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Generation of viable human embryos in a protein-free embryo culture (ART-7b) medium enhances clinical pregnancy rate and prevents disease transmission in assisted reproduction

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ABSTRACT

Objective: To perform a clinical trial to evaluate human embryos generated in the protein-free medium (PFM).

Materials and methods: The quality of embryos generated in medium with (control; Medi-Cult™) and without protein (test; ART-7b) and their viability were compared. Sperm was prepared in control or test media as appropriate. Effect of different antibiotic supplements in the PFM was investigated. The efficacy of the PFM following frozen-storage for 2 years was also determined. Investigations and clinical trial in the human were performed with approval of the hospital management.

Results: The fertilization rate and the quality of embryos (IVF and ICSI) in the test PFM were either statistically higher or similar in comparison to control medium containing protein. The clinical pregnancy rate from embryos generated in the PFM (with +ve fetal heart beat) was 54.7% (52/95) in women 39 years and below. The clinical pregnancy rate from embryos generated in frozen-thawed PFM or that supplemented with different antibiotics was similar to fresh test medium.

Conclusion: An efficacious PFM has been formulated which could be useful in the elimination of transmission of protein-bound pathogens and prions to IVF patients.

Keywords: albumin, day 2, embryos, PFM, ultra micro-droplet, ART-7b

Almost half a century ago Wes K. Whitten (1,2) formulated the first chemical medium for culture of mouse embryos. The medium was supplemented with donor serum proteins and the pH was maintained using a bicarbonate/CO_2 buffer system. For many years pre-implantation embryos were also cultured in medium supplemented with donor serum. In the human however, serum supplementation was later shown to have detrimental effects on embryos in culture (3). This problem was overcome by culturing human embryos in serum-free media containing serum albumin (4,5). The serum albumin for commercial or in-house culture media preparation was obtained from two main donor sources, namely, human serum albumin (HSA) or bovine serum albumin (BSA). In some countries BSA is not used for culture of human embryos.

Serum proteins used in culture medium obtained from donors have the potential to transmit diseases to patients undergoing assisted reproduction treatment such as AIDS, CJD, hepatitis (6) and possibly other hitherto unknown diseases. Stringent purification and sterilization procedure used in preparations of donor albumin cannot exclude with absolute certainty the

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transmission of unknown pathogens (7) or dangerous prions. Certain prions (eg: CJD prions) cannot be eliminated even by extreme heat.

Proteins serve important physiological roles in the culture medium and confer the useful physical attributes of increased viscosity and lubrication that promotes ease of handling and prevent stickiness of the embryo to the walls of dishes and catheters.

Protein-free or chemically defined media for mouse, rabbit, non-human primates and humans have been reported [Mouse (8-11); Rabbit (12); non-human primate (13); human: 14-17).

The author recently formulated a PFM for culture of human embryos following an extensive and systematic investigation initially in the mouse and then in the human (14). The protein-free medium was developed to specifically eliminate the potential of transmission of disease through protein-bound pathogens/prions in culture medium and for application in embryonic research. The completely chemically defined nature of the PFM would lend itself to research into embryonic metabolism. This PFM (ART-7b) was evaluated using mouse embryos, human sperm and, 1PN and 3PN human embryos. A clinical trial performed with human ICSI embryos generated in the same PFM resulted in excellent results. The preliminary observation of clinical trials on the PFM employing conventional IVF and ICSI techniques was communicated earlier (15, 16).

The present report on 114 patients is a continuation of an earlier study (15, 16). It attempts to answer the questions as to whether the ART-7b PFM (PFM): (i) could support spermatozoa penetration and fertilization of the egg during conventional IVF, (ii) is as efficient as medium containing serum albumin in the generation of viable and quality embryos, (iii) could elicit viable pregnancies from embryos generated under completely protein-free culture conditions? Other investigations performed include the determination of (iv) the effect of freezing and cold storage on the efficacy of the PFM and (v) whether gentamycin can replace penicillin and streptomycin in the culture medium. In addition, the effect of addition of protein supplement (HSA) to the PFM in the presence and absence of a macromolecule was also investigated.

MATERIALS AND METHODS

Human Spermatozoa Preparation

Husbands of female patients produced semen by masturbation. Spermatozoa were prepared for insemination by the standard swim-up technique in the respective culture media for control (Medi-Cult^{TM}) and test (ART-7b) media separately. Spermatozoa for experiments on the effect of protein deficiency on oocyte fertilization and embryo development were prepared in the protein-free ART-7b medium. The spermatozoa preparation was incubated under a gaseous phase of 5% CO_2 in air at 37^{o}C until use.

