *in vitro*.

D. Embryo Culture Media Designed by Simplex Optimization

In a radical departure from the traditional methods for designing embryo culture media, Lawitts and Biggers (1991; 1992) applied the principles of simplex optimization to determine the optimal concentration of each component. They began the process with a generating medium, based on M16 and CZB, containing NaCl, KCl, potassium phosphate (KH_2PO_4), magnesium sulphate ($MgSO_4$), lactate, pyruvate, glucose, bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA), and glutamine. Ten other test media were derived from the generating medium, each containing a high concentration of one of the components. These eleven media formed the START simplex. Four cycles of the simplex optimization process showed that high concentrations of NaCl, pyruvate, KH_2PO_4, and glucose were detrimental to mouse embryo development (Lawitts and Biggers, 1991). A further 16 cycles of optimization resulted in the formulation of Simplex Optimization Medium (SOM) which was marked by a low NaCl concentration, and was able to overcome the mouse 2-cell block (Lawitts and Biggers, 1992). In a subsequent study, it was found that blastocyst development was improved by increasing the concentration of KCl from 0.25 mM to 2.5 mM and this modified version was called KSOM (Erbach *et al*. 1994). Of particular interest, glucose was found to have no inhibitory effect on mouse embryo development in KSOM, even at a relatively high concentration (Summers *et al*. 1995). Embryo development was further improved by the addition of amino acids to KSOM (KSOM-AA) (Ho *et al*. 1995; Biggers *et al*. 2000; Summers *et al*. 2000).

In addition to many studies on mouse embryos, KSOM, with or without amino acids, has been shown to support the development of cattle (Liu and Foote 1995), rabbit (Liu *et al*. 1996), rhesus monkey (Weston and Wolf 1996), pig (Machaty *et al*. 1998), rat (Zhou *et al*. 2003), and human (Biggers and Racowsky 2002, Figure 3) embryos.

For more detailed and extensive reviews of the history of the development of embryo culture media, see Biggers (1998), Hammer (1998), and Summers and Biggers (2003).

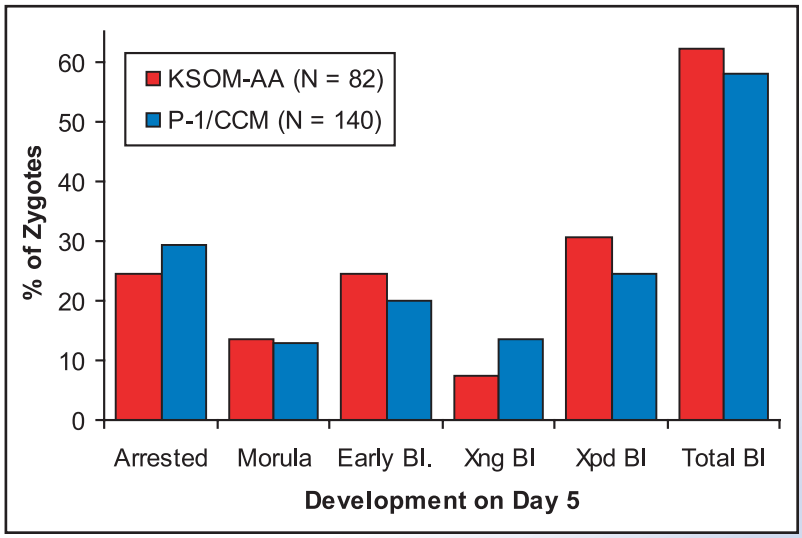


Figure 3. Development of human embryos cultured in KSOM-AA from Day 1 to Day 5, or in P-1 from Day 1 to Day 3 and CCM from Day 3 to Day 5. (From Biggers and Racowsky 2002).

II. The Development and Use of global® Medium in Human ART

Based on the reports of the successful culture of mouse embryos in KSOM-AA, Klaus Wiemer and his colleagues showed that a modified version could support high rates of development of Day 3 human embryos to the blastocyst stage (Wiemer *et al*. 2002; Anderson *et al*. 2002). The medium was subsequently further modified for human use, and marketed as global®.

