Int. J. Dev. Biol. 42: 885-893 (1998)

Culture of human preimplantation embryos to the blastocyst stage: a comparison of 3 media

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ABSTRACT Following culture for 2 days in Earle's balanced salt solution (EBSS), human embryos which remained after transfer were cultured in one of 3 media for 4 days, from the 2- to 4-cell stage to the blastocyst stage. Sibling embryos were divided equally between treatments. Throughout the 4 day culture period, embryos were assessed for morphology and development, as well as uptake and production of energy substrates. Cell numbers in the inner cell mass and trophectoderm were determined for embryos which reached the blastocyst stage. No significant effect was observed in the extent or rate of development to the 8-cell, morula or blastocyst stage between treatments. Uptake of pyruvate was related to concentration in the medium and no differences in glucose uptake were observed between media. Endogenous energy metabolism, as measured by lactate production, was significantly higher in Ham's F12 than in EBSS from day 3.5 onwards. Blastocyst cell numbers were also increased; 79.6±7.7 in Ham's F12 (n=17) and 57.8±5.2 in EBSS (n=19), $p<0.05$. Of the embryos which reached the blastocyst stage by day 5, 36% (14/25) had degenerated by day 6 in EBSS compared to only 19% (5/27) in Ham's F12 ($p=0.06$). Slightly higher rates of embryo survival between day 5 and 6 in Ham's F12 may account for the observed increase in blastocyst cell number. The results do not suggest that improved embryo development can be obtained using human tubal fluid or Ham's F12, in preference to EBSS during early cleavage stages, but the use of Ham's F12 may improve embryo survival at later stages of development.

KEY WORDS: *IVF, human, preimplantation embryo, culture medium, blastocyst*

Introduction

The requirements for successful human preimplantation embryo development are poorly understood. Although it has been possible for some time to culture human embryos from the 1-cell to the blastocyst stage (Steptoe *et al.,* 1971), the number of embryos that reach this stage has remained low. Sub-optimal culture conditions are thought to contribute to these poor rates of development and consequently, following *in vitro* fertilization (IVF), embryos are transferred to the uterus as soon as fertilization and early cleavage have been observed. Transferring embryos at early stages however, allows little time to assess viability, and may result in the transfer of non-viable embryos. Further, the human embryo *in vivo* would not be expected to enter the uterus until day 3 or 4 after fertilization (Croxatto *et al.,* 1972) and transfer of IVF generated embryos to the uterus at early stages may compromise their survival. It is desirable therefore, to transfer embryos at late preimplantation stages, when continued development has been observed and the embryo would naturally expect to be in the uterus. If later transfers are to be successful however, it is necessary to determine the optimal conditions for culture through preimplantation stages.

Abbreviations used in this paper: IVF, *in vitro* fertilization; EBSS, Earle's balanced salt solution; HTF, human tubal fluid; MEM, minimal essential medium; FD, follicular diameter; hMG, human menopausal gonadotrophin; hCG, human chorionic gonadotrophin; E2, oestradiol; LDH, Lactate dehydrogenase; NAD(H), nicotinamide adenine dinucleotide (reduced form); NADP(H), nicotinamide adenine dinucleotide phosphate (reduced form); ATP, adenosine triphosphate; EDTA, ethylenediaminetetraacetic acid; HEPES, N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonoc acid]; EHM, Earle's handling medium; DNP, dinitrophenol; BSA, bovine serum albumin; TE, trophectoderm; ICM, inner cell mass; UV, ultra violet.

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0214-6282/98/$10.00

Printed in Spain

Balanced salt solutions such as Earle's (EBSS), Human Tubal Fluid (HTF; Quinn *et al.,* 1985) and T6 (Whittingham, 1971) are widely used for embryo culture, as they are inexpensive and relatively easy to prepare. Although they have been found to support embryo development from the 1-cell to the blastocyst stage, they are by design, nutritionally incomplete. It seems unlikely therefore, that balanced salt solutions could give optimal rates of embryo survival, but unless a significant improvement in the rate of embryo development can be demonstrated using complex media, simple media are likely to remain popular for embryo culture.

Commercial media, specifically for embryo culture have become available in recent years, but their formulations are mainly based on balanced salt solutions rather than any known requirements for embryo culture. They overcome the need to spend time on media preparation but in doing this, they take away the strict control which can be maintained over the culture conditions. The same problem occurs with the more complete media, as they are too complex to be made efficiently in the laboratory. These media are specifically designed for tissue culture and until recently they were thought unnecessary for embryo culture (Edwards *et al.,* 1981). The problem with the complex media may be that they contain more ingredients than are necessary to support optimal rates of development, in contrast to balanced salt solutions which probably contain too few. The balance may be somewhere between the two.

One of the first media to be specifically designed for human embryo culture was HTF (Quinn *et al.,* 1985), based on the ionic composition of human fallopian tubal fluid. Until then, T6, a medium developed for mouse embryo culture (Whittingham, 1971), was widely used for human embryos *in vitro*. It was suggested that HTF was more suitable for early embryo culture as it improved the pregnancy rate after IVF when compared to the more traditional T6 (Quinn *et al.,* 1985). Others however, were quick to question this claim, suggesting that no difference in pregnancy rate could be obtained by using HTF in preference to T6 (Cummins *et al.,* 1986). Despite these results, HTF was accepted as a reliable medium for embryo culture, perhaps because it gave similar results to the more traditional media and could be prepared simply and reliably in the laboratory. Further, as HTF had been specifically devised for human embryo culture, it may have been preferred over media devised for other purposes.