Ovarian Stimulation and Oocyte Retrieval

Down-regulation was performed by subcutaneous injections of gonadotrophin releasing hormone agonist (Buserelin; Suprefact; Hoescht, Frankfurt, Germany). The injections commenced in the mid-luteal phase until menstruation or down-regulation was achieved as evidenced by blood estradiol, progesterone and LH levels that have reached baseline values and when endometrial thickness was 4mm or less. Follicle recruitment was initiated with injections of follicle stimulating hormone (FSH; Metrodin; Serono, Rome, Italy) administered for three days. Following this, human menopausal gonadotrophin (Pergonal 500; Serono, Rome, Italy) was administered according to the response of the patient to stimulate follicular development. Ovulation induction was performed with an injection of a 10,000 IU of human chorionic gonadotrophin (hCG; Pregnyl; Organon, Oss, Holland) after achieving follicular size of 16mm or more. Oocyte retrieval (OR) was performed by ultrasound guided vaginal aspiration at 36h post hCG injection.

Randomization of human oocytes

Oocytes were apportioned equally for individual treatments without bias. Oocytes collected during follicle aspirations were alternatively placed into two dishes, one containing control and the other test media so that no bias occurred in the allocation of human oocytes for the

control and test groups.

The study was approved by the IVF Unit Management and the Chairman of the Hospital after deliberation and investigation into the composition of the medium, demonstration of its non-toxicity to human spermatozoa; its non-toxicity to and its capability to support development of mouse zygotes to the blastocysts stage; and its non-toxicity to human embryos as evidenced by its capability to support development of unipronuclear and triploid human zygotes in vitro.

Insemination and Culture Techniques

In Vitro Fertilization (IVF)

Oocytes were inseminated individually in 200ul micro-droplets of equilibrated culture medium under embryo-tested mineral oil (M8410, Sigma Chemicals, USA) previously adjusted to contain 100,000/mL motile spermatozoa. The inseminated oocytes were cultured in an atmosphere of 5% CO_2 in air at 37^{o}C. The oocytes were denuded 18-22h later with denuding pipettes (ART No.1670, International Medical Products BV, Zutphen, Holland). Only protein-free ART-7b medium was used for experiments on the effect of PFM on fertilization while the control medium containing protein were used for the control group.

Intracytoplasmic sperm injection (ICSI)

ICSI was performed by standard methods (18) using a protocol developed in the authors' laboratory (16).

Determination of Fertilization

Fertilization was determined 18h to 20h after IVF. The oocytes were considered fertilized when two distinct pronuclei were visible. Fertilized oocytes were cultured in ultra micro-droplets of equilibrated medium under mineral oil in an atmosphere of 5% CO_2 in air at 37^{o}C. Cleavage and embryo quality was assessed about 48h post-insemination.

Ultra Micro-Droplet (UMD) Culture

Soon after ICSI, the injected oocytes were cultured for 48h in ultra micro-droplets (UMD) cultures as previously described (16, 19). While in conventional IVF, the fertilized oocytes were cultured for 24h in UMD cultures. Test and control embryos were cultured in appropriate culture media to obtain cleaved day 2 embryos. Briefly, UMDs were in the order of 1.5 to 2uL of culture media for culture of 3 to 9 human embryos. These were made using a Stripper^{TM} (Midatlantic Diagnostics, USA) or Flexipet^{TM} (Cook IVF, Australia) pipette in culture dishes previously filled with equilibrated mineral oil. The dishes were incubated under a gaseous phase of 5% CO_2 in air at 37^{o}C. The media were not renewed on a daily basis. Excess embryos were frozen-stored.

An extremely low volume employed was to prevent dilution of autocrine and paracrine factors (APFs) released by the embryos that could improve the quality and viability of the embryos (20-23). Indeed a separate study has shown that the clinical pregnancy rate from day 2 embryos generated in continuous UMD cultures for 48hrs in MediCultTM medium following ICSI was about 62% (41/66; unpublished observation). This observation suggests the possible involvement of APFs in the generation of quality embryos and thus illustrates the usefulness of the continuous UMD culture procedure.

Determination of Day 2 Cleaved Embryo Quality

The quality of human day 2 embryos was determined as described previously (16, 19). Since most healthy day 2 human embryos generally attain the 4-cell stage, an average blastomere number (ABN) of 4 and above was considered excellent in a range of 2 to 6. The embryos were graded (average embryo grade; AEG) according to a scale of 1 to 4, where the numerical 1 denoted poor quality, and 4, excellent quality. Three laboratory personnel graded embryos collectively to avoid bias with the use of a television monitor. Excess embryos of grade 3 and above were cryopreserved.