As shown in the following graphs, global® medium has been successfully used for the culture of early human embryos from Day 1 to Day 3 (Figures 4-5), as a "blastocyst medium" for culture from Day 3 onward (Figures 6-8), and as a single medium for culture from Day 1 to Day 5, with a change to fresh medium at Day 3 (Figures 9-11).

A. Culture of Human Embryos in global® from Day 1 to Day 3

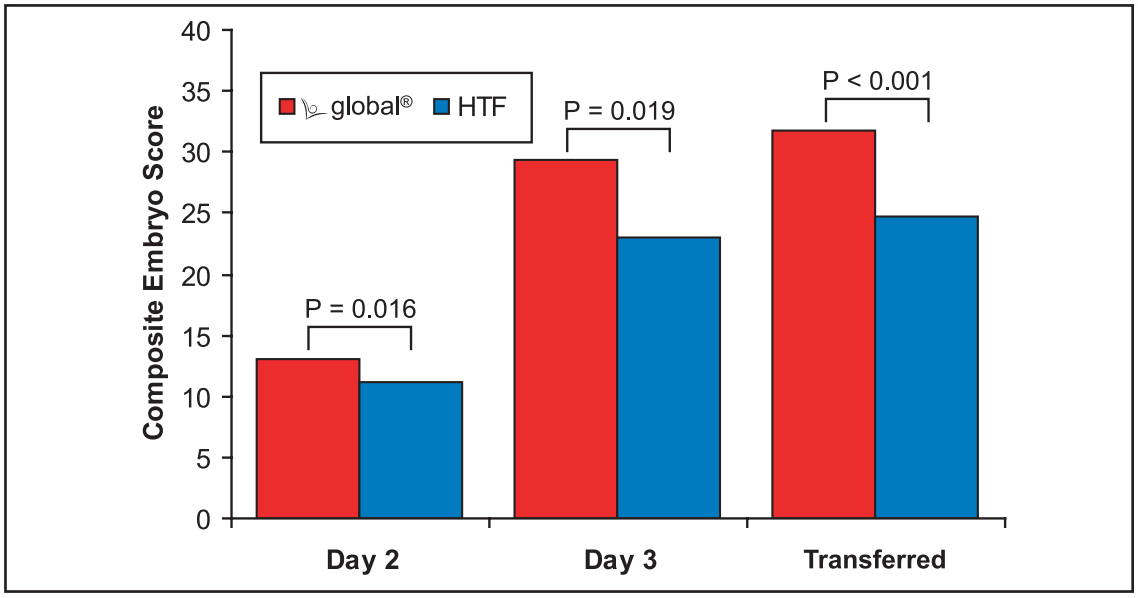


Figure 4. Development of human embryos cultured in global® or HTF from Day 1 until transfer on Day 3. The composite embryo score was significantly greater for embryos cultured in global® than for those cultured in HTF on Day 2, on Day 3, and for embryos transferred. (Neal *et al*. 2004)