Other attempts to improve the culture media used for human IVF, and therefore improve rates of embryo survival, have met with moderate success. Various studies (Lopata and Hay, 1989a,b; Younglai *et al.,* 1992; Olar and Potts, 1993) have shown that embryo development is improved with the use of more complete media such as Ham's F10 or minimal essential medium alpha (α-MEM), when compared to EBSS or HTF. Further evidence that complex media may favor embryo development was provided in the study by Muggleton-Harris *et al.* (1990), who reported high rates of development in Ham's F12 compared to 4 other media. More recent studies (Barnes *et al.,* 1995; Gardner and Lane, 1997) have led to the development of new, less complex and serum free media which have been shown to support good rates of blastocyst development and implantation following transfer (Gardner *et al.,* 1998).

With the exception of the work of Gardner *et al.* (1998), the studies mentioned here have failed to influence traditional methods of embryo culture for a number of reasons. First, the small number of embryos used in some studies are not sufficient to overcome variations in rates of development which may occur naturally or between patients. Second, studies based on embryos which are not suitable for freezing may not be applicable to normally developing embryos. Finally, experiments carried out over a limited time period, cannot fully evaluate the effects of culture media on development. It is possible that the effects of the medium would not be expressed for some time after the initiation of culture. Furthermore, since the embryo experiences a change of environment *in vivo*, when it passes from the fallopian tube into the uterus, different media may be required at different stages *in vitro*. Further evidence that a succession of media may be required comes from the work of Hardy *et al.* (1989b) which showed that the substrate requirements of human embryos change throughout preimplantation stages.

The present study compares embryo development, from the 2- to 4-cell stage on day 2 post insemination to the blastocyst stage on day 6, in a simple balanced salt solution (EBSS), to development in HTF and a more complete medium, Ham's F12. As well as morphology and development, the work examines the effects of culture medium on blastocyst cell number, and uptake and production of energy substrates to determine patterns of usage in the various media.

Results

Embryo development and morphology

In the initial experiment, forty normally fertilized embryos were cultured in each of the three media tested (Table 1). Although the number of embryos reaching the blastocyst stage was similar

TABLE 1

EMBRYO DEVELOPMENT IN VARIOUS MEDIA

Medium	Osmolarity (mOsmol/Kg)	No. of Embryos (n=)	No. reaching the 8-cell stage (%)	No. reaching the morula stage (%)	No. reaching the blastocyst stage (%)
EBSS	284±2	40	35 (88)	32 (80)	23 (58)
HTF	284±2	40	34 (85)	26 (65)	19 (48)
Ham's F12	300	40	33 (83)	27 (68)	19 (48)

Differences between treatments are not significant at the level of $p<0.05$.

between treatments, many of the embryos cultured in Ham's F12 appeared morphologically abnormal with no distinct structure, and had an appearance similar to that of osmotically shocked embryos. Typically, in EBSS and HTF, the arresting embryos became fragmented and dark in appearance, and further cell division was not observed. This type of arrest also occurred in Ham's F12, but in a proportion of the embryos, morphological assessment of the embryos was very difficult (Fig. 1). Some of these abnormal embryos appeared to cavitate (Fig. 1), but allocation of cells to the TE or ICM was not apparent. Indeed, it was generally impossible to determine how many cells such embryos contained, as cell membranes could not be seen.

In a second experiment, the osmolarity of the Ham's F12 was adjusted from 300±2 to 284±2 mOsmol/Kg to determine whether rates of development to the blastocyst stage could be improved. EBSS and HTF are routinely used at an osmolarity of 284±2 mOsmol/Kg and under these conditions give reasonable rates of development. Forty three embryos were cultured in each of the media and the results were similar to those observed without adjusting the osmolarity of the Ham's F12 (Table 2). The unusual morphology that had previously been observed with the Ham's F12 continued to occur, but not as frequently. The number of embryos reaching the 8-cell, morula and blastocyst stages, were slightly, but not significantly different than observed in the early part of the study, and were again similar between treatments. The proportion of embryos developing to the blastocyst stage was not significantly increased as a result of lowering the osmolarity of the Ham's F12 (Table 2).

Since the proportion of embryos reaching each developmental stage was similar in experiments (data compared using Fisher's Exact Test), the figures were amalgamated to determine the overall effects of these media on development. The majority of embryos that reached the blastocyst stage did so by day 5, and no differences were observed between treatments (Table 3). In addition, a small number of embryos reached the blastocyst stage by day 6 in each of the three media. The number of day 5 blastocysts that had arrested by day 6 varied between media, with the highest number of arrests occurring in EBSS (14/39, 36%). Only 8/33 (24%) of embryos that had reached the blastocyst stage by day 5 in HTF arrested by day 6, and even fewer (5/32, 16%) arrested in Ham's F12. Although more than twice as many embryos arrested in EBSS when compared to Ham's F12, this difference did not quite reach significance (p=0.06).

Energy substrate utilization

Where possible, pyruvate and glucose uptake and lactate production were measured for each individual embryo in the study. In HTF medium it was impossible to measure differences in lactate concentration between incubation and control drops due to the high background level of sodium lactate (21.7 mM). Similarly, glucose uptakes could not be measured in Ham's F12 as this medium contained 10 mM glucose. The concentration of substrates in the media are shown in Table 4.