Test Medium Formulation

Investigations initially focused on the determination of optimal concentrations of various media components. The various experiments performed to formulate the PFM have been described (15,16). Patency application is being negotiated for the ART-7b PFM.

Culture Media

The test ART-7b PFM contained amino acids, antioxidants, osmolytes, vitamins and minerals. It was prepared in-house, used fresh or stored frozen at –20°C (frost-free) for 2 years before use. The control media containing protein (HSA) was the commercial Medi-Cult ™ medium (Medi-Cult a/s, Lerso Parkalle 42, Copenhagen 2100, Denmark). The exact composition of control and test media employed is not disclosed for commercial reasons. Some experiments on the ART-7b PFM medium were performed to determine the effect of protein supplement (HSA) in the presence or absence of a macromolecule.

Institutional Review

The IVF Unit Management of the hospital approved a clinical trial for the transfer of embryos generated in the protein-free ART-7b medium. The efficacy and safety of the ART-7b medium for human application has been earlier ascertained (14-16).

Specific Experiments

Experiment 1

Immediately after ICSI, sibling human oocytes were apportioned equally and cultured in UMD of either the ART-7b PFM (test group) or in the control (Medi-Cult™) media. The fertilization rate and subsequent development of the fertilized oocytes up to the day 2 cleavage stage were recorded and compared between the two groups.

Experiment 2

Sibling human oocytes were apportioned equally for the test and control groups. The test group was inseminated with spermatozoa prepared in the PFM in 200uL micro-droplets of the ART-7b PFM. The fertilization rate and subsequent development of the fertilized oocytes up to the day 2 cleavage stage was compared with that of oocytes and embryos cultured under similar conditions or following intracytoplasmic sperm injection (ICSI) in the control (Medi-Cult™) medium. The spermatozoa of the control group were prepared in the control medium. In this investigation, conventional IVF was performed in both test and control oocytes. However ICSI is performed for control oocytes only, if the patient had no previous history of IVF fertilization to avoid fertilization failure and disappointment to the patient. The zygotes were cultured in UMD cultures of respective test and control media for 24h to obtain day 2 cleavage stage embryos.

Experiments 3a and 3b

This experiment (Experiment 3a) investigated the differences in the fertilization rate and subsequent development of the fertilized sibling oocytes up to the day 2 cleavage stage when cultured under similar conditions in fresh control (Medi-Cult™) and frozen-thawed ART-7b media. A similar but separate experiment (Experiment 3b) was conducted to examine whether there was a difference between fresh control and frozen-thawed ART-7b media.

Clinical Trial

A clinical trial of embryos generated in the PFM was performed. One hundred and fourteen patients were selected for the study at random. In this clinical trial 81 patients received embryos generated in PFM supplemented with penicillin and streptomycin while the remaining 33 received embryos generated in PFM supplemented with gentamycin. This experiment was performed to compare the difference in clinical pregnancy rates of non-sibling day 2 cleavage stage embryos generated in ART-7b PFM when supplemented with either penicillin and streptomycin or gentamycin. This experiment was intended to elucidate possible differences between different antibiotic supplements used in the embryo culture procedures.

In separate experiments involving 69 patients, the effect of protein supplementation (HSA; made up to a final concentration of 5mg/ml PFM; In Vitro Care. Inc, USA) in the ART-7b PFM in the presence or absence of a macromolecule on the clinical pregnancy rate was determined. The macromolecule was intended to confer lubrication attribute to the PFM.

All couples that underwent assisted reproduction treatment in the author's centre (conventional IVF, ICSI and TESE-ICSI procedures) using Medi-Cult™ (control) medium

Table 1. Quality of day 2 human sibling embryos generated by ICSI in protein-free (ART-7b) medium

Medium	Fertilization %	Arrested at 1-cell stage	Blastomere Mean (±SD)	Grade Mean (±SD)	% ≥ 4 Blastomeres	% ≥ 3 grade
ART-7b (Protein-free)	77.8 (196/252)	2.8	3.8 (1.2)	2.9 (0.7)	71.4	63.5
Medi-Cult* (+ protein)	69.4 (175/252)	6.3	3.3 (1.1)	2.8 (0.8)	54.8	58.6
Significance	p=0.0432	p=0.0877	p=0.001	p=0.0802	p=0.0020	p=0.4125

[Embryo grade: 4=excellent; 3=good; 2= fair; 1=poor]
*Control embryos generated by ICSI or IVF

for the years 1998 through 2000 served as controls for comparing pregnancy rates between patients that received embryos generated using media with and without supplemental serum proteins.

