The Choice of Culture Media and Strategies: Advantages & Potential Risks

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Maintenance of Embryo Viability

Embryos *in vitro* are exposed to significantly more stress than is experienced *in vivo*

Embryo viability *in vitro* is dependent on:

- composition of culture medium
- quality of culture medium
- physico-chemical characteristics of culture medium
  - pH
  - osmolarity
  - tempererature
  - gaseous environment: O_2 & CO_2
- culture conditions

In Vitro Consequences of Stress

- Delays in development
- Arrest in development often prior to activation of the embryonic genome
- Altered embryo physiology, particularly metabolism
- Altered gene expression patterns
- Altered imprinting status of specific genes
- Altered viability

Constitution of Culture Media for ART

- Water
- Ions
- Energy substrates
- Amino Acids
- Vitamins
- Nucleic acid precursors
- Proteins
- Growth Factors/Hormones
- Antioxidants
- Chelators
- Antibiotics
- pH indicator
- ?Lipids
- ?ECM molecules

Culture Medium

- Simple culture medium

or

- Complex culture medium

or

- Sequential culture medium

Simple Culture Media

Simple salt solutions with added energy substrates supplemented with serum or serum albumin

- Earle's Balanced Salt Solution
- Quinn's Human Tubal Fluid / Basal XI
- T6
- KSOM
- Menezo's B2
- M16
- BM1
- CZB

Historical Perspective on IVF

- human IVF was traditionally performed in simple culture medium i.e. EBBS, HTF, T6 culture media
- embryos were transferred on day 2 rather than continuing culture to beyond the time when the human embryonic genome is fully activated (day3) as in vitro blocks to development were commonly observed at this time in experimental models using embryos from the hamster, mouse and domestic animals

Deficiencies of Simple Media

- early attempts to culture to the blastocyst stage of development confirmed that these culture media were sub-optimal
- although an acceptable proportion of embryos cleaved to the blastocyst stage of development, viability was compromised - implantation rate 7%

Bolton et al., 1991

Complex Culture Medium

Complex medium were originally designed to support the growth of immortal cell lines in vitro and contain carbohydrates, amino acids, vitamins, nucleic acid precursors, transitional metals

- Ham's F10
- Ham's F12
- MEM/αMEM
- Waymouth's medium
- TCM 199
- Chang's Medium

Complex Culture Medium

Many contain components that have since been demonstrated to have an adverse affect on embryo viability:

- High levels of glucose
- Divalent metal ions
- Nucleotides
- Certain hormones

Design of ART Specific Culture Media

Two schools of thought:

- 'Back to nature' approach
  - Sequential culture media
    - Based on the collected works of Gardner, Lane, Pool, Quinn and associated co-workers
- 'Let the embryo choose' approach
  - Monoculture
    - Based on the collected works of Biggers and co-workers

Sequential Culture Media

Company	Media
Cook IVF	Sydney IVF Cleavage Medium / Sydney IVF Blastocyst Medium
Cooper Surgical	Quinn's Advantage Cleavage Medium / Quinn's Advantage Blastocyst Medium
Vitrolife	G-1 v5 Plus / G-2 v5 Plus
Medicult	EmbryoAssist / BlastAssist
FertiPro N.V.	Ferticult / Ferticult G3
InVitro Care Inc.	IVC-One / IVF-Two
Irvine Scientific	ECM / Multiblast Medium

Mono Culture Medium

Company	Medium
IVFonline	Global (KSOMaa)

Sequential Culture Medium Philosophy

1. Changing energy requirements of the embryo and the inhibitory effect of glucose on early development
2. Inhibitory effect of EDTA on blastocyst development and inner cell mass
3. Role of amino acids in preimplantation embryo development
4. Breakdown and metabolism of amino acids, particularly L-Glutamine, to ammonium and its negative impact on embryo quality

Sequential Medium

- from the time of ovulation to the time of blastocyst development *in vivo*, a period of 4-5 days, the human embryo encounters a changing environment
- Day 1 - Day 3 Early cleavage stages - Oviduct
- Day 3 - Day 5 Morula to Blastocyst stages - Uterus

Composition of Mammalian Fluids

Component	Human Oviduct Fluid	Human Uterine Fluid	Human Serum	Mouse Oviduct Fluid
Na	130	nd	145	139
Cl	132	nd	nd	165
K	21.2	nd	5.0	23.4
Ca	1.13	nd	1.13	1.71
Mg	1.42	nd	2.00	1.04
S	12.3	nd	nd	8.45
P	8.69	nd	nd	8.93
Pyruvate	0.24	0.10	0.10	0.37
Lactate	1.98	5.87	0.60	4.79
Glucose	1.11	3.15	5.00	3.40
Glutamine	0.30	nd	nd	0.20

