

Na+
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Glucose
Pyruvate
Lactate
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Leucine
Proline
Isoleucine
Tryptophan
Choline
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Ascorbate
FSH
GH
Estradiol
Insulin
SSR
SSS
hSA
BSA
IGF-1
GCSF
IL-6
NANOG
FactX
GanCF
XCZjk
SillyF
Cosmetin
Hoax-II

Culture media supplementation and culture conditions

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Hospital in Trondheim

Norway

Declaration of interests:

Worked for MediCult 1999-2002

Founder and shareholder, CellCura of Norway



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- XCZjk
- SillyF
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- Hoax-II

A case of natural gene transfer 😊

Image © 2007 NASA
Image © 2007 TerraMetrics

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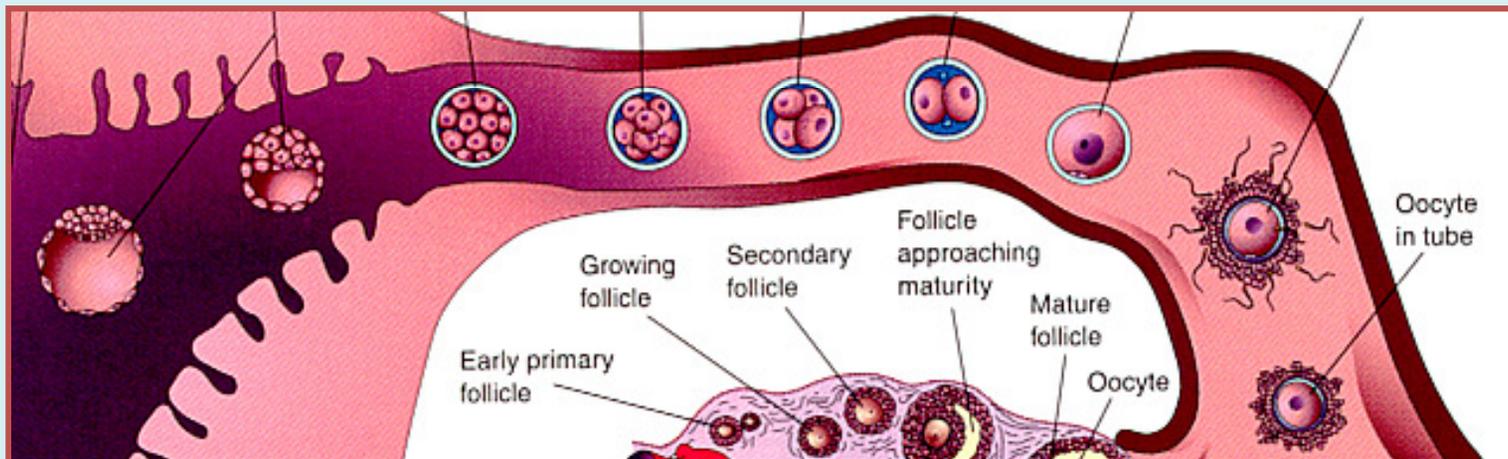
My talk

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- *In vivo vs. in vitro*: challenges
- Culture media for ART
 - Considerations when designing ART media
 - Pitfalls and problems
- Success criteria
 - ART and culture media
 - A case for a new set of success variables?
- I apologise
 - for omitting a few slides (not to overlap others)
 - for introducing a few new
 - for not telling you which culture media is the best
 - for running to the airport just after my talk

What is Embryo culture in ART?

We're trying to mimic to condition in the fallopian tube (?)



This is not easy and we have still not achieved this and it will be a long time before we're there

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What is Embryo culture in ART?

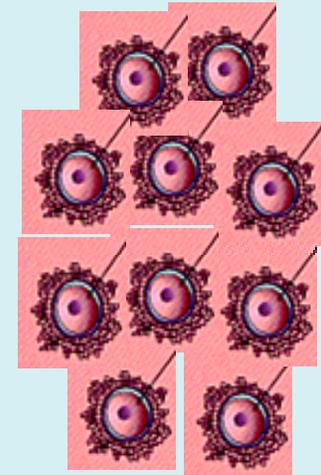
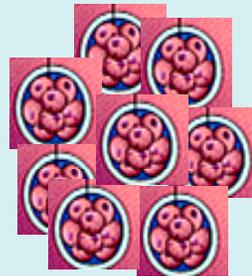
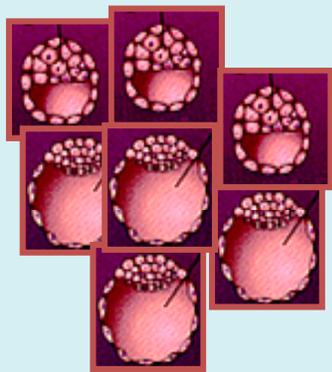
- The basic principles:
 - Facilitate fertilization and embryo development in vitro
 - “Normal” fertilisation
 - “Normal” embryo development
 - Select embryos for replacement
 - High birth rate/cycle low multiple birth rate
 - Render the embryos suitable for cryopreservation
 - Avoid factors that can have an influence embryo development after implantation (i.e. epigenetic effects)

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Pretending to be the fallopian tube

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BUT We have so many oocytes!