The uptake and production of substrates in the various media are shown in Figures 2 and 3. The number of observations is lower in Figure 2 than in Figure 3, as there were fewer embryos in this part of the study and some problems were encountered with the mains power supply at that time, which affected the assay procedure and caused some data to be lost. The observations in Figure 2 are

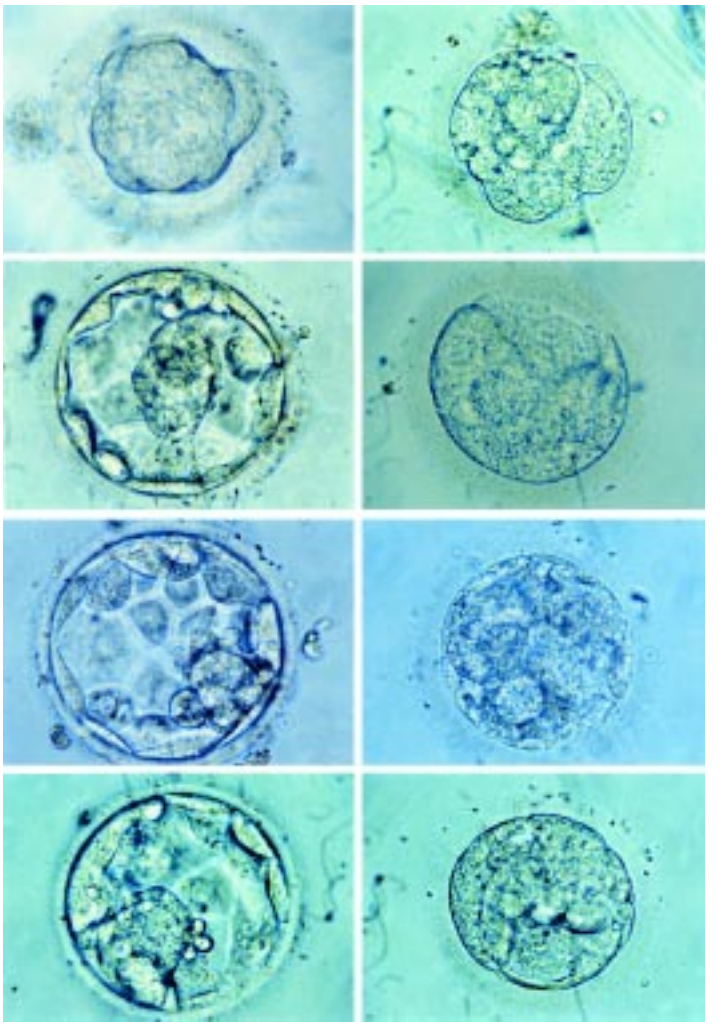

Fig. 1. Embryo morphology on day 5 (upper panel) and day 6 (remaining 3 panels) in EBSS (left panels) and Ham's F12 (right panels).

however similar to those in Figure 3, although they did not reach significance. Also, the uptake of pyruvate and production of lactate was more variable in Ham's F12 (Fig. 2) when the osmolarity was 300 mOsmol/Kg than when it was adjusted to 284 mOsmol/Kg (Fig. 3).

With the osmolarity of all 3 media at 284 mOsmol/Kg, the pattern of pyruvate uptake reflected, to a large extent, its concentration in the medium (Fig. 3). The lowest uptake, in HTF, reflected the lowest concentration of pyruvate in the media (0.33 mM). Uptake of pyruvate in EBSS was significantly higher than in HTF, reflecting a slightly higher concentration in this medium (0.47 mM). Finally, although uptake of pyruvate was significantly higher in Ham's F12 than in the other two media, it was not proportionally higher, considering that the pyruvate content of Ham's F12 (1.0 mM) is 2 and 3 times higher than that of EBSS and HTF respectively.

Glucose uptake was measured for embryos cultured in EBSS and HTF only. During the first culture period (day 2-3), significantly more glucose was taken up by embryos in HTF than by those in EBSS (Fig. 3). Thereafter, no differences in uptake were observed between treatments, despite the fact that HTF contains 2.5 times more glucose than EBSS (Figs. 2 and 3).

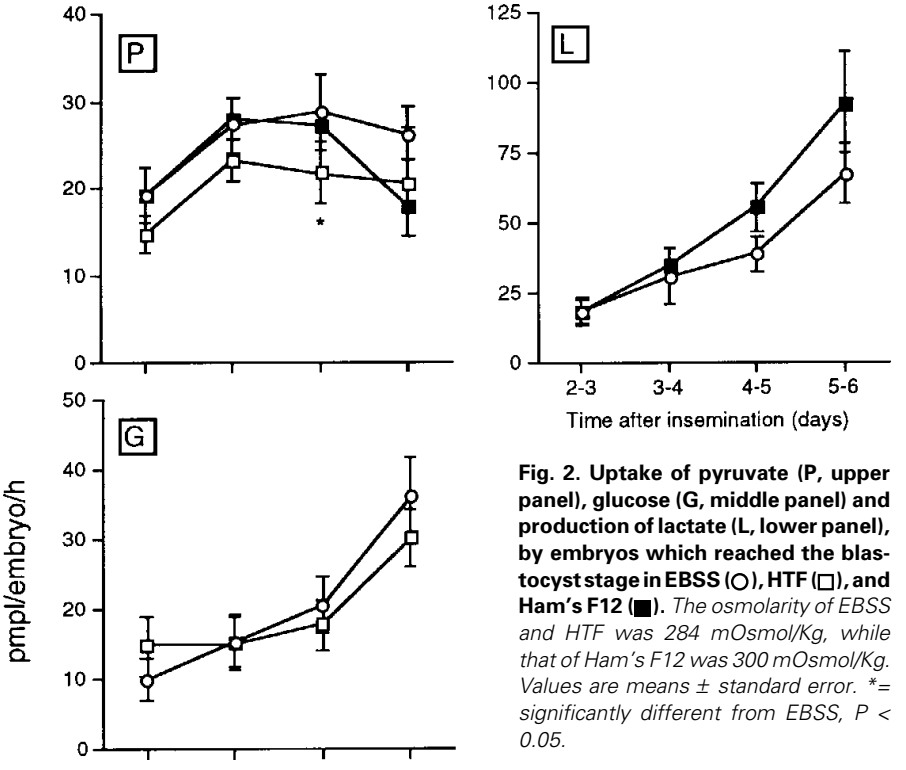

Fig. 2. Uptake of pyruvate (P, upper panel), glucose (G, middle panel) and production of lactate (L, lower panel), by embryos which reached the blastocyst stage in EBSS (○), HTF (□), and Ham's F12 (■). *The osmolarity of EBSS and HTF was 284 mOsmol/Kg, while that of Ham's F12 was 300 mOsmol/Kg. Values are means ± standard error. *= significantly different from EBSS, P < 0.05.*

With the exception of the first culture period, lactate production was significantly increased in Ham's F12, when compared to EBSS (Fig. 3). Lactate production increased in both media over time in culture, as did the differences in levels of production observed between media.