1. Energy Substrates

Pyruvate, Glucose and Lactate

- Uptake of pyruvate remains high throughout human preimplantation embryo development
- Shift in preference from pyruvate to glucose around the time of compaction
- Glucose does not support embryo development before the 8-cell stage
  - oocyte does not see high levels of glucose in vivo as cumulus readily metabolizes it to pyruvate and lactate
  - sperm utilize glucose
- Lactate does not support embryo development before the 2-cell stage
  - acts synergistically with pyruvate
  - only the L-isomer is biologically active
  - lactate is a weak acid and at concentrations ≥ 5mM induces a significant drop in pH_i

Culture Media - Carbohydrates

Component	Mono Culture	Sequential Culture	
	KSOMaa mmol/L	G1 mmol/L	G2 mmol/L
Na pyruvate	0.2	0.32	0.1
Na lactate	10.0	10.5	5.87
Glucose	5.56	0.5	3.15

Pros & Cons

- Oviduct fluid contains significant amounts of glucose
- Glucose is utilized during early preimplantation embryo development via the pentose-phosphate pathway for biosynthetic requirements and therefore omission is non-physiological
- Global medium utilizes glucose concentrations typically found in oviduct fluid
- Sequential medium reflects the change in concentrations of pyruvate and glucose from the oviduct fluid to uterine fluid

2. Chelator - EDTA

- Concentrations of EDTA as low as 0.005mmol/L are capable of overcoming 2-cell blocks to development in experimental animal species
- Sequential media formulations usually utilize a concentration of EDTA around 0.01-0.1mmol/L
- 0.01-0.1mmol/L EDTA increases development of human zygotes to blastocyst but reduces the cell numbers in resultant blastocysts when included for the entire culture period
- 0.1mmol/L EDTA for the entire culture period results in significantly reduced foetal body weight
- 0.01mml/L EDTA has a beneficial effect isolated to early cleavage stage embryos and is inhibitory after compaction; inhibits glycolysis which is utilized exclusively by the ICM

Culture Media - EDTA

Component	Mono Culture	Sequential Culture	
	KSOMaa mmol/L	G1 mmol/L	G2 mmol/L
EDTA	0.01	0.01	-

Pros & Cons

- KSOMaa medium proponents argue that EDTA for the entire culture period has no significant affect on pre- or post- implantation development provided it is used at a low concentration

  Biggers et al., 2008

- Even at 0.01mmol/L EDTA causes a reduction in blastocyst cell numbers and perhaps the requirement for EDTA could be eliminated if culture was performed in a more physiological O_2 concentration i.e. <8%

  Orsi & Leese, 2001

- Gylcolytic enzyme 3-phophoglycerate kinase is inhibited by 0.01-0.1mmol/L EDTA

  Lane & Gardner, 2001

3. Amino Acids

- oocytes and embryos contain specific transport mechanisms for amino acids
- readily metabolized by embryo which maintains an endogenous pool
- enhance embryo development to the blastocyst
- transient exposure of mouse zygotes (< 5 min) to medium lacking amino acids impairs subsequent development to blastocyst
- glutamine unquestionably the most important (utilized in preference to glucose)
- taurine/hypotaurine important for sperm motility

Sequential Media - Amino Acids

Gardner, Lane et al.

- non-essential amino acids (alanine, asparagine, aspartate, glutamate, glycine, proline and serine) and glutamine stimulate cleavage rate, blastocyst formation and hatching
- after compaction non-essential amino acids and glutamine no longer stimulate cleavage although they do increase blastocoel formation and hatching
- essential amino acids stimulate cleavage rates after the 8-cell stage and increase development of the inner cell mass in the blastocyst
- essential amino acids reduce the cell number of blastocysts from cultured zygotes

Non Essential Amino Acids	Mono Culture	Sequential Culture	
	KSOMaa mmol/L	G1 mmol/L	G2 mmol/L
Glutamine*	1.0	-	-
Alanyl-glutamine	-	0.5	1.0
L-Alanine	0.05	1.0	1.0
L-Asparagine	0.05	1.0	1.0
L-Aspartic Acid	0.05	1.0	1.0
L-Cysteine*	0.05	-	0.1
L-Glutamic acid	0.05	1.0	1.0
Glycine*	0.05	1.0	1.0
L-Proline*	0.05	1.0	1.0
L-Serine*	0.05	1.0	1.0
L-Tyrosine*	0.1	-	0.2
L-Arginine*	0.3	-	0.6
L-Histidine*	0.1	-	0.2
Taurine*	-	0.1	-