Statistical Analysis

Statistical analyses were performed using the statistical package, "StatistixTM". A value of $p<0.05$ or less was considered statistically significant.

RESULTS

Experiments 1 and 2

Table 1 and 2 show the characteristics of ICSI and conventional IVF fertilizations and resultant embryo quality in culture media with and without supplemented serum proteins. The quality of embryos generated by ICSI or IVF in test medium appear to be marginally superior to that generated in control medium. Table 3 shows data on a larger number of sibling embryos generated by both ICSI and conventional IVF. The fertilization and zygote arrest rates, and the quality of embryos generated in PFM is statistically superior to those generated in control medium containing serum proteins.

Experiment 3a and 3b

There were no differences with regard to all parameters tested between sibling embryos generated in fresh control medium containing serum proteins (Medi-Cult^{TM}) and the frozen-thawed ART-7b PFM. In this experiment, insemination was performed by either conventional IVF or ICSI. The fertilization rate, zygote arrest rate, and all parameters of embryo quality tested were statistically similar (Table 4).

Similarly, the fertilization rate of non-sibling oocytes after ICSI was similar (p=0.6767) in both the fresh (84.5%; 49/58) and frozen-thawed (88.6%; 62/70) ART-7b protein-free media. The number of zygotes that arrested at the 1-cell stage was similar (p=0.7852) in fresh protein-free (1.7%; 1/58) and frozen-thawed protein-free media (4.2%; 2/70). The average blastomere number of cleaved day 2 embryos was also similar (p=0.2092) in fresh protein-free (3.6; n=57) and frozen-thawed protein-free media (3.2; n=68).

Clinical trial

The results of the clinical trial are given in Tables (5 and 6). The average number of embryos transferred was 3.1 (358/114).

Table 2. Quality of day 2 human sibling embryos generated by conventional IVF in protein-free (ART-7b) medium

Medium	Fertilization %	Arrested at 1-cell stage	Blastomere Mean (±SD)	Grade Mean (±SD)	% ≥ 4 Blastomeres	% ≥ 3 grade
ART-7b (Protein-free)	85.3 (116/136)	2.2	3.4 (1.0)	3.1 (0.9)	58.4	74.3
MediCult* (+ protein)	79.2 (118/149)	8.1	3.4 (1.0)	2.7 (0.8)	56.2	58.1
Significance	p=0.2352	p=0.0521	p=0.8650	p=0.0011	p=0.8465	p=0.0166

[Embryo grade: 4=excellent; 3=good; 2= fair; 1=poor]
*Control embryos generated by ICSI or IVF

Table 3. Quality of day 2 human sibling embryos generated by conventional IVF or ICSI in protein-free (ART-7b) medium

Medium	Fertilization %	Arrested at 1-cell stage	Blastomere Mean (±SD)	Grade Mean (±SD)	% ≥ 4 Blastomeres	% ≥ 3 grade
ART-7b (Protein-free)	80.4 (320/398)	2.8	3.7 (1.1)	3.0 (0.7)	65.0	68.0
Medi-Cult (+ protein)	73.1 (293/401)	7.0	3.4 (1.0)	2.8 (0.8)	55.4	58.4
Significance	p=0.0178	p=0.0092	p=0.0011	p=0.0007	p=0.0219	p=0.0205

[Embryo grade: 4=excellent; 3=good; 2= fair; 1=poor]

The clinical pregnancy rate in women 39 yrs and below was 54.7% (52/95), in women 40yrs and above was 15.8% (3/19) and the overall clinical pregnancy rate in all age groups combined was 48.2% (55/114). The pregnancy rate in the control group (all age groups combined) was 31.0% (469/ 1515; p= 0.0002). Of these, the proportion of pregnancies in the test group that proceeded to term was 76% (38/50; 5 patients lost to follow-up) while 24% aborted (12/50). In the control group the proportion of pregnancies that proceeded to term was lower at 66.9% (303/453; p=0.0001; 16 patients lost to follow-up) and 33.1% aborted (150/453; p=0.0001). The proportion of treated women in the test group that delivered was 35% (38/109; 5 patients lost to follow-up), while in the control group it was 20.2% and (303/1499, p=0.0005; 16 patients lost to follow up).

The overall implantation rate of embryos generated in PFM was 24.3% (83/342) and the embryo viability rate was 15.5% (53/342, i.e. 53 babies were born to 38 mothers).