We have to select prior to implantation!
This is not the case naturally, so we do not have much help from nature



Design of embryo culture media

- Basically 3 types of media:
 - Relatively simple salt solutions
 - EBSS, Universal-IVF, IVF-medium, HTF's, P1
 - “Old”-culture media intended for tissue culture
 - Ham F-10/F-12, M-199, α -MEM
 - Not necessarily beneficial for human IVF/ICSI
 - “Physiological” media
 - Formulation approximating the *in vivo* conditions
 - Sequential or mono
 - Most of these media also contains substances which have specific roles *in vitro* (antibiotics, pH-indicator, antioxidants, EDTA)

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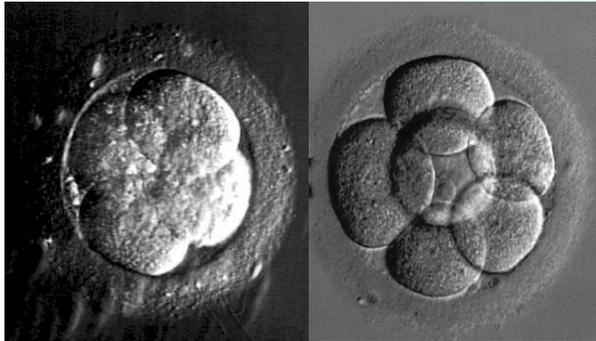
A good culture medium?

- Big difference between:
- Continuous culture of somatic cells
 - Needs “everything”
 - A complete diet
 - *May adapt to adverse culture conditions!*
- Short term culture of reproductive cells
 - Marginal growth, limited requirements for nutrients
 - *Adverse culture conditions may have adverse effects!*

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Embryo culture in ART

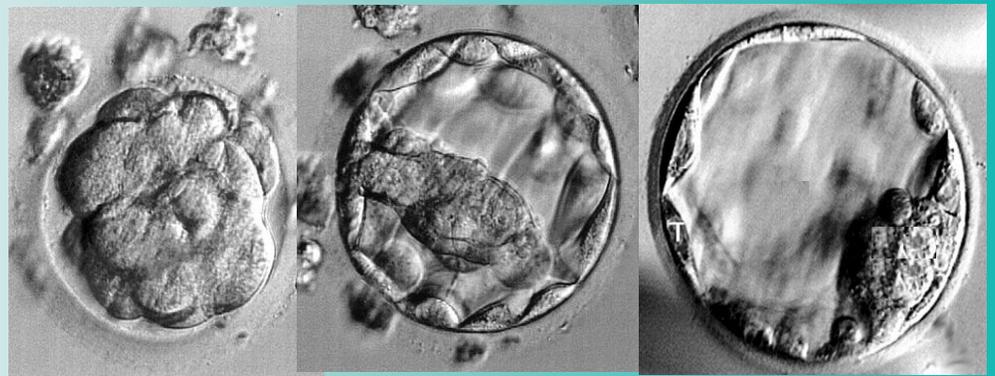
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“Autonomous phase”

“Environment dependent phase”

Sequential medium?
Single medium?
Still debated....



Design of embryo culture media

Different approaches

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Ca²⁺
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Mn
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Hoax-II

- **“Hands –off approach”**

- The silent embryo
- Do not stress it
- Permissive formulation
 - Close to physiological
- Absence of
 - Toxic compounds
 - Bioactive compounds
 - Hormones, growth factors....

- **“Hands-in approach”**

- Stress/stimulate the embryo
- Add bioactive components
 - Only the good embryos will respond?
- Absence of toxic components

The quiet embryo

- Henry Leese, York:
 - It seems that is it the quiet embryos that implant
 - Metabolically “silent”
- The less good embryos have a higher metabolic rate
 - Stressed ?

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A good culture medium?

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Hoax-II

- You must first define your criteria:
- **1) Performance?**
 - Clinical results – pregnancies
 - Safety
 - Documentation
- **2) Manufacturer**
 - Documentation
 - Design philosophy
 - Ok manufacturer, informative, good documentation, Reliable and convenient supply
 - Distributor, transport
 - Shelf life

Clinical results

What is your clinical success criteria?

- To obtain :
 - A high fertilization rate ?
 - Fast growing and good looking embryos?
 - Maximum number of good embryos ?
 - A high pregnancy rate from fresh transfers?
 - A low miscarriage rate
 - A high cumulative delivery rate (fresh + frozen)
 - A low rate of multiple pregnancies/deliveries
 - Or.....?
- Your success criteria will to a large extent:
 - define your selection parameters
 - have a strong influence on your culture strategy.

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Clinical results as endpoints

The inherent problem

- The hen or the egg or..?



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What's relevant endpoints when evaluating a culture media/culture systems

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Cl+
H₂CO₃⁻
Ca²⁺
K+
Mn
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PO₄
Glucose
Pyruvate
Lactate
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- In human ART
 - A high birth rate per embryo replaced is what we are aiming for, but:
- Surrogate endpoints
 - Fertilization rate (2PN rate)
 - Cleavage rate (correct kinetics)
 - Embryo morphology (empirical)
 - Correct number of chromosomes (karyotype)
 - Expression patterns (transcriptome)
 - Metabolism (metabolome)
- None of these will singly be a good predictor of embryo quality



What's relevant endpoints when evaluating a culture media/culture systems

- Is Animal models of any value?
- Pro:
 - Can do proper studies with animals
 - Large prospective studies
 - Follow up of off-spring
 - Can generate a lot of high quality data
- Con
 - Relevance for the human situation?
 - Which animal model?
 - Mouse
 - Bovine
 - Primate
 - Large differences between both species and within different strains

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Composition of culture media

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Cl-
H₂CO₃⁻
Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
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Hoax-II

- **Small ions**

- Na, K, Ca, PO₄
- HCO₃,

- **Energy substrates**

- Carbohydrates
 - Pyruvate, lactate, glucose
- Carboxylic acids

- **Amino acids?**

- **Macromolecules**

- **Others**

- Cofactors?
- Fe .. trace metals?
- Vitamins?
- Hormones?
- Growth factors?

