The choice of culture media and strategies: advantages and potential risks

Etienne Van den Abbeel PhD

Maribor 2010

Introduction

ART success: birth of one healthy child

Transfer of one embryo: SET and sFRET

The Road for Single Embryo Transfer = blastocyst transfer?

Lane and Gardner (2007)
Papanikolaou et al (2006)
Blake et al (2004)

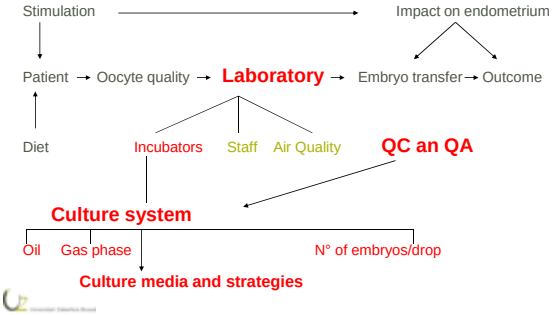
Introduction

The Road for Single Embryo Transfer:

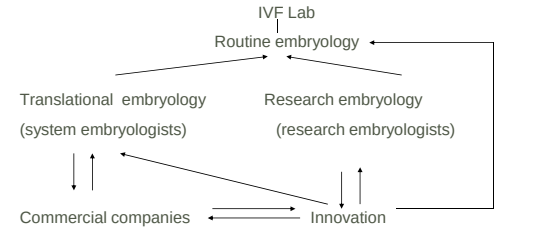
Three laboratory areas that warrant consideration and discussion

Optimizing embryo development in culture
Selecting the most viable/normal embryo for transfer
Optimizing cryopreservation procedures

Holistic approach of IVF



Holistic approach of IVF



Maintenance of Embryo Viability

Embryos grown in vitro vs in vivo show differences in:

→ Growth dynamics

→ Compaction pattern

→ Polarity

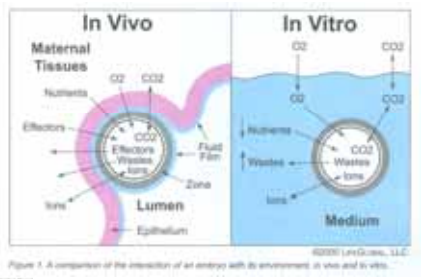
→ Blastocysts quality

→ Hatching process

→ Freezability

Maintenance of Embryo Viability

Embryos grown in vitro vs in vivo



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
  - temperature
  - gaseous environment: O_2 & CO_2
- culture conditions

In Vitro Consequences of Stress

Potential short term responses

- Epigenetic modifications
- Altered intracellular signaling
- Metabolic stress
- Gene expression changes
- Apoptosis
- Cell proliferation disturbed

Potential long term consequences

- Reduced implantation
- Unbalanced fetal/placental allocations
- Altered maternal nutrient provision
- Abnormal fetal growth
- Abnormal birth weight and postnatal growth
- Cardiovascular and metabolic syndromes

(Role of reproductive tract fluids in developmental origins of health and disease, Leese et al, 2008)

In Vitro Consequences of Stress

Primordial follicle
Antral follicle
IVM/Superovulation
IVF
Zygote
8-cell
Blastocyst

Impact stress factors

Culture Medium

- Simple culture medium

or

- Complex culture medium/culture systems

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
- 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 mostly 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
- Menezo B2

Complex Culture Medium

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

Co-culture systems

Kattal et al: Role of co-culture in human IVF: a meta-analysis Fertil Steril (2007)

Conclusion: The pooled data on co-culture demonstrate a statistically significant improvement in blastomere number, implantation rates and ongoing pregnancy rates

Benefit of co-culture:

- Release of embryotrophic factors
- Detoxicification of the culture medium

Disadvantages

- Possible transmission of disease (requirement for autologous tubal/endometrial cells)
- Complexity/Workload