The clinical pregnancy rates from embryos generated in PFM supplemented with the penicillin/streptomycin or gentamycin antibiotic supplements were similar in all age groups combined (50.6%; 41/81 vs 42.4%; 14/33 respectively; p=0.5570). If patients 40yrs and above were excluded, the clinical pregnancy rate in young women (39 yrs and below) in both groups still remained comparable (p=0.4346). The clinical pregnancy rate in the young women in the gentamycin group was 73.6% (14/19) while in the same age group supplemented with penicillin-streptomycin it was similar at 55.1% (38/69).

The clinical pregnancy rate from embryos generated in frozen-thawed PFM was 52.9% (18/34). The clinical pregnancy rate in the group in which PFM was supplemented with protein in the presence of the macromolecule was in order of 63.2% (12/19) in young women (39 yrs and below) and 57.1% (12/21) in all age groups combined. Similarly the group in which PFM was supplemented with proteins in the absence of the macromolecule the clinical pregnancy rate was 60.5% (26/43) and 54.2% (26/48) respectively.

DISCUSSION

The present investigation has demonstrated the ART-7b PFM to maintain normal homeostasis in the preimplantation early cleavage-stage embryo evidenced by the generation of viable human embryos.

Table 4. Quality of day 2 human sibling embryos generated by IVF or ICSI in frozen-thawed PFM and fresh medium containing protein.

Medium	Fertilization %	Arrested at 1-cell stage	Blastomere Mean (±SD)	Grade Mean (±SD)	% ≥ 4 Blastomeres	% ≥ 3 grade
ART-7b (Protein-free)	78.6 (81/103)	2.5	3.8 (1.2)	3.1 (0.9)	71.4	68.4
Medi-Cult (+ protein)	75.3 (64/85)	8.5	3.7 (1.1)	2.6 (0.8)	70.9	50.8
Significance	p=0.7119	p=0.3015	p=0.1652	p=0.2325	p=1.000	p=0.0506

[Embryo grade: 4=excellent; 3=good; 2= fair; 1=poor]

Table 5. Summary of embryos transferred and, implantation, viability and delivery rates following protein-free embryo culture.

Test Population (-protein)	
Total No. of Patients	114
Total No. of Patients Clinically Pregnant	55 (48.2%; p=0.0002)
No. of Clin. Pregnant Patients lost to Follow-up	5
No. of Patients that Aborted	12 (24%; p=0.0001)
No. of Patients that Delivered	38
Delivery Rate	35% (38/109; p=0.0005)
Proportion of pregnancies that proceeded to term	76% (38/50; p=0.0001)
Total No. of Embryos Transferred	358
Average No. of Embryos Transferred	3.1
Implantation Rate	24.3% (83/342)*
No. of Babies Delivered	53 (2 triplets; 13 twins; singletons 21)
Embryo Viability Rate	15.5% (53/342)
Control Population (+protein)	
Total No. of Patients	1515
Total No. of Patients Clinically Pregnant	469 (31.0%)
No. of patients lost to follow-up	16
No. of Patients that Aborted	150 (n=453; 33.1%)
No. of Patients that Delivered	303
Delivery Rate	20.2% (303/1499)
Proportion of pregnancies that proceeded to term	66.9% (303/453)

* Figures corrected for the 5 patients that were lost to follow-up

One of the main benefits of the PFM is the elimination of the potential of transmission of disease-borne pathogens or dangerous prions to patients undergoing assisted reproduction treatment. This is because of the fact that the embryos are not exposed to potentially hazardous donor serum proteins. A chemically defined embryo culture medium is also useful for research into the nutrient requirement and metabolism of embryos.

In addition to pathogens and prions, serum may contain embryotoxic factors, as in patients with endometriosis, recurrent abortion and those suffering from unexplained infertility (24). These embryotoxic factors, the nature of which remains to be elucidated, have been shown to be detrimental to embryos in vitro (24,25). Moreover, the use of serum or albumin raises another problem, that of reproducibility. Batch to batch variation in sera or albumin is well recognized. An inability to control quality of different batches of sera will affect quality of embryos generated. Problems associated with embryotoxicity and or non-reproducibility of the quality of serum proteins could be avoided if a chemically defined medium is utilized instead of culture media containing donor proteins.

Serum proteins in embryo culture medium are source of fixed nitrogen and nutrients, an antioxidant and a chelator of metal ions. The physiological functions of albumin and plasma proteins are well documented. The albumin moiety prevents membrane peroxidation and thus has a direct role in membrane stability. Other functions include capillary membrane permeability and in osmo-regulation. It provides 80% of the total colloid osmotic pressure in plasma and is involved in the transport of carbon dioxide and acts as a pH buffer. It also accounts for the greatest (95%) portion of the non-bicarbonate buffer value of plasma. Serum proteins could serve as a source of energy; e.g. alanine could be deaminated to pyruvate, which can be either converted to acetyl-CoA or glucose and glycogen. Albumin help solubilize lipids and transports hormones, vitamins and metals. It serves as reservoirs for the release and use of these components.