Blastocyst cell numbers

Differential labeling of 66 out of 140 blastocysts was attempted; 24/51 in EBSS, 27/46 in HTF and 19/43 in Ham's F12. The labeling worked well in 19 of the EBSS blastocysts, 25 of the HTF blastocysts and 17 of the Ham's F12 blastocysts. In the remainder, the labeling was unevenly distributed and hence only the total cell count was obtained. Failure of labeling was probably due to degeneration of the blastocyst, with subsequent disruption of the trophectoderm seal, or adhesion of cellular fragments/debris to the outer surface of the trophectoderm, preventing even binding of the antibody and hence, leading to uneven cell lysis. The total number of cells in the Ham's F12 blastocysts (80±8) was significantly higher (p<0.05) than in the EBSS blastocysts (58±5) and slightly higher than those in HTF (69±6) (Fig. 4). The cell number of the HTF blastocysts was numerically higher, but not significantly different than the EBSS blastocysts. Specifically, the distinction in cell number between media was in the inner cell mass: 19±2 in EBSS, 26±3 in HTF and 26±3 in Ham's F12 (significantly higher than EBSS, p<0.05). Trophectoderm cell numbers were similar between treatments (Fig. 4).

Discussion

Early attempts to maintain human preimplantation embryos *in vitro* involved culture in balanced salt solutions such as Earle's, or more complete media such as Ham's F10. The requirements for culture from the fertilization and early cleavage stages to the blastocyst stage were unknown, and the first successful formation of human blastocysts *in vitro* was as a result of using a succession of media, rather than one single medium for embryo culture (Steptoe *et al.,* 1971). Attempts to define which medium best supported embryo development were limited by a shortage of human embryos for research, and by the poor rates of development observed. Later, it was proposed that blastocyst rates were as good in simple balanced salt solutions, such as Earle's, as in more complete media like Ham's F12 (Edwards *et al.,* 1981). Earle's medium is still widely used today, suggesting that little or no advance in embryo culture techniques has come about in recent years.

Studies that have proposed the use of alternative media for embryo culture have met with little success. There is a general reluctance in infertility clinics to change established methods for embryo culture unless a significant increase in embryo viability can be expected, and until recently this has not happened. Reported increases in rates of development to the blastocyst stage (Muggleton-Harris *et al.,* 1990; Olar and Potts, 1993) and improvements in embryo health (Younglai *et al.,* 1992) remain unsubstantiated. In these studies, the small numbers of embryos used, as well as the marginal improvements observed and the low rate to blastocyst in control media have meant that traditional embryo culture media remain in use. However, the work of Gardner *et al.* (1998) suggests that with the continued development of new media higher implantation rates and lower rates of multiple pregnancies can be achieved following IVF.

TABLE 2

EMBRYO DEVELOPMENT IN VARIOUS MEDIA, WITH THE OSMOLARITY OF HAM'S F12 ADJUSTED TO 284 MOSMOL/KG

Medium	Osmolarity (mOsmol/Kg)	No. of Embryos (n=)	No. reaching the 8-cell stage (%)	No. reaching the morula stage (%)	No. reaching the blastocyst stage (%)
EBSS	284±2	43	39 (91)	33 (77)	28 (65)
HTF	284±2	43	37 (86)	36 (84)	27 (63)
Ham's F12	284±2	43	39 (91)	35 (81)	24 (56)

Differences between treatments are not significant at the level of p<0.05.

HTF was one of the first medium specifically designed for human embryo culture (Quinn *et al.,* 1985). Until recently, its use had not been fully evaluated, particularly for culturing those embryos which remained after transfer. An early report concluded that HTF gave identical fertilization and implantation rates to T6 (Cummins *et al.,* 1986), but later, only 34 (14.5%) out of 234 inferior quality embryos reached the blastocyst stage in HTF compared to 104 out of 392 (26.5%) in α-MEM (Lopata and Hay, 1989a,b). In the work described here, no significant differences were observed in the number of embryos developing to the 8-cell, morula and blastocyst stages between media. This finding is not in agreement with the study of Muggleton-Harris *et al.* (1990), who demonstrated that higher numbers of blastocysts could be obtained when using Ham's F12 in preference to other media. The data presented by of Muggleton-Harris *et al.* (1990), are difficult to evaluate however, mainly due to the small number of embryos used. The high levels of development in Ham's F12 could be due to random or biological variation, and there was not a sufficient number of embryos (range 3-5) cultured in the other media to make any valid comparisons. Further, the embryos used in the study were not siblings divided between treatments, and the authors failed to state exactly how many blastocysts were obtained in Ham's F12.