A variation on Co-culture systems: grouped culture

Observations

- A single embryo cultured in a four-well plate or test- tube, any factor produced by the embryo will become ineffectual due to dilution (Gardner and Lane, 2004)
- Culture of embryos in reduced volumes and/or in groups increases the effective concentration of embryo derived factors, facilitating their action in either a paracrine or autocrine manner (Gardner and Lane, 2004)
- The quality of human, bovine and mouse embryo growth is improved when embryos are cultured in groups rather than separately (Moessner and Dodson, 1995, Salahuddin et al, 1995, Ahern et al, 1998)
- Cooperative interactions exist among embryos (Paria and Dey, 1990)
- Embryo development is influenced by embryo density and incubation volume (Lane and Gardner, 1992)

In-vivo culture

ZIFT: Zygote intra-Fallopian transfer
Benefit?

Encapsulation (*in vivo* culture) technology:
(Blockeel et al: an in vivo culture system for human embryos using an encapsulation technology: a pilot study (Hum Reprod, 2009)

Culture in an *in utero* culture system comprised of a perforated silicon hollow tube
Conclusion: feasible and safe, "suggests" superior embryo quality, pregnancies obtained

Sequential Culture Media

Company	Media
Cook IVF	Sydney IVF Cleavage Medium / Sydney IVF Blastocyst Medium
Cooper Surgical	Sage
Vitrolife	G-1 v5 Plus / G-2 v5 Plus
Medicult	EmbryoAssist / BlastAssist
FertiPro N.V.	Ferticult / Ferticult G3
Irvine Scientific	ECM / Multiblast Medium

Composition of culture medium: debate

Differences between oviduct and uterus in mammalian embryos (Lane et al, 2007)

Component	Oviduct	Uterus
Glucose concentration	0.50mM	3.15mM
Pyruvate concentration	0.32mM	0.10mM
Lactate concentration	10.50mM	5.20mM
Oxygen concentration	8%	1.5%
Carbon dioxide concentration	12%	10%
pH	7.5	7.1
Glycine concentration	2.77	19.33
Alanine concentration	0.5	1.24
Serine concentration	0.32	0.80

Human embryo culture in-vitro: media and strategies

Three types of protocol:

- Uninterrupted culture using one medium ***(nonrenewal mono culture medium protocol)***
- Interrupted culture using one medium but renewed on D2/D3 ***(renewal mono culture medium protocol)***
- Interrupted culture where two media of different composition are used sequentially ***(sequential media protocol)***

Culture medium and strategies: debate

Questions

- How can we maximize the development of embryos in vitro?
  - → Can a single culture medium be developed which supports development throughout the pre-implantation period?
  - → Should the composition of the medium be changed to correspond with the physiological changes which occur as development proceeds?

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

culture medium and strategies: debate

Areas of debate

- Glucose: to be or not to be
- Role of amino acids
- L glutamine and ammonium
- Role of EDTA

Glucose to be or not to be

Observations

- Uptake of pyruvate remains high throughout human pre-implantation embryo development
- Shift in preference from pyruvate to glucose around the time of compaction
- Glucose/phosphate inhibition before compaction?
- Glucose alone 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 alone does not support embryo development before the 2-cell stage
  - → acts synergistically with pyruvate

Debate

Sequential culture: requirement for changing glucose

- Sequential medium reflects the change in concentrations of pyruvate and glucose from the oviduct fluid to uterine fluid (Pre compaction: pyruvate/post compaction: glucose)

Mono culture (Global): no requirement for changing glucose

- Oviduct fluid contains significant amounts of glucose
- Glucose is utilized during early pre-implantation embryo development via the pentose-phosphate and hexosamine pathway for biosynthetic requirements and therefore omission is non-physiological
- Global medium utilizes glucose concentrations typically found in oviduct fluid
- Glucose inhibition is a dogma

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

Role of amino acids

Observations

- Functions of AA: precursors of biosynthesis, energy source, regulator, buffer, chelator
- 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
- Taurine/hypotaurine important for sperm motility