Proteins therefore perform a number of crucial functions. The formulation of the PFM will involve the substitution of the protein moiety with other components. These components could perform functions similar to that of the protein moiety. Clearly, a single component cannot fulfill all the functions of serum protein. However many components with varying properties collectively or synergistically could perform most of the functions of serum protein in the culture medium. Attempts at substituting serum albumin in culture medium should take into consideration the role of proteins in vivo and as well as its physical attributes which are useful for embryo handling and manipulation in vitro. The early experiments of this investigation primarily researched and identified culture medium components that could replace one or more functions of the protein in culture. The findings of

Table 6. Summary of clinical pregnancies from day 2 embryos generated in ART-7b protein-free medium

CPR in women 39 years and below (PFM)	54.7% (52/95)
CPR in women 40yrs and above (PFM)	15.8% (3/19)
CPR Overall (all age groups) (PFM)	48.2% (55/114)
CPR (PFM; pen & strep; all age groups)	50.6% (41/81)
CPR (PFM ; gentamycin; all age groups)	42.4% (14/33)*
CPR (PFM; pen & strep; ≤ 39yrs)	55.1% (38/69)
CPR (PFM; gentamycin; ≤ 39yrs)	68.4% (13/19)*
CPR (frozen-thawed PFM)	52.9% (18/34)
CPR in women 39 years (PFM + protein with MM)	63.2% (12/19)*
CPR (all age groups; PFM + protein with MM)	57.1% (12/21)*
CPR in women 39 years (PFM + protein w/o MM)	60.5% (26/43)*
CPR (all age groups; PFM + protein w/o MM)	54.2% (26/48)*

(CPR = Clinical Pregnancy Rate); (MM=Macromolecule)
* Not significantly different from corresponding group

these experiments enabled the formulation of the ART-7b PFM (15,16).

Briefly, individual embryo culture medium components such as amino acids (alanine, aspartate, glutamate, glutamine, glycine, serine, taurine), antioxidants and chelators (N-acetyl cysteine, bilirubin, butylated hydroxyanisole, catalase, citric acid, desferrioxamine, EDTA, reduced glutathione, glutathione peroxidase, glutathione transferase, magnolol, pentoxyfylline, probucol, quinacrine, sodium selenite, superoxide dismutase, tocopherol, uric acid), alternate energy sources (fructose, glutamine, sodium pyruvate), osmolytes (mannitol, myoinositol), vitamins (ascorbic acid, cyanocobalamin, folic acid, tocopherol) and elemental iron were investigated systematically. Their optimum concentrations were determined using mouse zygote assay. A number of novel media were then formulated based on the findings and elucidation of optimal concentrations of various culture media components. This approach is in keeping with the "let the embryos choose" principle described by Summers and Biggers (26). The efficacy of these media was then determined using mouse zygotes and subsequently tested with human 1PN and 3PN, initially, and later with normal diploid zygotes with consent. The entire study required the use of about 6,000 mouse zygotes and about 2,000 human oocytes that took about seven years to complete. The present investigation into the nutrient requirement of the embryo, initially mouse, later human, and the eventual formulation of the protein-free ART-7b medium for human therapeutic assisted reproduction treatment required about 50 experiments and a clinical trial. The findings of the investigations in the mouse and preliminary findings in the human have been reported elsewhere (15,16) and are not presented in this communication.

The present clinical trial on the generation of human embryos in the ART-7b PFM following conventional IVF or ICSI has, resulted in excellent clinical pregnancy rates similar to the previous findings of the author (15, 16). The clinical pregnancy, delivery and abortion rates were significantly better in the test group compared to the control. Also, the proportion of pregnant women in the test group that delivered was similar to the world average reported for cleavage stage embryo transfers (27).

The present study has revealed that: (i) penetration of spermatozoa into oocytes during conventional IVF, gamete interaction and subsequent fertilization in the human following both conventional IVF or ICSI are not impaired in the PFM, (ii) the resultant early cleavage stage embryos are viable and capable of eliciting clinical pregnancies, (iii) the quality of early cleavage stage embryos generated in the PFM is in general superior to that generated in control medium containing serum proteins.

