Original Articles

A comparison of in vitro fertilization outcome by culture media used for developing cleavage-stage embryos

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Summary

Purpose: To determine whether removing glucose and phosphate from media used for developing cleavage-stage embryos improves outcome following transfer of fresh or frozen embryos. Furthermore the study would evaluate the efficacy of adding non-essential amino acids and glutamate to media.

Methods: Embryo development was rotated on a weekly basis in human tubal fluid (HTF), versus two media relatively devoid of glucose and phosphate (e.g., P1), with one having the addition of essential amino acids and glutamate (Quinn's Advantage Medium).

Results: For fresh cycles, the implantation rate was significantly higher for Quinn's. There was less fragmentation with P1 and Quinn's. For frozen cycles, the viable pregnancy, implantation rates and embryo quality were higher for Quinn's and P1 than HTF.

Conclusion: Removal of glucose and phosphate for day-2 embryos improves in vitro fertilization outcome after embryo transfer. It is not clear if adding certain non-essential amino acids and glutamate provides further improvement.

Key words: Culture media; Glucose free; Phosphate; Fresh embryo transfer; Frozen transfer.

Introduction

Tervit *et al.* formulated a culture medium similar in biochemical composition to sheep oviduct fluid [1]. The medium supports the development of a high proportion of cattle [1], sheep and goat [2] embryos to the blastocyst stage. Based on the analysis of composition of human tubal fluid (HTF) [3, 4], a medium was developed to improve the pregnancy rates (PRs) with human in vitro fertilization (IVF) [5]. This was named HTF after human tubal fluid.

For years HTF has been one of the most commonly used media for IVF and was the one exclusively used by the Cooper Center for IVF for over ten years. Human tubal fluid can be considered a simple medium which is a balanced salt solution with added carbohydrate energy sources, e.g., pyruvate, lactate, and glucose [5]. At the time that HTF was developed the majority of embryo transfers (ETs) occurred at the 2-day stage.

The cleavage stage embryo of several mammals does not appear to utilize glucose as an energy source to any great extent [6, 7]. In fact, some data suggest that glucose may retard the development of the cleavage-stage embryo in several mammalian species [8-11]. These studies support the concept that premature utilization of glucose by cleavage stage embryos is associated with an impairment of oxidative capacity, resulting in reduced embryo development [11]. This led to the development of a medium that is relatively glucose free [12].

The glucose-phosphate interaction is also important. Decreased oxygen consumption is found in embryos grown in media containing glucose and phosphate together. The theory is that glucose/phosphate enhances the glycolytic pathway consuming all the common intermediates, thus rendering the mitochondrial oxidation pathway non-functional. Therefore some media also have phosphate removed [12].

In contrast to the embryo, sperm depends on glucose as an energy source. Thus if a glucose-free medium is used it cannot be used until fertilization has taken place. The fertilization medium must have glucose [11].

Amino acids are important regulators of mammalian preimplantation development. The addition of specific amino acids in embryo culture media has been shown to alleviate the "culture blocks" to mammalian embryo development [13-16]. Though media lacking amino acids allowed some embryos to reach the blastocyst stage, it was not optimal without the addition of amino acids. Some studies have suggested that the addition of amino acids improved the efficiency for development of the zygote past the 2- and 4-cell block in culture [13, 17].

Some studies have postulated that the amino acids alanine, asparagine, aspartate, glutamate, glycine, proline, and serine, which are not essential for growth of somatic cells in culture, stimulated the development of F1 mouse zygotes to the blastocyst stage within 72 hours of culture, the time at which the mouse blastocyst is found in vivo [13]. Interestingly the group of essential amino acids for somatic cell growth found in Eagle's medium (arginine, cystine, histidine, isoleucine, lysine, methionine, phenylalanine, threonine, tryptophan, tryosine and valine) had negative effects on embryo growth [13, 17-19] (interestingly they may be stimulators for later

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embryos). The benefits of non-essential amino acids and glutamine prior to compaction may be related to their effect on regulating energy metabolism, osmolytes, and buffering of Ph [11].