- Physiological concentrations

- Different needs for gametes, zygotes and different embryo stages

- Some, all of them?

- Concentrations?

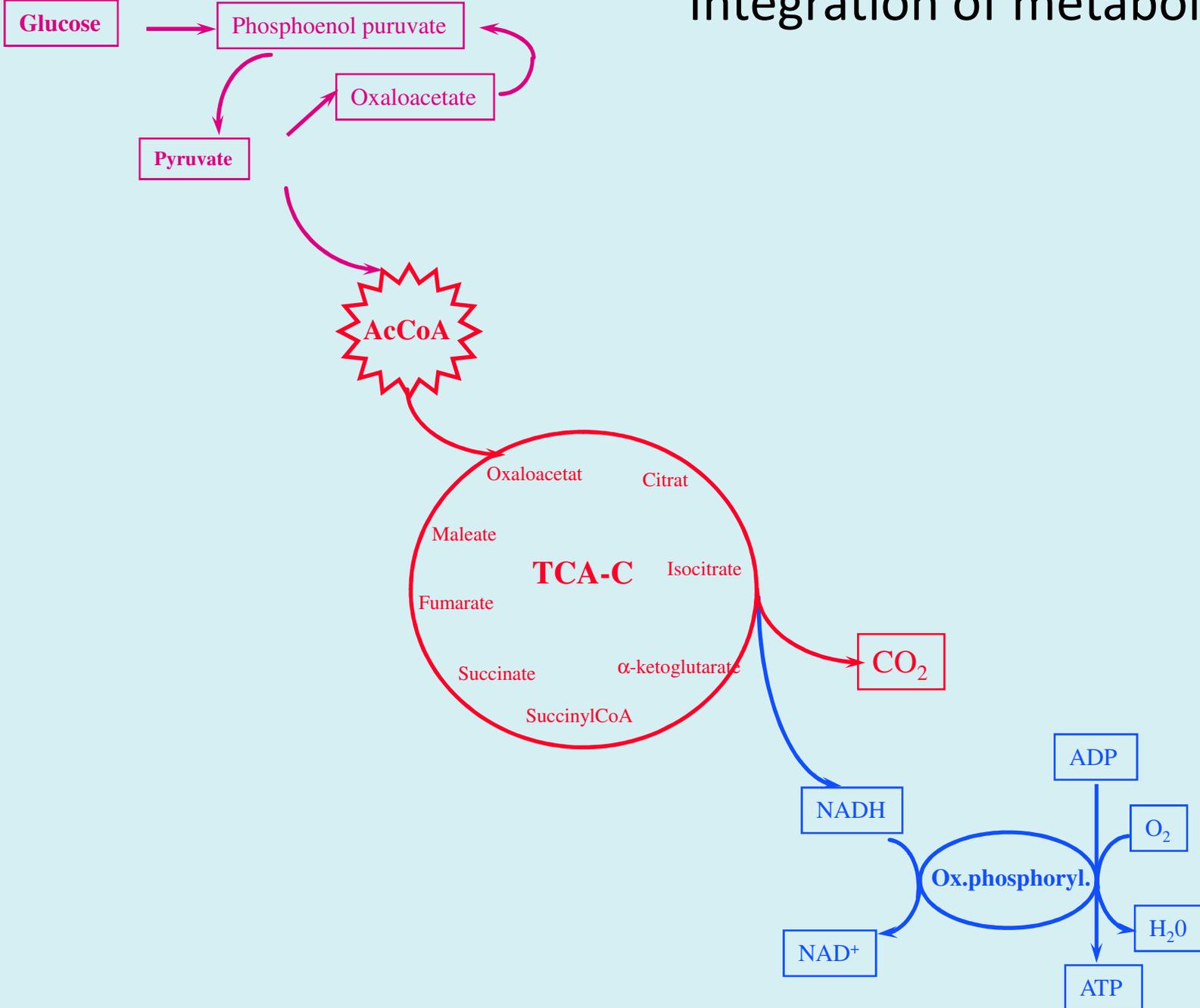
- hSA, Hyaluronic acid....??

- Needed?

- Beneficial?

- Detrimental?