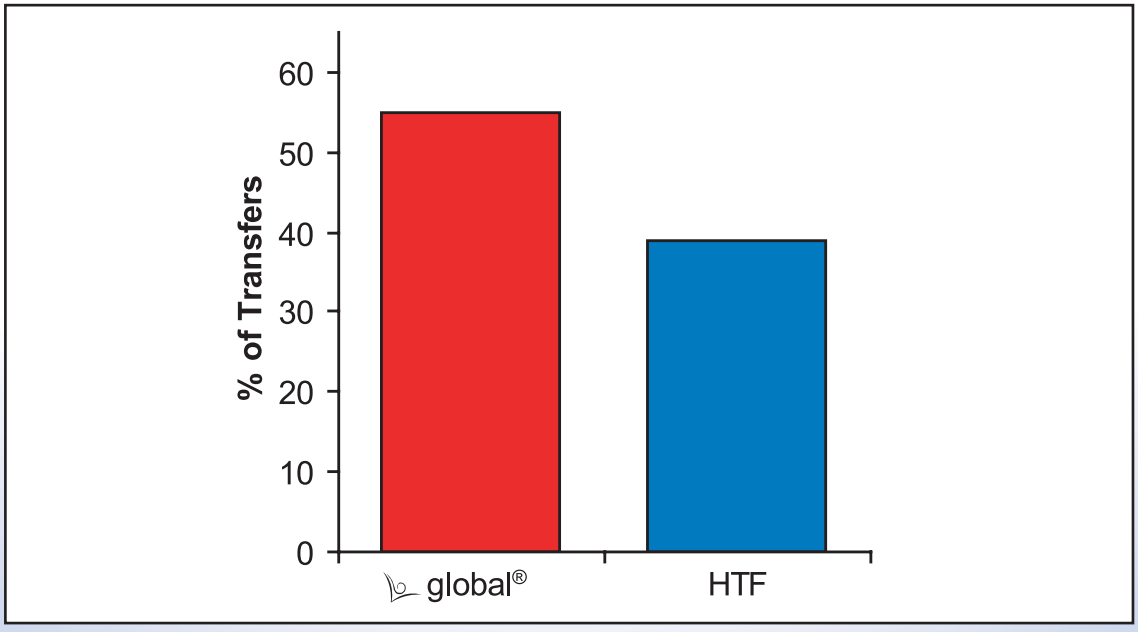


Figure 5. Pregnancy rates from human embryos cultured in global® or HTF from Day 1 to Day 3 and transferred on Day 3. There was no significant difference in pregnancy rates between culture media. (Neal *et al*. 2004)