The high fertilization rate during conventional IVF in the PFM medium is direct evidence to indicate that protein deficiency in the medium will not impair sperm capacitation, fertilization, and embryo development in vitro and subsequent viability in utero. It is curious that in spite of a deficiency of proteins in the medium, sperm capacitation, penetration of sperm into the oocyte and fertilization occurred. A plausible explanation is that human seminal plasma contain the fertilization promoting peptide (FPP) (28). The FPP has been shown to stimulate capacitation in ejaculated human spermatozoa (29). Therefore the FPP was probably responsible for the capacitation

of spermatozoa soon after ejaculation. Furthermore, the cumulus-corona cells adhering to the oocytes will release proteins into the culture medium, which could then be utilized for sperm fertilization and embryo growth. This provides further strength to the argument that serum proteins are not required in embryo culture medium.

It has been demonstrated that the PFM can be stored frozen for extended periods of time. The clinical pregnancy rate following transfer of embryos generated in the frozen-thawed PFM (52.9%; 18/34) is similar to that obtained from embryos generated in fresh (46.3%; 37/80) medium in all age groups combined. In the present study the frozen-thawed PFM have been stored for up to 2 years at -20^{o}C. In spite of which viable embryos have been generated. The PFM appeared clear following thawing, except in one of 120 tubes, a minute quantity of a light pinkish brown precipitate was noted. This precipitate dissolved when mixed. This could be an artifact as other tubes were not affected.

Supplementation of different antibiotics in the PFM does not appear to influence the clinical pregnancy rate. The clinical pregnancy rates from embryos generated in PFM supplemented with penicillin/streptomycin (50.6%; 41/81) and gentamycin (42.4%; 14/33) were statistically similar (p=0.5570) in patients of all age groups combined. The clinical pregnancy rates remained similar (p=0.4346) when corrected for age. In younger women of 39 years and below, the clinical pregnancy rates for embryos generated in PFM supplemented with penicillin/streptomycin was 55.1%(38/69) and gentamycin 68.4%(13/19). The addition of serum proteins to the PFM in the presence or absence of the macromolecule did not significantly alter the clinical pregnancy rate. This goes to suggest that the PFM without protein is equally efficacious as would a medium with protein.

In conclusion, the present study has demonstrated that sperm penetration into oocyte during conventional IVF, and fertilization following ICSI or conventional IVF is not compromised in the human in PFM. Based on these findings it is reasonable to assume that capacitation of spermatozoa may occur and the acrosome reaction may not be affected in the absence of protein in the medium. Pregnancies were also not compromised when embryos generated in the ART-7b PFM were transferred, with a clinical pregnancy rate of 55% in women 39 years and below. Overall the ART-7b PFM is equally efficacious if not superior to medium containing serum proteins in the generation of viable human embryos. The routine use of PFM in human assisted conception procedures will eliminate the potential risk of transmission of diseases through protein-bound pathogens or dangerous prions.

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REFERENCES

1. Whitten WK. Culture of tubal ova. Nature;1956: 177:96
2. Whitten WK. Culture of tubal ova. Nature 1957;179:1-1082
3. Hemmings R, Lachapelle MH, Falcone T, Miron P Ward L, Guyda H. Effect of follicular fluid supplementation on the in vitro development of human pre-embryos. Fertil Steril 1994; 62:1018-21.
4. Menezo Y. Culture media and embryo metabolism: the influence of serum addition. In: In Vitro Fertilization and