Integration of metabolism

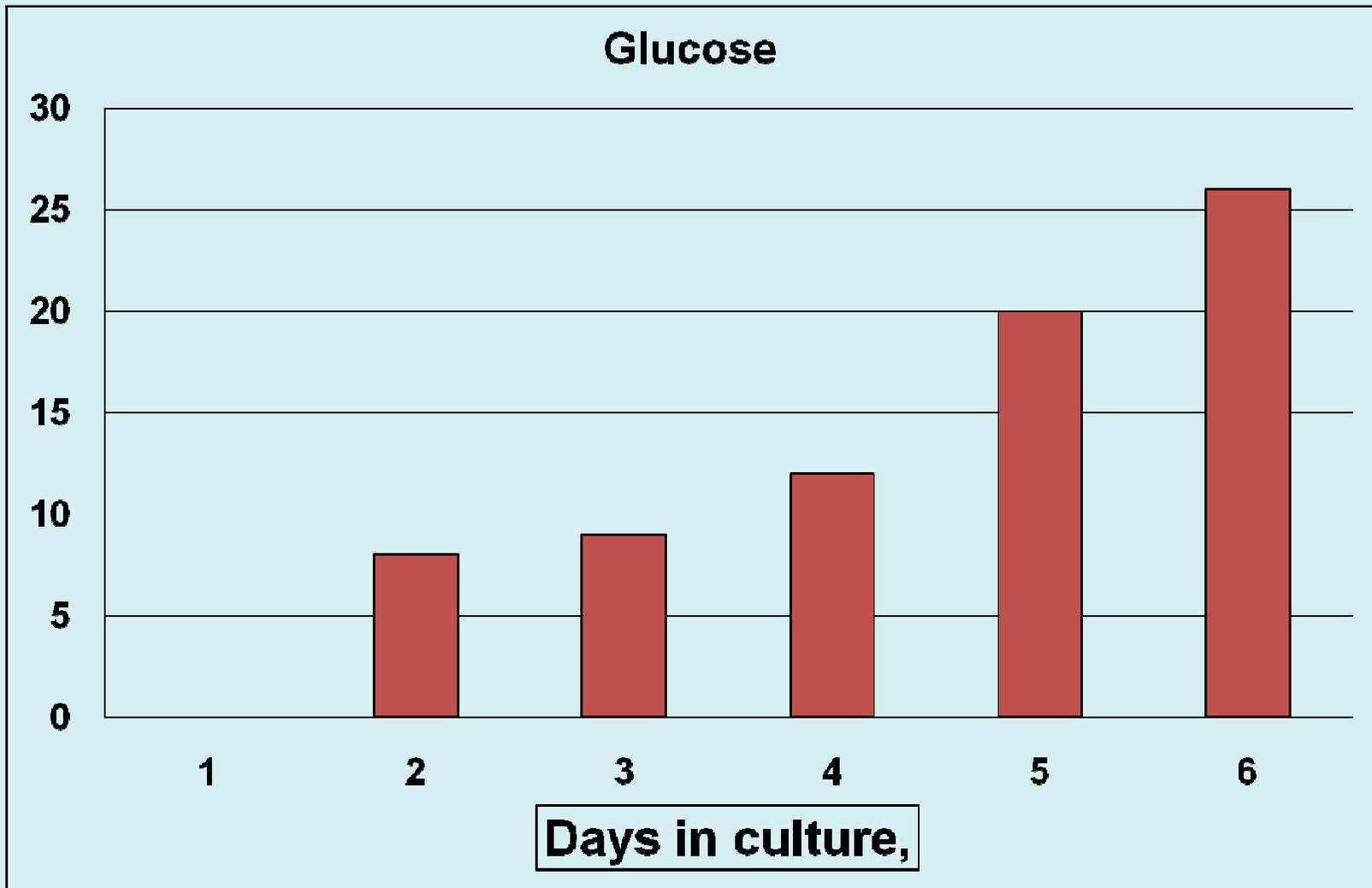


- Na+
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- Ca²⁺
- K+
- Mn
- Mg
- PO₄
- Glucose
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Utilisation of Glucose by human embryos

Schematically after Hardy & Leese, 1989

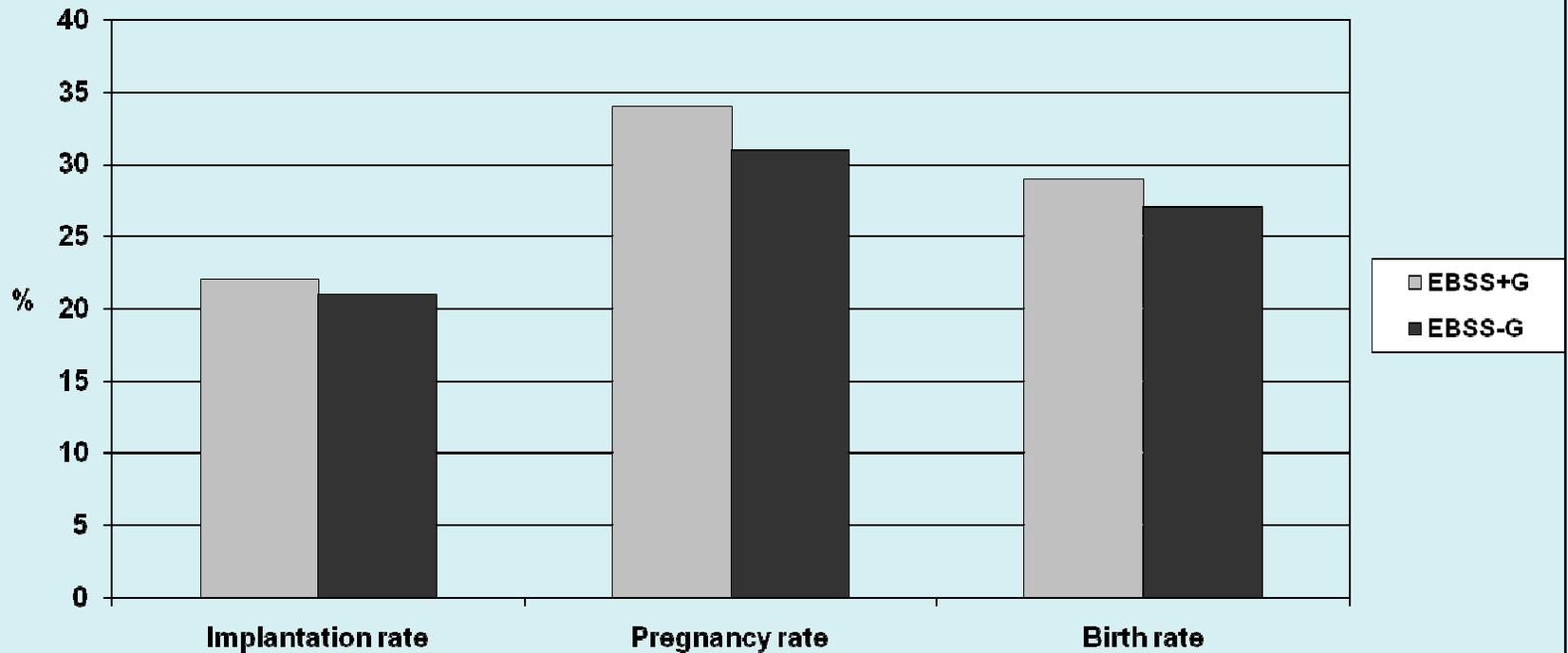
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Glucose free culture medium?