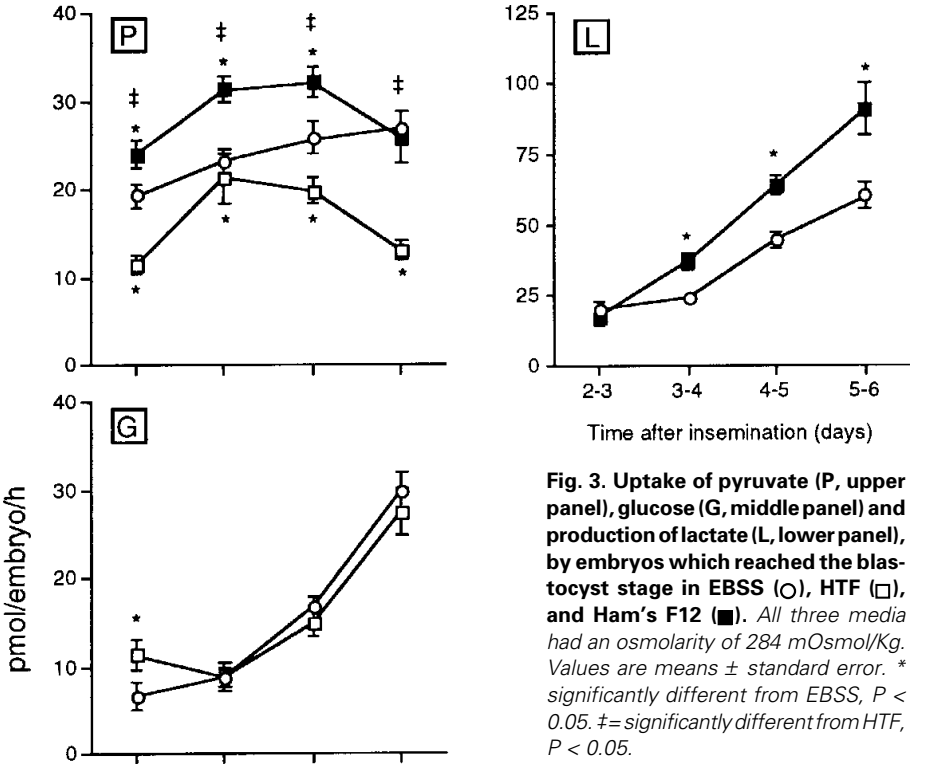

Fig. 3. Uptake of pyruvate (P, upper panel), glucose (G, middle panel) and production of lactate (L, lower panel), by embryos which reached the blastocyst stage in EBSS (○), HTF (□), and Ham's F12 (■). *All three media had an osmolarity of 284 mOsmol/Kg. Values are means ± standard error. * significantly different from EBSS, P < 0.05. ‡= significantly different from HTF, P < 0.05.*

In the present study (Tables 1 and 2), the embryos that had been cultured in Ham's F12 contained slightly more cells (79.7±5.2) than the embryos in HTF (69.26±6.1) and significantly ($p<0.05$) more cells than those in EBSS (57.7±5.2) (Fig. 4). Specifically, the overall increase in cell number reflected an increase in ICM cells where numbers were 26±2.7 in Ham's F12 compared to 19±1.8 in EBSS ($p<0.05$). The most likely cause for this increase in ICM cell number was the improved rate of blastocyst survival between days 5 and 6 (Table 3). The number of embryos reaching the blastocyst stage was similar between media on day 5 and day 6, but more than twice as many day 5 blastocysts had arrested by day 6 in EBSS when compared to Ham's F12 (Table 3). Thus, when the cell numbers of the blastocysts were determined on day 6, the blastocysts in Ham's F12 appeared to have contained a higher proportion of embryos that had reached the blastocyst stage by day 5, and continued to develop until day 6, as compared to either HTF or EBSS. Blastocysts in Ham's F12 are likely to have had a higher average cell number than the blastocysts in the other media, in which more of the day 5 blastocysts had arrested by day 6. Our observation that embryos at the blastocyst stage survive better in the more complex medium is consistent with the results of Olar and Potts, (1993), who concluded that blastocysts obtained in a complex medium (α-MEM) were healthier than those obtained in HTF.

TABLE 3

EMBRYO SURVIVAL BETWEEN DAYS 5 AND 6

Medium	Total no. of embryos (n=)	No. reaching the blastocyst stage by day 5 (%)	No. of day 5 blastocysts arresting between day 5 and day 6 (%)
EBSS	83	39 (47)	14 (36)
HTF	83	33 (40)	8 (24)
Ham's F12	83	32 (39)	5 (16)

Differences between treatments are not significant at the level of $p<0.05$.

A possible explanation for the difference in cell number observed between treatments being specifically manifested in the ICM is provided by the work of Hewitson and Leese (1993) who showed that the TE cells act as a transport epithelium in the blastocyst, enriching the fluid in the blastocoel. In Ham's F12, a complete medium, the fluid in the blastocoel is likely to be more enriched than in that of embryos developing in EBSS. It is possible therefore, that an enriched blastocoel has led to improved development of the ICM in embryos cultured in Ham's F12.

Hardy *et al.* (1989b) showed that pyruvate is the preferred substrate of early human embryos, and this finding has been confirmed here. Only over the final culture period, when the embryos would have reached the blastocyst stage, was the uptake of glucose beginning to surpass that of pyruvate (Fig. 3). In the mouse embryo, there is a decline in pyruvate uptake and a rise in glucose uptake at late preimplantation stages (Gardner and Leese, 1986), but such a dramatic shift in patterns of substrate uptake was not observed here in the human embryo, where embryos continued to consume appreciable quantities of pyruvate at the blastocyst stage.