B. *Culture of Human Embryos in global® from Day 3 to Day 5 or 6*

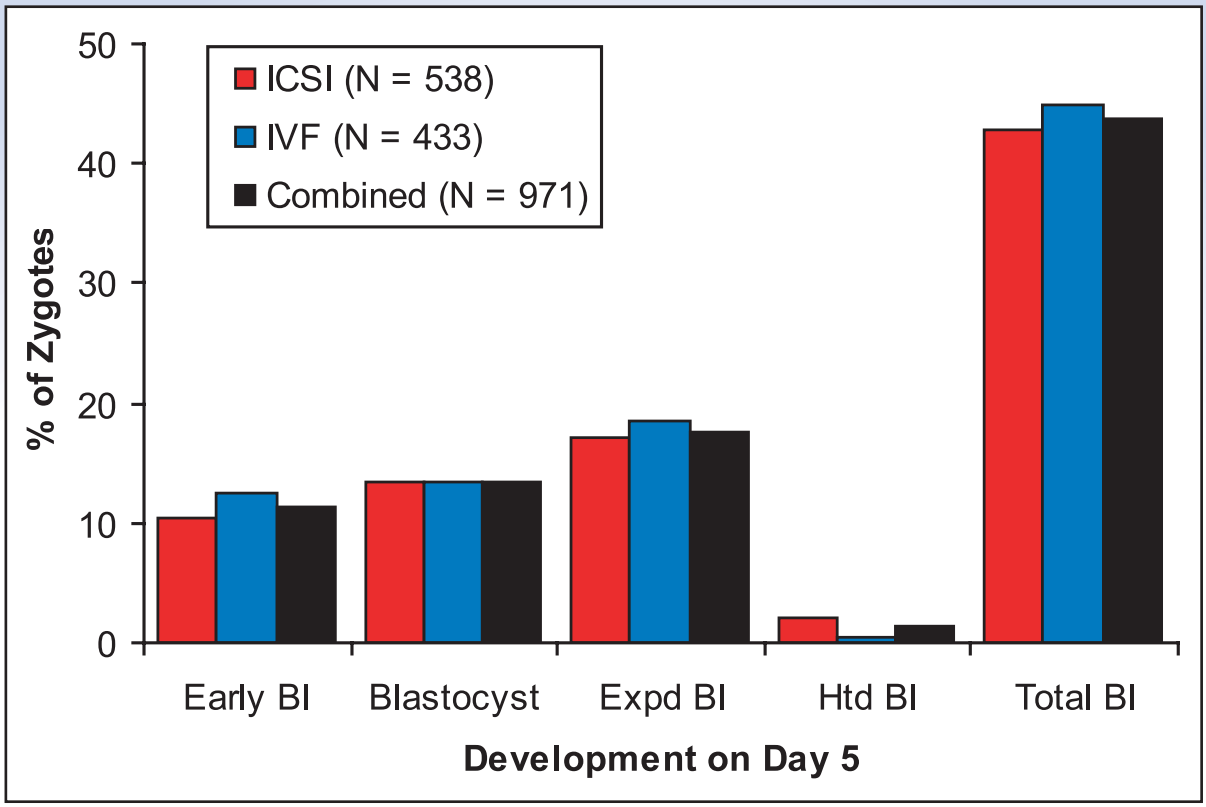


Figure 6. Development of human embryos cultured in HTF from Day 1 to Day 3, followed by culture in global® from Day 3 until transfer on Day 5. (Wiemer *et al*. 2002)

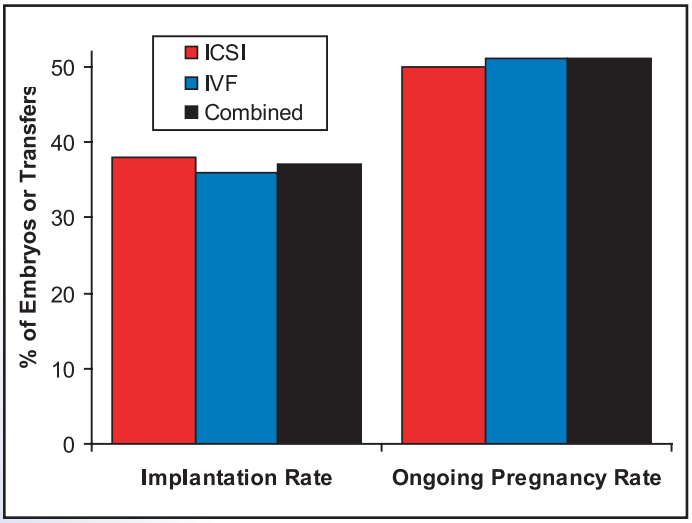


Figure 7. Implantation and pregnancy rates of human embryos cultured in HTF from Day 1 to Day 3, followed by culture in global® from Day 3 until transfer on Day 5. (Wiemer *et al*. 2002)

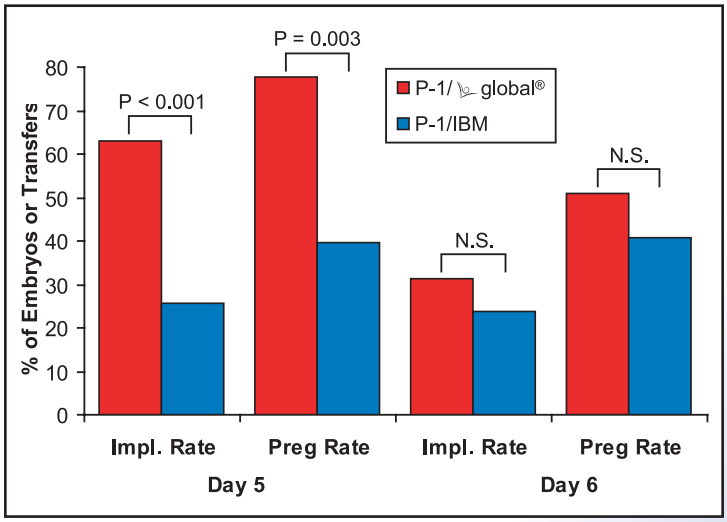


Figure 8. Implantation and pregnancy rates of frozen-thawed human blastocysts. The embryos were cultured in P-1 from Day 1 to Day 3, and then either in global® medium or in IBM before being frozen on Day 5 or Day 6. The embryos were then transferred after thawing, in a subsequent cycle. The implantation and pregnancy rates for embryos frozen on Day 5 were significantly greater for embryos cultured in global® than for those cultured in IBM. There were no significant differences in implantation or pregnancy rates between the culture media for embryos frozen on Day 6. (Unpublished data from Dr. T.B. Pool, Fertility Center of San Antonio, San Antonio, TX, USA)