Na+
Cl+
H₂CO₃⁻
Ca₂+
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
Glutamine
Glutamate
Alanine
Leucine
Proline
Isoleucine
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Prospective clinical trial in Leeds

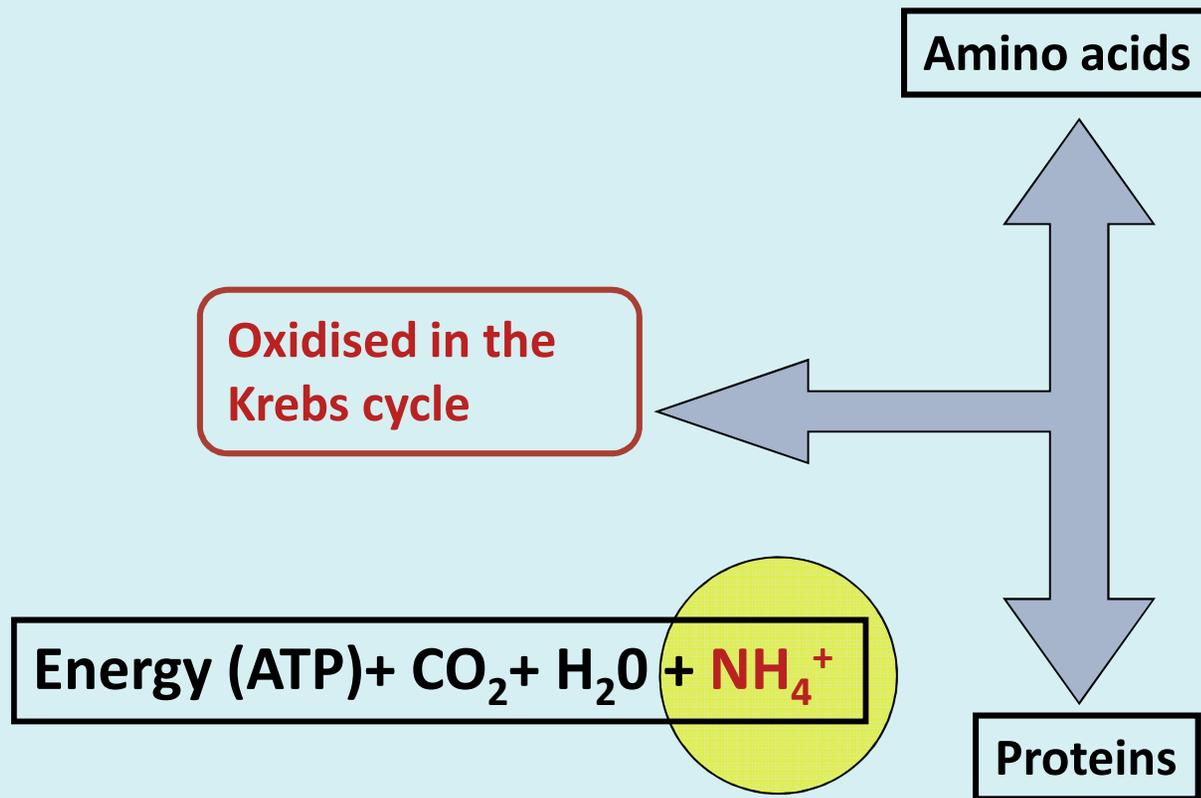


No significant differences

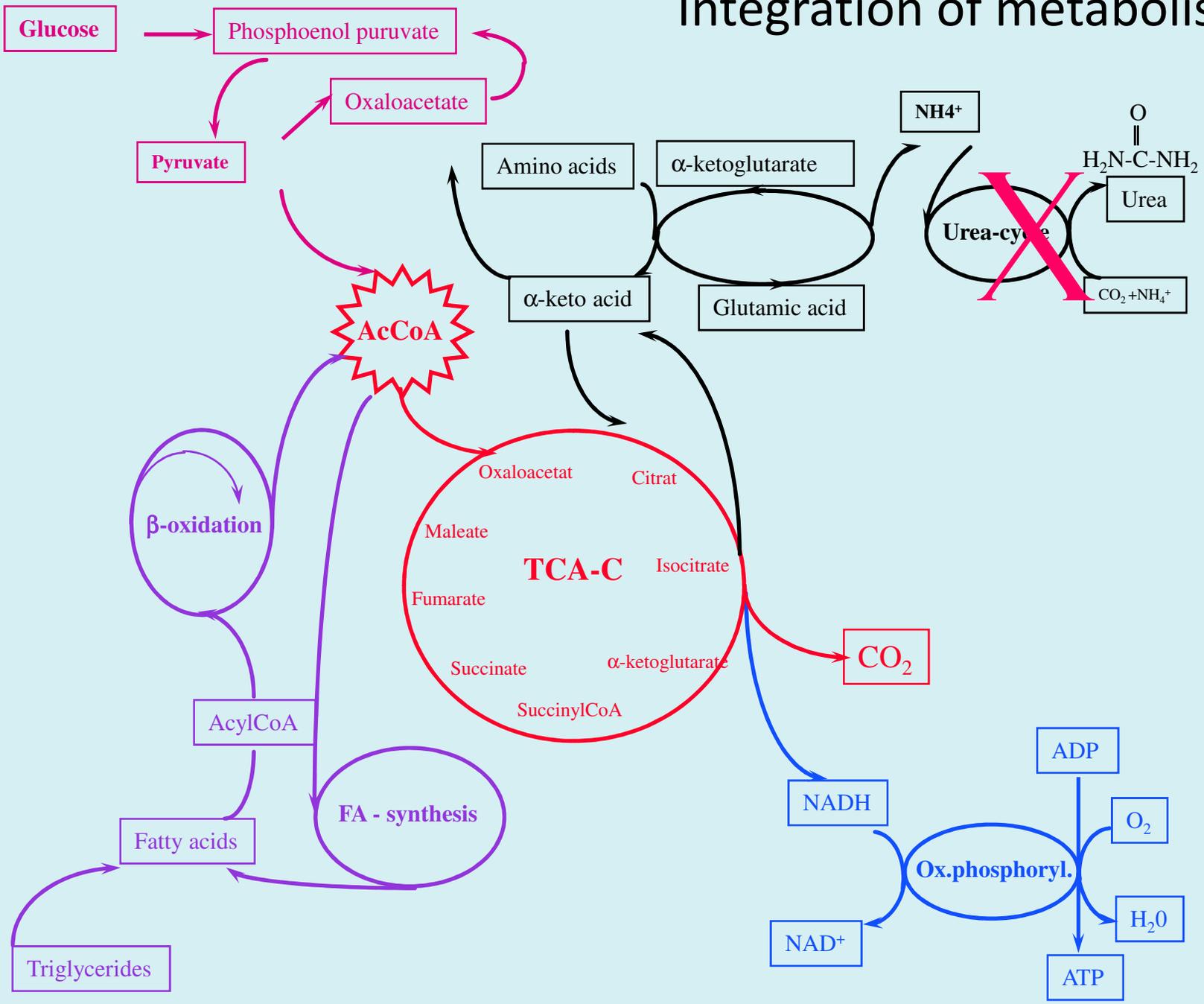
Coates et al Fertil Steril 72 229-232 1999

What about amino acids in the medium?

Na+
Cl-
H₂CO₃⁻
Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
Glutamine
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Integration of metabolism