A potential negative effect of amino acids is that they may be metabolized by the embryo or may undergo spontaneous breakdown to toxic ammonium [13, 18, 20]. After 48 hours of culture the addition of the enzyme glutamate dehydrogenase, the substrate 2 ketoglutarate, and the co-factor NADH can be added to culture medium to convert the ammonium present to glutamate [21].

Another factor added to some media are chelators. The most commonly used one is EDTA which was demonstrated to overcome the 2-cell block in mouse embryos [22]. EDTA is present in several embryo media [12, 14, 23-25]. EDTA may increase embryo development by the chelation of toxic heavy metal ions that are present in the media and may inhibit premature utilization of glycolysis by cleavage stage embryos [20, 26].

With the advent of improved media, e.g., HTF which allowed a high percentage of embryos to reach day 3 in culture, many IVF centers switched from day-2 to day-3 transfers. This change would theoretically allow better embryo selection by eliminating those that might have 2-cell block and possibly by placing into the uterus an embryo that is at least one day closer to the blastocyst stage that normally enters the uterus in vivo. Though media, e.g., HTF, have provided adequate pregnancy rates, it is possible that even for day-3 transfers, using glucose/phosphate-free medium, e.g., P1, or using one that is relatively glucose free but has added the non-essential amino acids, glutamate, and EDTA, e.g., Quinn's Advantage Medium, may theoretically improve IVF outcome to a greater extent. The study presented here prospectively compared outcome of fresh and frozen ET using embryos developed in HTF, P1, or Quinn's Advantage Medium.

Materials and Methods

Each of the three trial media (HTF, P1, Quinn's) were used exclusively in the laboratory for one week, then rotated each successive Monday to another one of the three media. The alterations made to the ingredients of HTF (fertilization medium) vs Quinn's Advantage (cleavage and fertilization medium) vs P1 (cleavage medium) are seen in Table 1. The rotations lasted for 27 weeks. Embryos were cultured in organ culture dishes (Falcon 3037, Thomas Scientific) with 1 ml of medium in the well covered by 1 ml of washed mineral oil (Squibb, Princeton, NJ). The moat contained 2 ml of medium for rinsing and humidity.

Table 1. — *Comparison of ingredients of the three mediun used.*

Ingredient	Quinn's fertilization	Quinn's cleavage	HTF	P1
Glucose	2.8 mMol	0.1 mMol	2.78 mMol	0
EDTA	0.01 mMol	0.01 mMol	0	0
Magnesium	2.0 mMol	2.0 mMol	0.2 mMol	0.2
Taurine	0.1 mMol	0.1 mMol	0	0
Citric acid	0.0005 mMol	0.0005 mMol	0	0
Alanyl-glutamine	1.0 mMol	1.0 mMol	0	0
Essential amino acids	various	various	0	0
Phosphate	0	0	0.37 mMol	0

Following oocyte retrieval, oocytes were either conventionally inseminated with 25,000 motile sperm per oocyte, or had intracytoplasmic sperm injection (ICSI) performed, depending on the partner's sperm parameters. HTF was used for insemination stage for P1 since HTF contains higher glucose (Quinn's has its own insemination medium, called fertilization medium, for the insemination stage containing higher glucose concentration than the cleavage medium). Oocytes were assessed for fertilization at 16-21 hours post-insemination and moved to fresh culture dishes. Embryos selected for transfer all had assisted hatching performed the morning of the transfer, using zona drilling with acidified Tyrode's medium. Transcervical embryo transfer was done on day 3 of culture, using a Wallace catheter (Marlow Surgical) and ultrasound guidance.

All embryo stimulation cycles and transfer cycles performed during the study period were included, irrespective of etiology for IVF, age, and type of controlled ovarian hyperstimulation used in the retrieval cycle. If cryopreserved embryos needed to be thawed for frozen embryo transfers (ETs), they were cultured in the same type of medium as when they were frozen for consistency in data collection.

Outcome measures included clinical pregnancy rates (PRs), viable PRs, and implantation rates per ET. Rates were compared by medium type using chi-square analysis. A p value of $\leq .05$ was considered significant.