C. *Culture of Human Embryos in global® from Day 1 to Day 5 or 6*

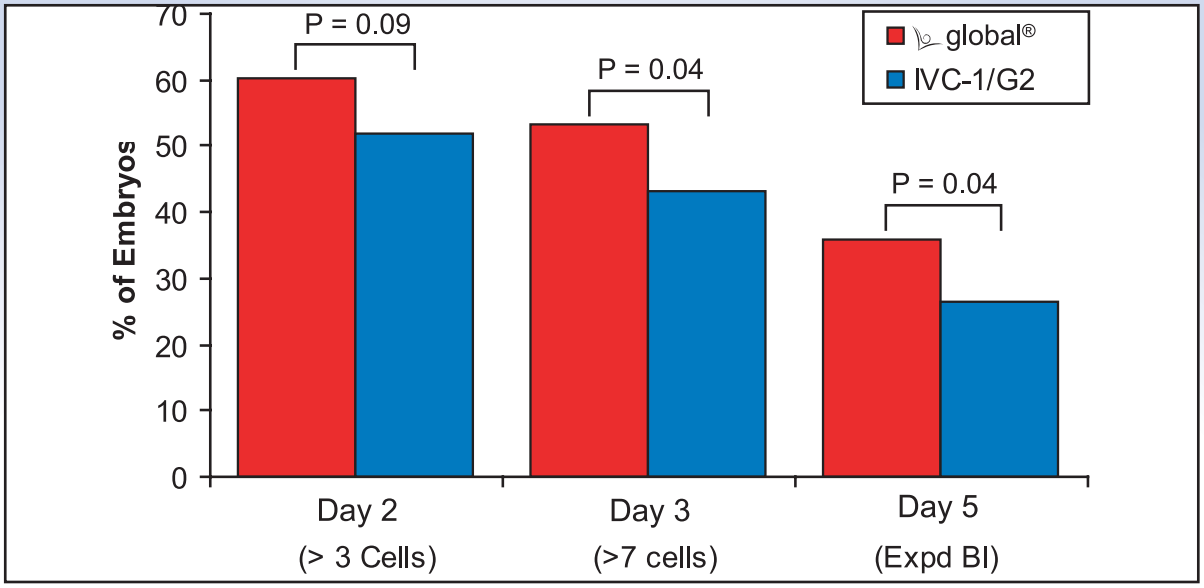


Figure 9. Development of human embryos cultured in global® from Day 1 to Day 3 and Day 3 to Day 5, or in IVC-One from Day 1 to Day 3 and then in G2 from Day 3 to Day 5. The proportions of embryos > 7 cells on Day 3, and at the blastocyst stage on Day 5 were significantly greater for those cultured in global® than for those cultured in IVC-One/G2. (Freeman and Rieger 2004)