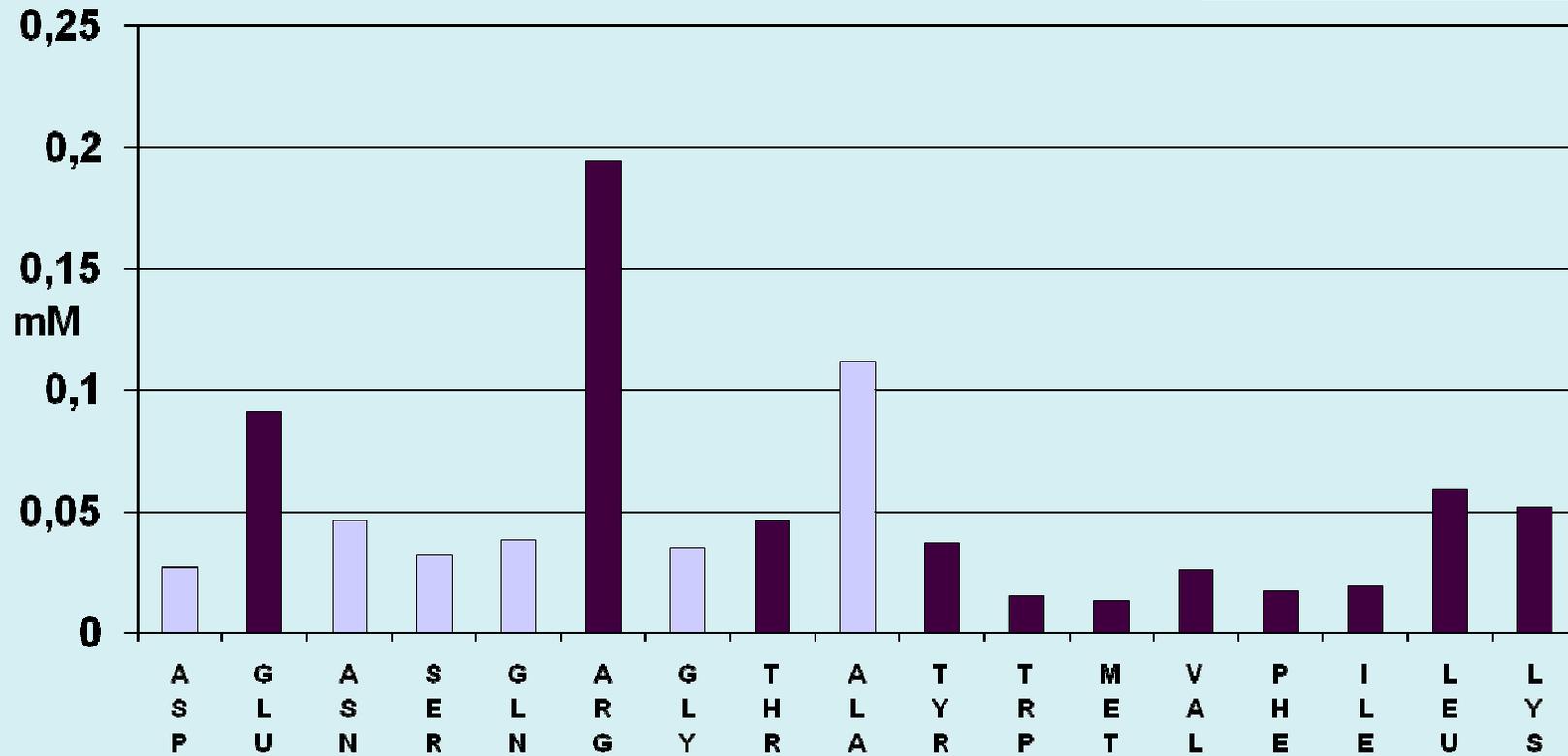
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Amino acids in the Human tubal fluid

Na+
 Cl-
 H₂CO₃⁻
 Ca²⁺
 K+
 Mn
 Mg
 PO₄
 Glucose
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 Lactate
 Citrate
 Glutamine
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Perfused human Fallopian tube