The results suggest that the uptake of pyruvate is influenced by its concentration in the medium (Fig. 3) with the highest uptake occur-

ring in Ham's F12. Pyruvate is likely to be an essential component of the culture medium, but the concentration at which optimal development occurs has not yet been determined. The effects of pyruvate concentration cannot be examined here as the media were supplemented with various other energy substrates such as glucose, lactate and amino acids. However, the relative concentration of substrates in the medium may be important if there is a limiting endogenous supply of metabolic intermediates, such as phosphate or ADP. During the first culture period (day 2-3) for example, embryos cultured in HTF consumed more glucose than embryos in EBSS (Figs. 2 and 3), perhaps compensating for the low level of pyruvate uptake in HTF. Thereafter, no differences were observed in glucose uptake between EBSS and HTF, even though HTF contains 2.78 times more glucose than EBSS. It should not be necessary therefore, to culture embryos in media with high concentrations of glucose, as the human embryos in this study were able to satisfy their requirement for glucose at a medium concentration of 1 mM. Indeed, evidence from animal models (Chatot *et al.,* 1989) suggest that glucose may be inhibitory to early embryo development. Similar effects of glucose on human preimplantation development have been observed (Conaghan *et al.,* 1993)

The production of lactate by embryos in Ham's F12 was higher than in EBSS (Figs. 2 and 3), possibly as a result of the very high concentration of glucose (10 mM) in the medium. We considered this unlikely however, as it was observed here that glucose uptake was not affected by levels in the medium (Fig. 2). The uptake of pyruvate in Ham's F12 was high, but pyruvate is unlikely to be converted to lactate. A possible contributor to these high levels of lactate production therefore, is amino acid metabolism (Leese *et al.,* 1993).

The abnormalities of development that were observed in Ham's F12 (Fig. 1) were not observed in other embryos, including siblings, which were cultured in EBSS or HTF. In Ham's F12, a number of embryos took on an unusual appearance, which made it difficult to establish the number of blastomeres, and whether or not an embryo had arrested. Some of these embryos appeared to cavitate, but did not exhibit a morphologically distinct ICM or TE. Initially it was thought that these embryos showed signs of osmotic shock, in that cell membranes were no longer visible, although shrinkage of the cytoplasmic volume did not occur. Thus, the osmolarity of the medium, 300 mOsmol/Kg, was reduced to 284 mOsmol/Kg which was similar to that of the other media. Even with the reduced osmolarity however, the abnormal morphology continued to be a feature in the Ham's F12, but not as frequently as at the higher osmolarity.

TABLE 4

CONCENTRATIONS OF SUBSTRATES IN EARLE'S BALANCED SALT SOLUTION, HUMAN TUBAL FLUID AND HAM'S F12

	Concentration of substrate (mM)		
	Pyruvate	Glucose	Lactate
EBSS	0.47	1.0	0.0
HTF	0.33	2.7	21.7*
Ham's F12	1.0	10.0*	0.0

* The high levels of these substrates in the medium prevented determination of uptake or production by the embryos.

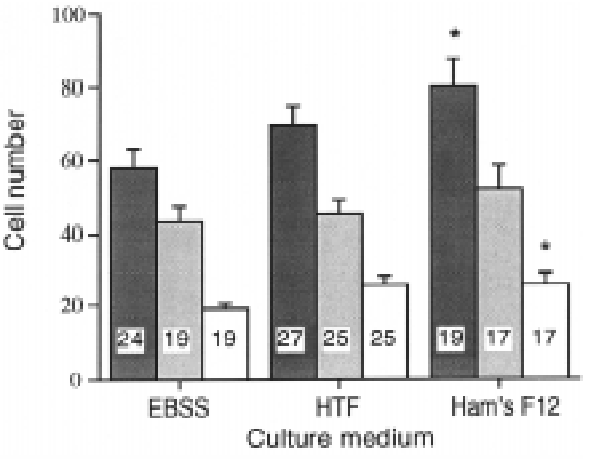

Fig. 4. Cell number for embryos which developed to the blastocyst stage in EBSS, HTF and Ham's F12 medium. ■ *Total cell number ± standard error.* ▩ *Trophectoderm cell number ± standard error.* □ *Inner Cell Mass cell number ± standard error. The number of embryos successfully labeled and counted in each group is shown within the bars. *= significantly higher than EBSS value, p < 0.05.*

Although the recommended osmolarity for EBSS and HTF is 284 mOsmol/Kg, there is a notable absence of studies on the effects of osmolarity on human embryo development. Human plasma has an osmolarity of about 290 mOsmol/Kg and perhaps this has contributed to the value used for embryo culture. This would suggest that the osmolarity of Ham's F12, which is not markedly different from plasma, should not adversely affect embryo development. In mouse embryos, osmolarity affects development only slightly across a broad optimum centered at 260-280 mOsmol/Kg (Brinster, 1965).

Apart from osmolarity therefore, some other component of the medium, which was not present in EBSS or HTF, was likely to be affecting the morphology and development of these embryos. One possible candidate is hypoxanthine, which has been shown to inhibit the development of human embryos cultured in Ham's F10 (Bastias *et al.,* 1993). The mechanism of this inhibition is unclear, but hypoxanthine, which is found in follicular fluid, is thought to be involved in the maintenance of meiotic arrest in the pre-ovulatory oocyte (Eppig *et al.,* 1985) and may therefore affect the developing embryo. Mehta and Kiessling, (1993) were able to improve rates of mouse embryo development using a modified Ham's F10 in which ethylenediaminetetraacetic acid (EDTA) had been added and hypoxanthine had been removed

Another factor which may have affected development, is glucose, which is present in Ham's F12 at 10 times the concentration (10 mM) of EBSS (1 mM). Glucose has been shown to inhibit embryo development in many species, including the mouse (Chatot *et al.,* 1989) and human (Conaghan *et al.,* 1993) at concentrations of only 1.0 mM and 0.5 mM. It is possible therefore, that the high concentration of glucose in Ham's F12 is contributing to the anomalies of development.

Finally, Ham's F12 contains sulphates of copper, iron and zinc, which are not present in EBSS or HTF. Cations of copper and iron are thought to be involved in oxygen free radical formation, and in particular, production of hydroxyl radicals during the oxidation of ferrous iron by hydrogen peroxide. The effects of oxygen free

radicals can be widespread, and include the oxidation of proteins leading to their degradation, reaction with fatty acids (lipid peroxidation) leading to membrane breakdown, enzyme inactivation and DNA damage. Using a modified Hams F10 supplemented with EDTA and in which hypoxanthine had been omitted, Mehta and Kiessling, (1993) achieved a significant improvement in mouse embryo development. The divalent metal cations are believed to have a high affinity for EDTA and are therefore not available for radical formation.