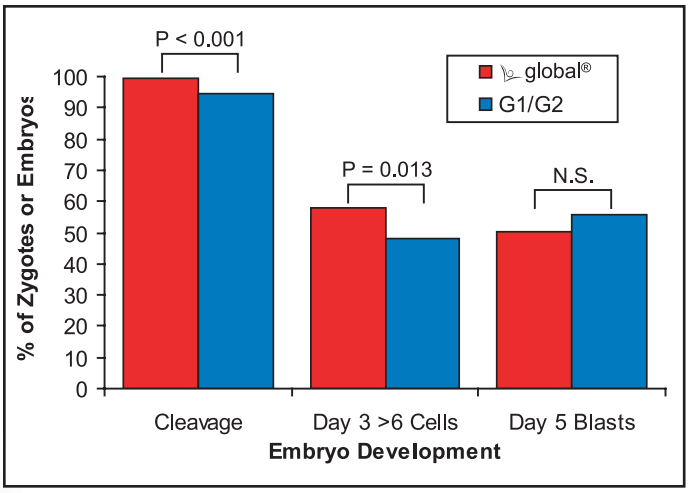


Figure 10. Development of human embryos cultured either in global® from Day 1 to Day 3 and from Day 3 to Day 5, or in G1 from Day 1 to Day 3 and then in G2 from Day 3 to Day 5. Cleavage rates and the proportion of embryos > 6 cells on Day 3 were significantly greater for embryos cultured in global® than for those cultured in G1. (Unpublished data from Dr. A.J. Carrillo, The Fertility Center, Louisville, KY, USA)

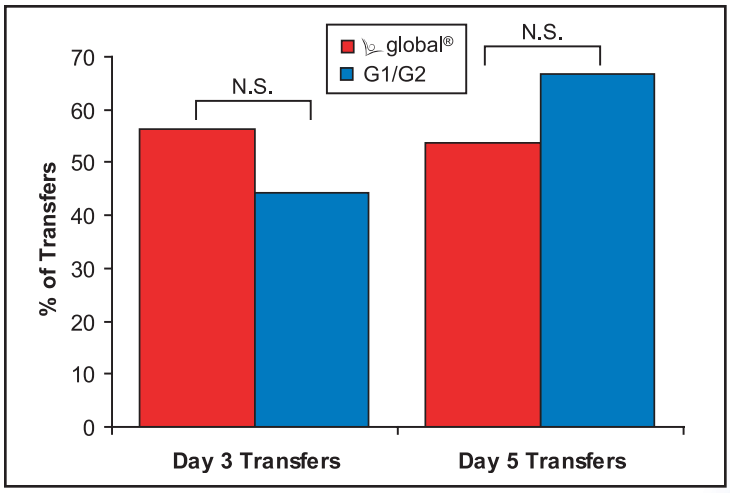


Figure 11. Pregnancy rates of human embryos cultured in global® from Day 1 to Day 3 and Day 3 to Day 5, or in G1 from Day 1 to Day 3 and in G2 from Day 3 to Day 5. The embryos were transferred on Day 3 or Day 5. There were no significant differences in pregnancy rates between culture media for embryos transferred on Day 3 or on Day 5. (Unpublished data from Dr. A.J. Carrillo, The Fertility Center, Louisville, KY, USA)

III. Conclusions

- The results presented above clearly demonstrate that global® can support the development of human embryos at all stages from the zygote to the blastocyst.
- The use of global® as a single medium from Day 1 onward reduces the possibility of stress associated with changing the medium composition at Day 3.
- Equally important, the use of a single medium for all phases of embryo culture reduces the requirement for maintenance and quality control of media, and reduces the chances for error in the clinical human ART laboratory.

Acknowledgements

Dr. Thomas B. Pool of the Fertility Center of San Antonio, San Antonio, TX, USA, and Dr. Alberto J. Carrillo of The Fertility Center, Louisville, KY, USA graciously provided their unpublished data. Drs Klaus Wiemer, Jacques Cohen, and Catherine Racowsky provided constructive comments and suggestions.

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From the Desk of Our Scientific Team

Questions about global® Medium answered by the Team

How can global®, a single medium, fully support both early cleavage of human embryos, and subsequent development to the blastocyst stage?

It has been suggested that the chemical environment of the oviduct is different from that of the uterus and therefore human embryos must be sequentially cultured in (at least) two different media to reflect the difference in the in-vivo environments. However, it is very doubtful that the measurements of the composition of oviduct and uterine fluids reflect the micro-environment of the embryo *in vivo*. Moreover, the environment in culture is physically very different from that in the reproductive tract, and embryo culture media must be designed to optimize embryo development in culture. Moving the embryo to a second medium is a stress upon the embryo. Many clinical studies have shown that single media, most notably global®, support the development of human embryos as well or better than do sequential culture media systems.

Why does global® medium contain glucose?