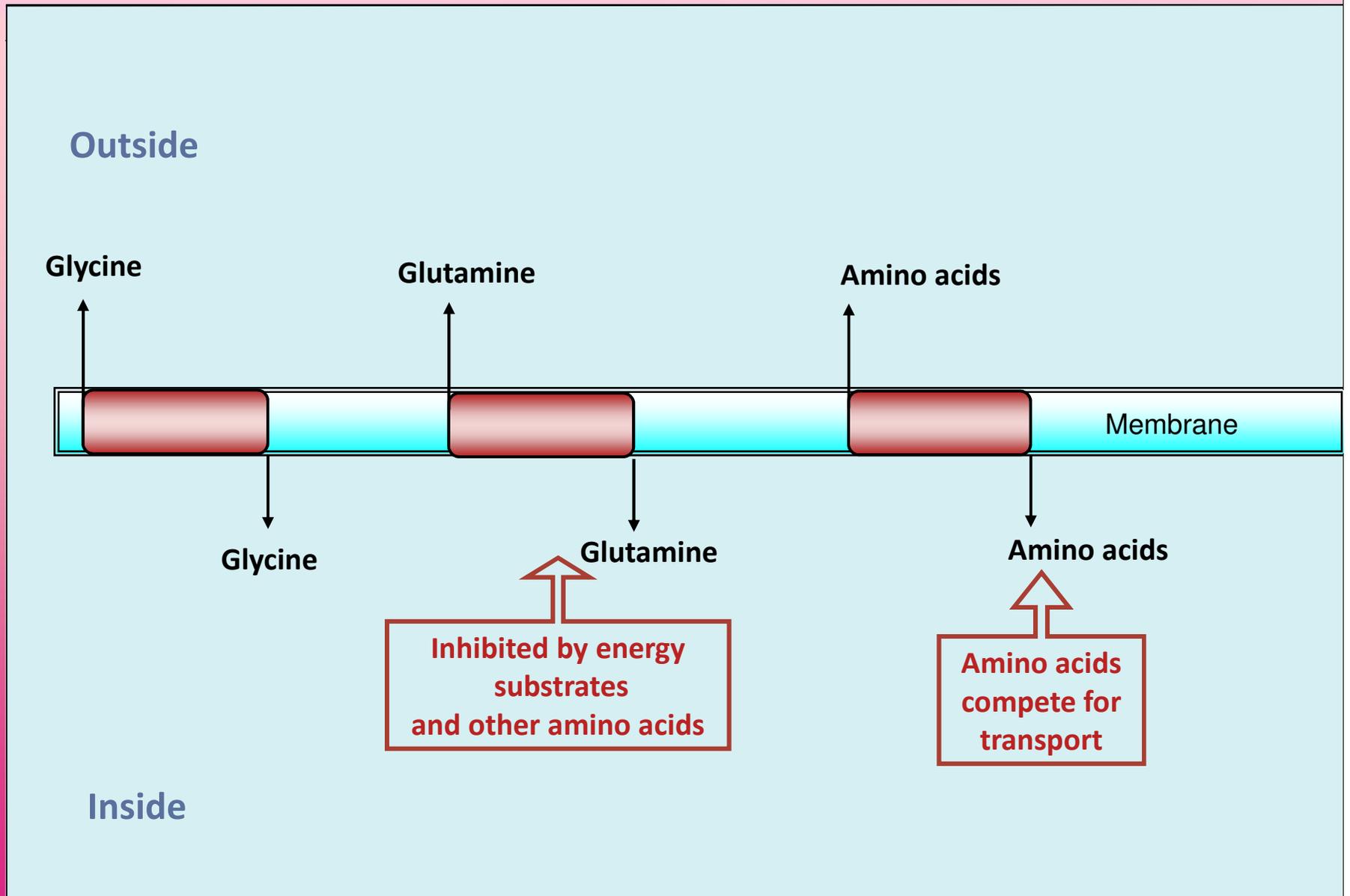
Essential AA
 Non-Essential AA



Tay et al. Human Reprod 12, 2451-2456, 1997

Amino acid transport across the membrane

Na+
Cl-
H₂CO₃⁻
Ca²⁺
K+
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PO₄
Glucose
Pyruvate
Lactate
Citrate
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Serum supplements to IVF-media (my best guesstimate 😊)

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- Mg
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- Hoax-II

10 These are not inert supplements!!

9

8 Contamination:

7 biochemical

6 microbiological

5 chemical

4

3 Bioactive substances

2

1 Whatnot...



2008

1 1 1 1 1 1 1 1 1 1 2 2 2 2

Growth factors, vitamins, cofactors and hormones ?

Na+
Cl+
H₂CO₃⁻
Ca²⁺
K+
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Glucose
Pyruvate
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Alanine
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Hoax-II

- GCSF
- Interleukins
- hCG
- LIF
- IGF's
- Growth hormone
-
- Prostaglandins
- Steroids
- Thyroid hormones
- NAD+, FAD+

- How to select?
 - Presence in the genital tract?
 - Expression patterns?
 - In vitro effects?
 - Growth, cell biology?
 - Clinical trials
 - Endpoint?
- One, some or all for them?
- When.. at what stage?
- Concentration?

You should really know what you are doing before you start add growth factors and hormones to the ART culture media!!

Challenges In Vitro

Na+
Cl+
H₂CO₃⁻
Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
Glutamine
Glutamate
Alanine
Leucine
Proline
Isoleucine
Tryptophan
Choline
B12
B2
Ascorbate
FSH
GH
Estradiol
Insulin
SSR
SSS
hSA
BSA
IGF-1
GCSF
Il-6
NANOG
FactX
GanCF
XCZjk
SillyF
Cosmetin
Hoax-II

- **Antioxidants?**

- Combat the Reactive Oxygen Species (ROS)
 - Can be generated by
 - Sperm cells, Leucocytes..
 - Will occur as normal by-product of normal cellular metabolism
 - The composition of the culture media can influence the rate of ROS generation
 - Most commercial media contain unspecific and some contain also specific antioxidants

- **O₂ concentration**

- 5% vs. 20% O₂ ?? - still debated ..
 - Pendulum now swings towards 5%?

- **Grouped or single culture**

- No consistent data

Challenges - In vitro

Na+
Cl+
H₂CO₃⁻
Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
Glutamine