Embryo Transfer, Edited by Feichtinger, W. and Kemeter, P., Palermo, Cofese, 1984;p.159
5. Menezo Y, Testart J. and Perrone, D. Serum is not necessary in human in vitro fertilization, early embryo culture, and transfer. Fertil Steril 1984;42, 750-5
6. van Os, HC, Drogendijk Aat C, Fetter WPF et al. The influence of contamination of culture medium with hepatitis B virus on the outcome of in vitro fertilization pregnancies. Am J Obstet Gynecol 1991;165, 152-9
7. Truyen U, Parrish CR, Harder TC et al. There is nothing permanent except change. The emergence of new viral diseases. Vet. Microbiol. 43, 103-122. 1995 Cited in: Parinaud J, Vieitiez G, Milhet P. et al. Use of plant enzyme preparation (coronase) instead of hyaluronidase for cumulus cell removal before intracytoplasmic sperm injection. Hum Reprod 1998; 13:1933-5
8. Brinster RL. Studies on the development of mouse embryos in vitro. III. The effect of fixed-nitrogen source. J Exp Zool 1965; 158:69-78
9. Cholewa JA, and Whitten WK. Development of two-cell mouse embryos in the absence of a fixed-nitrogen source. J Reprod Fertil 1970; 22:553-5
10. Spindle A. Beneficial effect of taurine on mouse zygotes developing in protein-free culture medium. Theriogenology 1995;44: 761-72
11. Biggers JD, Summers MC and McGinnis LK. Polyvinyl alcohol and amino acids as substitutes for bovine serum albumin in culture media for mouse preimlantation embryos. Hum Reprod Update 1997; 3: 125-35
12. Li J, Foote RH, Liu Z. et al. Development of rabbit zygotes into blastocysts in defined PFM and offspring born following culture and embryo transfer. Theriogenology 1996;47: 1103-13
13. Schramm RD and Bavister BD. Development of in-vitro-fertilized primate embryos into blastocysts in a chemically defined, protein-free culture medium. Hum Reprod 1996;11:1690-7
14. Ali J. Formulation of a protein-free culture system for the culture of human embryos: preliminary findings and pregnancies. In: Programme and abstract book of the 16th Annual Scientific meeting of the Fertility Society of Australia, 2-4 December 1997, Adelaide, Australia, p34 (Abstract)
15. Ali J. Investigation into the nutrient requirement of the human embryos: Successful formulation and clinical trial of a novel protein-free embryo culture medium. Emirates Med J 2000; 18:195-202
16. Ali J, Shahata MAM, Al Natsha SD. Formulation of a protein-free medium for human assisted reproduction. Hum Reprod 2000; 15:145-56
17. Parinaud J, Milhet P, Vieitiez G et al. Use of a medium devoid of any human or animal compound (SMART2) for embryo culture in intracytoplasmic sperm injection. J Assist Reprod Genet 1999;16:13-6
18. Palermo G, Joris H, Devroey P, Van Steirteghem AC. Pregnancies after intracytoplasmic sperm injection of single spermatozoon. Lancet 1992; 340(8810):17-8
19. 19 Ali J. A Practical Guide to Mouse Pre-implantation Embryology and Human Assisted Reproduction Technology. JN Shelton, Ed. Ladybrook Publishers, 2003; Perth, Australia
20. Paria BC, Tsukamura H and Dey SK. Epidermal growth factor-specific protein tyrosine phosphorylation in preimplantation embryo development. Biol Reprod 1991;45: 711-8
21. Paria BC and Dey SK. Preimplantation embryo development in vitro:cooperative interactions among embryos and the role of growth factors. Proc Natl Acad Sci USA 1990; 87: 4756-60
22. Canesco RS, Sparks AE, Pearson RE et al. Embryo density and medium volume effects on early murine embryo development. J Assist Reprod Genet 1992;9:454-7
23. Almagor M, Bejar C, Kafka I et al. Pregnancy rates after communal growth of preimplantation human embryos in vitro. Fertil Steril 1996; 66: 394-7
24. Miller KA, Pittaway DE and Deaton JL. The effect of serum from infertile women with endometriosis and early embryonic development in a murine in vitro fertilization model. Fertil Steril 1995; 64: 623-6
25. Fein A, Yacobovitch R, Torchinsky A et al. Evaluation of serum-associted embryotoxicity in women with reproductive disorders. J Assist Reprod Genet 1995; 12:305-11
26. Summers MC and Biggers JD. Chemically defined media and the culture of mammalian preimplantation embryos: historical perspective and current issues. Hum Reprod Update 2003; 9:557-82
27. De Mouzon J and Lancaster P. World collaborative report on in vitro fertilization. Preliminary data for 1995. J. Assist. Reprod. Genet. 1997;(suppl.) 14, 250S-265S
28. Cockle SM, Aitken A, Beg F, Morrell JM, Smyth DG. The TRH-related peptide by pyroglutamylglutamylprolinamide is present in human semen. FEBS Lett 1989; 252:13-7
29. Green CM, Cockle SM, Watson PF et al. A possible mechanism of action for fertilization promoting peptide, a TRH-related tripeptide that promotes capacitation and fertilizing ability in mammalian spermatozoa. Mol Reprod Dev 1996; 45:244-52.

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Advances in embryo culture conditions, especially the use of sequential media, have allowed a prolongation of embryo culture prior to transfer [4, 5]. A blastocyst culture until day 5, which was first performed in livestock breeding and later in human IVF, offers the advantage of selecting embryos which have completed the crucial steps of compaction and blastulation. Furthermore, an increase in pregnancy rates (PR) was observed after prolonged embryo culture until the blastocyst stage [2, 6,7,8,9,10,11,12,13,14,15,16].Â Culture of embryos was performed in an EmbryoScopeÂ© (Vitrolife) at 37 Â°C, 6.4% CO2 and 5.0% O2.Â a Comparison of clinical and ongoing pregnancy rates of day 4 and day 5 embryo transfers.