The concern about glucose in embryo culture medium originated from the observation that the cleavage of hamster embryos was inhibited by glucose when present in the medium with phosphate. This has shown not to be the case for a variety of media, and a variety of species. If fact, some source of glucose is absolutely required for development of the embryo at all stages. It was for this reason that glucose was included in the basic set of constituents used in the simplex optimization development of KSOM-AA, the medium upon which global® is based.

Does ammonium build up in global® medium?

The major potential source of ammonium in embryo culture medium is glutamine. It is for this reason that glycyl-glutamine is used as the source of glutamine in global®. It has been shown that there is very little or no accumulation of ammonium in embryo culture media that contain glycyl-glutamine rather than glutamine.

Why does global® medium contain EDTA?

EDTA was first shown to be beneficial for the culture of mouse embryos by Abramczuk *et al.* in 1977, and has been included in most embryo culture media since then. The exact mechanism of action of EDTA is not clear, but is generally thought to chelate iron and other divalent cations which can induce the production of toxic reactive oxygen species.

Why does global® medium contain Phenol Red?

Phenol Red is a pH indicator and is included in global® to serve as a rapid visual check that the CO_2-bicarbonate buffer system is functioning. However, the exact pH should be systematically monitored with a pH meter (see below). It has been suggested that Phenol Red may be toxic to embryos but there is absolutely no scientific evidence to support that suggestion. The one study that specifically examined the effect of Phenol Red, showed that it had no effect on the development of hamster embryos.

What CO_2 concentration should be used with global® medium?

In most cases, a 5-6% concentration of CO_2 in the incubator will produce a pH of 7.2 to 7.4 in HSA-supplemented global® medium. However, the exact concentration of CO_2 required to produce the optimum pH of 7.3 depends on several factors, including the altitude and the characteristics of the HSA used for protein supplementation. Consequently, we strongly recommend that each laboratory determine and use the exact concentration of CO_2 that is required to produce a pH of 7.3 in HSA-supplemented global® medium.

Why does global® medium become a darker red after the bottle is opened?

During manufacture, global® is bubbled with CO_2. Each time the bottle is opened, CO_2 escapes from the medium into the head space and from there into the atmosphere. Consequently, the medium in the bottle becomes slightly more basic and the Phenol Red indication becomes a darker red. This has no effect on the medium. When the medium is placed in a CO_2 incubator, it absorbs the CO_2, and the proper pH is established.

Why does global® medium not contain HSA?

HSA is not included in global® for two reasons. First, HSA is a biological component and consequently the quality control requirements are different from those for the chemical components. Second, the optimum concentration of HSA in embryo culture medium depends on the laboratory procedures, which differ between IVF laboratories. By not including HSA in global, each laboratory can supplement it with HSA to the concentration that is optimal for the laboratory's conditions.