In summary, the work presented here has assessed the development of human preimplantation embryos, from early cleavage stages to the blastocyst stage, in various media. The number of embryos reaching the 8-cell, morula and blastocyst stages was not influenced by the culture media, but in Ham's F12 some morphological abnormalities were observed. It is suggested therefore, that for culture of early cleavage stage embryos, EBSS or HTF should be used in preference to Ham's F12.

Once the embryos had reached the blastocyst stage, rates of survival were best in Ham's F12, suggesting that the requirements of late preimplantation embryos are more complex than those of early cleavage stages. Ham's F12 contains a variety of nutrients, vitamins and minerals, but in addition contains ingredients which may impair embryo development, such as hypoxanthine, glucose and metal sulphates. Perhaps a complete medium such as α-MEM, which does not contain these ingredients, would support higher rates of embryo development than were seen here.

The data from this study is in conflict with the study of Muggleton-Harris *et al.* (1990) who suggested that optimal rates of development through preimplantation stages could be achieved with the use of Ham's F12 in preference to other media. Here, using large numbers of embryos, divided between treatments, Ham's F12 only improved rates of embryo survival when the embryos had reached the blastocyst stage. The results of this study therefore, support the hypothesis that a succession of media may well be required for human preimplantation embryo culture, and that the requirements of the embryo may change at late preimplantation stages. Also, the study has defined criteria by which blastocysts can be assessed *in vitro* during the use or development of new media. These criteria, together with implantation and pregnancy rates that have also been used as end points in similar studies (Bongso *et al.,* 1994; Gardner *et al.,* 1998), should allow rigorous assessment of future developments in culture media for human preimplantation embryos.

Materials and Methods

Source of human embryos

The embryos used for this study were those remaining after transfer, and were obtained with approval from patients undergoing IVF treatment. Ethical permission for the study was granted by the Human Fertilization and Embryology Authority for Human IVF, and the Ethics Committees of the collaborating Institutions; the Royal Postgraduate Medical School and the University of York.

The superovulation, oocyte collection and patient management protocols have been described previously (Rutherford *et al.,* 1988). Ovarian stimulation was induced with daily injections of human menopausal gonadotrophin (hMG; Pergonal, Serono), following pituitary desensitization with continued administration of a Gonadotrophin hormone releasing hormone agonist (Buserelin; Hoechst). Follicular diameter's (FD's) and serum E2 levels were monitored at regular intervals and 10,000 IU human chorionic gonadotrophin (hCG; Profasi, Serono) was administered when a minimum of three follicles had attained a mean FD of 17 mm and serum E2 levels were greater than 3,500 pmol/l. Oocytes were collected trans-vaginally 34-36 h later (day 0).

The methods used for handling of gametes and embryos are a modification of those described by Hillier *et al.* (1984). Up to the time of embryo transfer, gametes and embryos were cultured in 1 ml EBSS (Gibco, Product no. 042-4050) made with ultra pure water (Fresenius water for injections, Fresenius Health Care Group, Basingstoke) and supplemented with 25 mM sodium bicarbonate, 0.47 mM pyruvate, antibiotics and 10% v/v heat-inavated maternal serum. Cultures were performed in a gas phase of 5% CO_2, 5% O_2 and 90% N_2 in a humidified atmosphere at 37°C.

Four to 6 h after collection, oocytes were individually transferred into fresh test tubes, containing approximately 100,000 sperm in 1 ml of the same medium. On the morning of day 1, (16-18 h after insemination), each oocyte was checked for the presence of pronuclei and polar bodies, after remaining cumulus and corona cells had been removed using a finely drawn Pasteur pipette with an internal diameter just larger than that of the oocyte. Only oocytes containing two pronuclei were classified as normally fertilized. Following a further 24 h culture period, up to 3 embryos at the most advanced stage of development and with the best morphology were selected for transfer. Embryos were transferred using a Wallace catheter (H.G. Wallace Ltd., Colchester. Product no. 1816) on day 2 post insemination.

Study design

Normally fertilized embryos from individual patients which had been cultured up to the 2- to 4-cell stage in EBSS, were allocated in equal numbers for culture in each of three media. These consisted of a modified EBSS (as described above, but with 1 mM instead of 5.55 mM glucose), Human Tubal Fluid [HTF; Quinn *et al.*, (1985)] or Ham's F12 (Sigma, product no. N 6760). Ham's F12 was supplemented with 1.176 g/l sodium bicarbonate (BDH, product no. 10247), Penicillin (64 mg/l, Sigma, PEN-K) and Streptomycin (50 mg/l, Sigma, S-6501). The pH was adjusted to 7.7 (in air and at room temperature) using 1 M HCl or 1 M NaOH.

HTF like EBSS, had an osmolarity of 284 mOsmol/Kg. Ham's F12 was initially used at the recommended osmolarity of 297 mOsmol/Kg, but subsequently, water was added to bring the osmolarity in line with the other media, at 284 mOsmol/Kg. All three media were supplemented with 10% v/v Human Serum Albumin (HSA, Zenlab 20; Blood Products Laboratory).

Incubation of embryos

Normally-fertilized embryos at the 2- to 4-cell stage (42 to 44 h after insemination) were denuded of all remaining cumulus cells by gentle manipulation using a series of finely drawn Pasteur pipettes. The procedure for incubating the embryos in drops was similar to that described previously by Hardy *et al.* (1989b). Embryos were washed in three changes of the appropriate medium and placed individually in a 5 μl drop of similar medium under filter-sterilized silicone fluid (Dow Corning 200/50 cs; BDH). Similar drops of medium incubated adjacent to the embryo-containing drops served as controls. All cultures were performed under a gas phase of 5% CO_2 in air. Following overnight incubation (day 3), embryos were removed from their drops and again washed three times into fresh drops of the same medium. This procedure was repeated daily, for four days in all, until the morning of day 6 (135 h after insemination).