Essential Amino Acids	Mono Culture	Sequential Culture	
	KSOMaa mmol/L	G1 mmol/L	G2 mmol/L
L-Isoleucine	0.2	-	0.4
L-Leucine	0.2	-	0.4
L-Lysine	0.2	-	0.4
L-Methionine	0.05	-	0.1
L-Phenylalanine	0.1	-	0.2
L-Threonine	0.2	-	0.4
L-Tryptophan	0.025	-	0.5
L-Valine	0.2	-	0.4

Pros & Cons

- Oviduct fluid contains all 20 amino acids
- Follicular fluid and oviduct fluid have concentrations of amino acids much lower than is present in sequential media formulations
- Global medium has amino acid concentrations similar to physiological levels
- Amino acid turnover studies indicate that all of the amino acids showing a net depletion from the 2c to blastocyst fall into the essential and conditional groups of amino acids rather than the non-essential group of amino acids, the most significant essential amino acid is leucine

4. Ammonium Production

- amino acids spontaneously break-down at 37°C and release ammonia
- ammonium is embryotoxic at concentrations as low as 75μM
- ammonium has been shown to decrease blastocyst cell numbers, reduce implantation rates, retard foetal growth and increase the incidence of exencephaly
- glutamine is the most labile

Sequential Media - Amino Acids

- To reduce ammonium toxicity culture medium containing amino acids should be changed every 48 hours

Pros & Cons

- addition of ammonium chloride to culture medium does not accurately simulate the effect of kinetic breakdown of glutamine in aqueous solution
- alanine provides a means of disposing of ammonia via glutamate dehydrogenase & alanine transaminase
- ammonium significantly reduced if glutamine replaced with the more stable alanyl-glutamine or glycyl-glutamine

Selected Abstracts Comparing Blastocyst Development in Single vs Sequential Media

The percentage human zygotes that develop into blastocysts over 5 to 6 days in global medium and in several sequential media observed in several clinics.

	Single medium (Global)		Sequential media		
Reference	Protocol	Blastocysts	Protocol	Blastocysts	P
Freeman and Rieger[a]	Renewed	36% (71/198)	IVC-1/G2	26% (52/199)	.036
Greenblatt et al.[b]	Renewed	53% (29/55)	G1/G2	38% (21/55)	.160
Angus et al.[c]	Renewed	58% (197/337)	GIII	50% (131/261)	.047
Kumagai et al.[d]	Renewed	54% (79/149)	Quinn's cleavage/Multiblast Irvine	48% (71/147)	.486
Matsubara et al.[e]	Renewed	46% (73/187)	BAS Medicult	39% (90/195)	.179
Sepulveda et al.[f]	Renewed	44% (117/268)	IrvineECM/Multiblast	33% (93/283)	.011
Zech et al.[g]	Renewed	44% (290/667)	G1.3/G2.3	35% (170/491)	.003

Biggers et al., 2008

Selected Abstracts Comparing Outcomes Following Culture in Single vs Sequential Media

The percentage of ongoing pregnancies that arise from blastocysts cultured from human zygotes.

	Single medium (Global)		Sequential media		
Reference[a]	Protocol	Pregnancies	Protocol	Pregnancies	P
Greenblatt et al.[b]	Renewed	43% (?)	G1/G2	42% (?)	–
Angus et al.[c]	Renewed	40% (12/30)	GIII	28% (8/29)	.412
Matsubara et al.[e]	Renewed	41% (7/17)	BAS Medicult	40% (6/15)	1
Zech et al.[g]	Renewed	49% (21/43)	G1.3/G2.3	40% (9/22)	.606

Comparison of General Features

Characteristic	Single Medium Non-renewed	Single Medium Renewed	Sequential Media
Embryo undisturbed	Yes	No	No
Accumulation of autocrine/paracrine factors	Yes	No	No
Replenishment of essential nutrients	No	Yes	Yes
Accumulation of toxins	Yes	No	No
Stress levels due to embryo manipulation	Low	Moderate	Moderate
Labour intensity	Low	Moderate	Moderate
Cost	Low	Moderate	High

Conclusions

- Culture medium formulations should not be considered independent of the culture environment as many of the physico-chemical aspects of the culture environment can influence the effectiveness of the selected culture medium
- The choice of which culture medium to use in your own laboratory should be dictated by performance within your own laboratory setting
  - Outcomes
  - Availability and Supply lines
  - ?Cost
- Although sequential culture media formulations are the most commonly used in human ART, the requirement for sequential culture media formulations is not absolute

Conclusions

- Current human ART culture media formulations have been improved significantly since the inception of IVF with significant improvements in ART outcomes
- Current media formulations can and should be further optimized
- The need for sequential media may be overcome with a change to non-static cultures which is one of the major causes of stress associated with embryo development *in vitro*