Results

The average age of the patients in each of the three culture media groups did not differ significantly (37.2 ± 4.88 years for HTF, 37.4 ± 5.77 years for P1 and 37.8 ± 5.34 years for Quinn's). Fertilization rates were also similar in the three groups (63.3% ± 33.5%, 67.0% ± 23.8%, and 65.7% ± 30.0%, respectively).

A comparison of outcome following IVF with fresh ETs is seen in Table 2. Though there were no significant differences in clinical or ongoing/delivered PRs between the three media (Table 2), there was a trend for lower PRs with HTF since the clinical PRs were approximately 40% higher with P1 and Quinn's Advantage than with HTF and the ongoing/delivered PRs were about 50% higher (Table 2). However, the implantation rates were significantly higher with Quinn's Advantage Medium compared to HTF (Table 2). The clinical ongoing/delivered PRs were very

Table 2. — *Comparison of outcome of fresh embryo transfer according to medium used for embryo culture.*

	HTF	P1	Quinn's advantage	p value
Number of transfers	68	113	100	
Average age (years)	37.2	37.4	37.8	
% fertilization	63.3	67.0	65.7	
Clinical PRs/transfer	32.4% (22)	44.2% (50)	43.0% (43)	.251
Multiple PRs/transfer	40.9% (9)	38.0% (19)	58.1% (25)	.131
Ongoing/delivered PRs/transfer	26.5% (18)	38.1% (43)	39.0% (39)	.195
Implantation rate/transfer	15.9% (32/201)	19.9% (70/352)	24.7% (324)	.048*

*Further comparison showed that the implantation rates were the same for HTF and P1 but higher for Quinn's Advantage.

similar with P1 vs Quinn's Advantage but the implantation rate was 20% higher with Quinn's Advantage (p = NS).

A comparison of outcome following frozen ETs found significantly higher ongoing/deliverd PRs and implantation rates with P1 and Quinn's Advantage when compared to HTF (Table 3). The differences could not be explained by age (average age at retrieval was 36.1 (HTF), 35.8 (P1), and 34.9 (Quinn's) while average age at transfer was 37.6, 36.0 and 35.9 years, respectively).

Embryo quality in fresh ETs was assessed by comparing average number of blastomeres per embryo and fragmentation grade (Table 4). Although the average number of blastomeres per embryos were similar for all three media, use of the Quinn and P1 media resulted in embryos with less fragmentation. The percent of embryos with ≤ 25% fragmentation (Grade A and B) was 67.5% for HTF, 82% for P1 and 81% for Quinn's ($p < .05$).

An increase in the number of Grade A and B embryos (0-25% fragmentation) was also observed in frozen ETs when P1 and Quinn's was used. While only 68.8% of the embryos were of Grade A and B when HTF was used, 81.9% of embryos cultured in P1 and 80.2% of embryos cultured in Quinn's were Grades A and B ($p < .05$). No differences in the average number of blastomeres per embryo were observed by culture media.

Table 3. — *Comparison of outcome of frozen embryo transfer according to medium used for embryo culture.*

	HTF	P1	Quinn's Advantage	p value
Number of transfers	190	26	31	
Average age at retrieval	36.1	35.8	34.9	
Average age at transfer	37.6	36.0	35.9	
Clinical PRs/ transfer	28.4% (54)	50.0% (13)	45.2% (14)	.026*
Multiple PRs/ transfer	31.5% (17)	30.8% (4)	64.3% (9)	.067
Ongoing/delivered/ transfer	26.3% (50)	46.2% (12)	45.2% (14)	.032§
Implantation rates/transfer	12.2% (74/609)	20.5% (17/83)	25.7% (80/324)	.001§

*Further comparisons showed that P1 and Quinn's advantage were the same, P1 higher than HTF, HTF and Quinn's Advantage the same.
§Further comparisons showed that P1 and Quinn's advantage were the same, both were higher than HTF.