Substrate assays

Following embryo culture, duplicate 2 μl samples were recovered from the incubation and control drops. One was diluted in 398 μl double distilled water and assayed for pyruvate and glucose content. The second 2 μl sample was diluted in 118 μl of 5 μM lactate standard solution and analyzed for lactate content. All chemicals used were obtained from Boehringer Mannheim (East Sussex, UK.) unless otherwise stated. The assays were a modification of those described by Gardner and Leese (1986), and Hardy *et al.* (1989b) and were performed on a CobasBio Autoanalyzer (Roche Products, Wellyn Garden City, Herts, UK) using a modification of the methods of Stappenbeck *et al.* (1990).

Pyruvate assay

Sixty µl of diluted medium was automatically sampled and added to 200 µl of 50 mM phosphate buffer [(30 mM K_2HPO_4 (BDH) and 20 mM NaH_2PO_4 (BDH)] (pH 7.0) containing 0.05 mM NADH. Ten µl of LDH (18.75 IU/ml) was added before fluorometric measurement of NADH. One, 3 and 5 µM pyruvate standards were used.

Glucose assay

Seventy µl of the diluted medium was sampled automatically and added to 200 µl of Tris buffer (24 ml of 0.1M Tris buffer (Sigma) (pH 8.0) added to 4.8 ml of 0.1 M $MgCl_2$ (BDH) and 0.5 ml of a NADP/ATP mixture made using 3 parts of 3 mM NADP to 2 parts of 10 mM ATP). Forty µl of enzyme solution (50 µl of Hexokinase/Glucose-6-phosphate dehydrogenase (3.2M) added to 4 ml of distilled water) was added before assay. The program used was a modification of that described by Stappenbeck *et al.* (1990), in that 5 readings were taken, one every 30 sec with the emission filter and excitation wavelength as described for the pyruvate assay. The increase in fluorescence due to the formation of NADPH, is proportional to the amount of glucose consumed in this reaction, and readings were calibrated using 2, 4 and 6 µM glucose standards.

Lactate assay

Forty µl of the diluted medium was sampled automatically and added to 200µl of buffer (6.7 ml of solution A (2 M glycine (Sigma), 0.8 M hydrazine sulfate (Sigma) and 10 mM EDTA (BDH)) added to 6.7 ml of 2N NaOH, 11 ml of double distilled water and 0.6 ml of 25 mM NAD (final pH 9.4)). After a 5 min pre-incubation, 20 µl of enzyme solution (400 µl of LDH (300U.mg^{-1}) added to 4 ml of distilled water) was added. The generation of NADH was related to 5, 10 and 15 µM lactate standards. Readings were taken every 30 sec for 20 min, using an emission filter and excitation wavelength as described by Stappenbeck *et al.* (1990) for their pyruvate assay.

Determination of blastocyst cell numbers

Embryos were labeled using the technique of Hardy *et al.* (1989a), which is based on that of Handyside and Hunter (1984). Embryo zonae were removed in acid Tyrode's solution, pH 2.4, and allowed to recover for 10 min in Earle's handling medium (EHM; as above but with only 4 mM sodium bicarbonate and supplemented with 21 mM HEPES (Calbiochem). The embryos were incubated on ice for 10-15 min. in 10 mM trinitrobenzensulphonic acid (Sigma) containing 4 mg/ml polyvinlpyrolidone (Calbiochem) in EHM. After washing 3 times in EHM, embryos were then placed for 10 min at 37°C in 0.1 $mg.ml^{-1}$ anti-DNP-BSA (ICN Immuno-Biologicals, High Wycombe, Bucks., UK). Following another 3 washes in EHM, the embryos were incubated for 15-30 min at 37°C in EHM supplemented with 0.01 $mg.ml^{-1}$ propidium iodide (Sigma) and 10% v/v guinea pig complement serum (Sigma). After 15 min, they were carefully observed until even lysis of the outer TE cells had occurred, and quickly transferred into 0.05 mM bisbenzimide (Hoechst 33258, Sigma) in absolute alcohol. Following overnight storage at 4°C, the embryos were washed in absolute alcohol for at least 30 min, and mounted in glycerol under a coverslip on a microscope slide.

Labeled nuclei were observed under a Leitz Laborlux D microscope (Leica UK) with λ Ploemopak incident-light fluorescence illuminator (Leica), incorporating a filter set made up of an UV excitation and band pass filter (A2; Leica) and an emission filter from a FITC filter set (I2, Leica). Using this combination of filters, TE nuclei labeled with propidium iodide and bisbenzimide appear orange, and ICM nuclei labeled with bisbenzmide appear green. After examining in whole mount for the degree and evenness of labeling, the embryo was disaggregated by applying gentle pressure on the coverslip with a pencil eraser. TE and ICM nuclei were counted.

Statistical analysis

Differences in the number of embryos developing to the blastocyst stage between different groups were compared using χ^2 analysis, with continuity correction and Fisher's Exact Test. Variation in blastocyst cell numbers between different treatment groups was compared by Analysis of Variance (Anova). Substrate uptake and production were expressed in pmol/embryo/h ± standard error of the mean. Differences between groups were compared using the Wilcoxon Rank-Sum (Mann-Whitney) test. All statistical analyses were performed using StatView II (Abacus Concepts Inc., Berkeley, CA).

Acknowledgments

We thank Karin Dawson and all the staff at the IVF Unit for their continuous encouragement, support and cooperation during the course of this work. We are grateful to Roger Pedersen PhD, University of California San Francisco, for critical reading of the manuscript. J.C. was supported by a grant from Prospect, UK.

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