Debate

Sequential media (Gardner et al - mouse studies): changing AA

- Non-essential amino acids (alanine, asparagine, aspartate, glutamate, glycine, proline and serine) and glutamine stimulate cleavage rate, blastocyst formation and hatching
- After 96 h of mouse embryo culture the best response of embryos was obtained with non-essential amino acids. Essential amino acids were inhibitory
- When culture was extended for more than 96h development, was inhibited even in the presence of non-essential amono acids
  → The delayed development was attributed to accumulation of ammonium
- 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

Debate

Monoculture (Global): not changing AA

- 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
- It is certain that no currently used media contain optimal concentrations of amino acids

Waymouth (1965): …" The terms 'essential' and 'non-essential' amino acid, rather commonly used, are not strict designations and should be accepted with scepticism unless carefully defined in a particular context" …

L glutamine and ammonium

Observations

- amino acids spontaneously break-down at 37°C and release ammonia
- ammonium is embryotoxic at concentrations as low as 75μM
- ammonium (ammonium chloride) 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

Debate

Sequential culture: renewal of medium required

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

Monoculture (Global)

- 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

Role of EDTA

Observations

- 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

Debate

No change in media

- KSOMaa medium (global media/monoculture) 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

Changing in media

- Even at 0.01mmol/L EDTA causes a reduction in blastocyst cell numbers

Orsi & Leese, 2001

- Glycolytic enzyme 3-phophoglycerate kinase is inhibited by 0.01-0.1mmol/L EDTA (ICM glycolysis inhibited – Fetal development reduced)

Lane & Gardner, 2001

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[3]	Renewed	30% (71/198)	IVC-1/G2	20% (52/199)	.030
Greenblatt et al.[5]	Renewed	53% (29/55)	G1/G2	36% (21/55)	.160
Angus et al.[1]	Renewed	58% (197/337)	QIII	50% (191/251)	.047
Kumagai et al.[4]	Renewed	54% (79/146)	Quinn's cleavage/Multiblast Irvine	46% (71/147)	.486
Matsubara et al.[6]	Renewed	46% (73/187)	BAS Medicult	39% (80/196)	.179
Sepulveda et al.[7]	Renewed	44% (117/268)	IrvineECM/Multiblast	33% (93/283)	.011
Zech et al.[8]	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 on the debate

- No important randomised trials to compare the different approaches
  - → Consensus is missing
- Importance of cryopreservation
- Commercialization
  - → Disclosure on composition?

  Lewis and Lewis (1911): ..." It is to be hoped that an artificial medium will be found as satisfactory as the plasma, for the advantages are obvious if one can work with ***a known medium*** in the investigation of the many new problems, which suggest themselves" ...

  - → Controversies
  - → Fragility of some widespread dogma's
  - → Generate open minded debate
- Further research needed (on human embryos?)
  - → Can we do better than nature?

General 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
- Although sequential culture media formulations are the most commonly used in human ART, the requirement for sequential culture media formulations is not absolute
- Current human ART culture media formulations have been improved significantly since the inception of IVF with significant improvements in ART outcomes

Future perspectives

- Current media formulations can and should be further optimized

  → Growth factors?
  → Cytokines?
  → Embryotrophic ligands

Future perspectives

- The need for sequential media/monoculture (static cultures) 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*

  → Microfluidics

Future perspectives

- In vitro culture and epigenetics?
  - → Khosla et al Culture of pre-implantation embryos and its long-term effects on gene expression and phenotype (Hum Reprod Update 7, 419-427, 2001)
  - → Katari et al DNA methylation and gene expression differences in children conceived in vitro or in vivo (Hum Molec Genetics 18, 3769-3778, 2009
  - → Dumoulin et al Effect of in vitro culture of human embryos on birthweight of newborns (Hum Reprod Adv Access, 2010)

Future perspectives

•Ecological clinical embryology

- •Critical re-evaluation of surrounding environmental factors, taking into account new concepts of environmental and epigenetic influences upon genotypes to produce phenotypes (Plancha, 2009)
- •Promote embryo metabolism which is 'quiet' rather than 'active' (Leese, 2002, 2008)
  - •Limit the concentrations of nutrients
  - •Trust the autonomy of the embryo