Table 4. — *Evaluation of embryo morphology according to medium used.*

	HTF	P1	Quinn's
Fresh embryo transfers			
Mean blastomere #	6.4 ± 1.7	6.4 ± 1.5	6.8 ± 1.5
% A (0%)	2.0	2.3	9.9
% B (1-25%)	65.5	79.7	71.1
% C (25-50%)	21.5	14.0	15.1
% D (> 50%)	11.0	4.0	3.9
Frozen embryo transfers			
Mean blastomere #	5.9 ± 1.6	5.9 ± 1.6	6.4 ± 1.9
% A (0%)	3.5	7.2	5.2
% B (1 ≤ 25%)	65.3	74.7	75.0
% C (25-49%)	26.8	18.1	17.7
% D (≥ 50%)	4.4	0.0	2.1

*$p < .05$ comparing percentage of A and B embryos to those of Grade C and D by culture medium.

Discussion

The quest to reduce the risk of multiple births has led to selecting only the best embryos to reduce the number of embryos transferred while not reducing the PRs. One of these methods involves developing the embryo to blastocyst before transfer [26, 27]. There is still debate as to whether transferring eight cell embryos on day 3 results in equal PRs to blastocyst transfer [28, 29] and some debate as to whether some embryos could have resulted in pregnancies if they had been transferred on day 3 but never made it to blastocyst stage [30, 31].

Suffice to say there are still many IVF centers in the world transferring embryos on day 3 but not at blastocyst stage. Attaining the blastocyst stage, however, requires the development of different medium that will adapt to the specific needs of the growing embryos. This study was designed to determine if some of these improvements in media to allow blastocyst transfer might be applied to improve IVF outcome following transfer of day-3 cleavage-stage embryos.

The evaluation of clinical PRs did not have sufficient power to show any significant improvement despite removal of glucose and phosphate (P1 medium) after fertilization of the embryo nor any benefit to the addition of non-essential amino acids or chelators (Quinn's Advantage). However there was a trend for lower clinical PRs with HTF. Similar conclusions were made for ongoing/delivered PRs. Significantly higher implantation rates, though, were found when embryos were grown in Quinn's Advantage Medium.

For frozen ETs, there was a significantly higher clinical PR for P1 compared to HTF and a trend for Quinn's Advantage to be superior to HTF. Both P1 and Quinn's Advantage Medium showed significantly higher ongoing/delivered PRs when compared to HTF as well as implantation rates. This suggests possibly that the new medium allow more embryos to be better quality thus improving the lot of supernumerary embryos that are cryopreserved. Alternatively, these new medium may allow embryos to better withstand the rigors of freezing and thawing. The reason for more frozen ETs using HTF is explained by the decision to use the same media to continue culture of the frozen-thawed embryos that was used during the IVF cycle. All frozen ETs during this 27-week period were evaluated and thus included those frozen in the medium used exclusively before this study, i.e., HTF. The pregnancy rates with frozen ETs as reported to SART have not changed over the last five years.

Thus these data strongly suggest that consideration be given to removing glucose and phosphate from media after fertilization to improve IVF outcome when transferring 3-day-old embryos. It is not clear whether the addition of non-essential amino acids, glutamate, and chelators, e.g., EDTA provides any improvement over merely removing glucose and phosphate. Though not significant, there was a trend for higher implantation rates with Quinn's Advantage Medium over P1 in both fresh (24.7% vs 19.9%) and frozen ETs (25.7% vs 20.5%). This trend

warrants at least a larger trial comparing medium with and without non-essential amino acids and chelators on PRs following transfer of day-3 cleaved embryos. It should be noted that a study designed as a sibling oocyte split in which oocytes were randomly assigned to HTF vs a new trial medium (similar to Quinn's Advantage) found a higher fertilization rate with the new trial medium (which we did not find), a higher cleavage rate, lower rate of uncontrolled division, higher number of blastomeres per embryo, a higher average embryo morphology score, and a higher embryo utilization rate [32].

The results found in the present study were based on the methodology of our culture system since we used 1 ml of medium in which to grow the embryos. Microdrop systems, in contrast, use only 5-10 microliters. Ammonia levels might not become toxic in our system because of the higher volume but could be toxic in smaller microdrops.

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