Stringent Quality Control Program

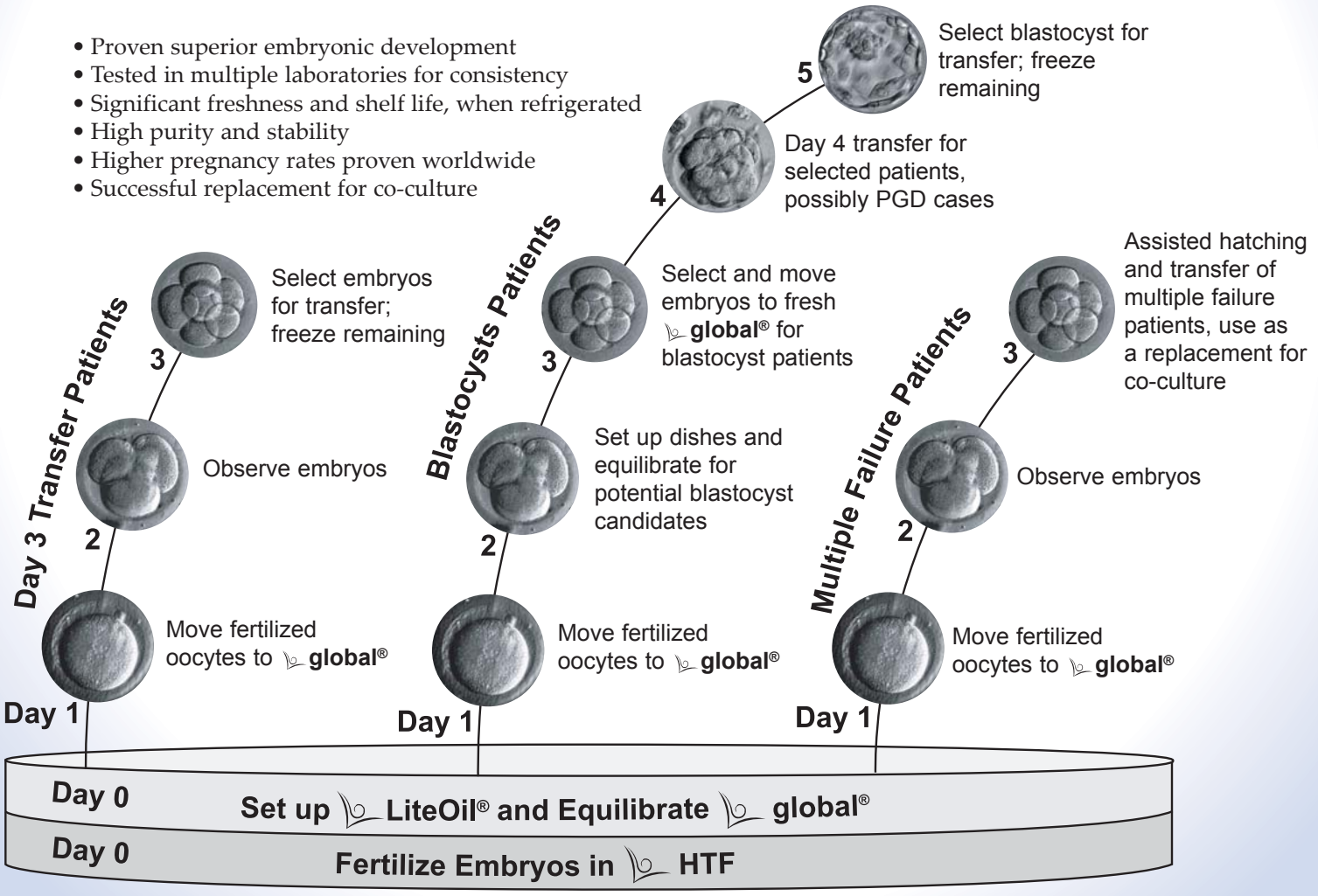
Quality is not just a statement, it is our ongoing focus and dedication. Our quality assurance program meets and exceeds all the international standards for medical devices and pharmaceuticals. High quality ingredients, water, manufacturing process, quality monitoring system. Lot-to-lot consistent results (pH, osmolality, LAL, MEA) proven by customers on comparative studies to assure compatibility and prevent osmolality or pH shock to gametes and embryos.

- Manufactured Every 2 Weeks
- All ingredients tested and qualified for human embryo use
- High Tech H20 Purification & Monitoring System meets USP standards
- cGMP Manufacturing Facility
- Clean room preparation
- Fresh overnight delivery
- FDA Compliant
- Increased results in clinical trials
- Advanced Formulations
- Extensive Research and Development
- Developed to meet nutrition requirements of gametes and embryos
- Sterile by membrane filtration
- Sperm survival tested (for sperm media products)
- Sterility tested (for each lot)
- 1-cell MEA tested >80% blastocyst* (for each lot)
- Endotoxin LAL tested by two different outside certified companies (for each lot)
- Physiochemical tested for each lot (pH & osmolality)
- Tested for shelf life stability

* Starting November 2005 we are moving from a 2-cell MEA to a 1-cell MEA as a more sensitive test.

Performance and Results

- Proven superior embryonic development
- Tested in multiple laboratories for consistency
- Significant freshness and shelf life, when refrigerated
- High purity and stability
- Higher pregnancy rates proven worldwide
- Successful replacement for co-culture



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