Glutamate
Alanine
Leucine
Proline
Isoleucine
Tryptophan
Choline
B12
B2
Ascorbate
FSH
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• Problem:

- Temperature
 - Oocytes do NOT tolerate hypothermia
- pH
 - Physiological pH regulation sensitive to CO₂ concentration
- Osmolality
 - Evaporation may occur
- Light
 - Direct sun light may harm culture media
 - UV-light may damage embryos

• Solution:

- Work fast
 - Work fast, work fast, work fast
 - Have some means for temperature control
- Work fast
- Add buffers (HEPES etc..)
- Work fast
- Cover with oil
- Add osmolytes?
- Varies from 260 to 290 mosm in different commercial media
- Avoid exposure to high energy light

Challenges - In vitro

Na+
Cl+
H₂CO₃⁻
Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
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FactX
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XCZjk
SillyF
Cosmetin
Hoax-II

• Problems:

• Micro organisms

- Follicular aspirates often contains vaginal flora
 - Cotell et al., Fertil. Steril., 66, 776-780, 1996.
- Semen preparations are in principle contaminated with micro organisms
- Operators and in vitro conditions may introduce micro organisms

• Embryos are sensitive to endotoxins

- Nagata and Shirakawa, Fertil. Steril., 65, 614-619, 1995

• Solution:

- Add antibiotics
 - Penicillin
 - Streptomycin
 - Gentamycin
- Good working routines
- Keep working surfaces, microscopes and incubators clean
- Adequate air quality

Challenges - In vitro

Na+
Cl+
H₂CO₃⁻
Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
Glutamine
Glutamate
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Hoax-II

- **Problem:**
- **Chemical contaminants**
 - From media components or media production
 - Volatile Organic Components(VOC)

- **Solution:**
- Source media from a producer with good Quality Control
- Air quality in lab and incubators
 - Clean or purge new equipment
- Use utensils and disposables that are intended for use in human ART

Laboratory hardware, utensils and consumables for the ART lab

Na+
Cl+
H₂CO₃⁻
Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
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- Some of it general purpose equipment
- Some of it adapted to ART
 - Not necessarily well adapted
- Some of it designed for the ART lab
 - Optimal design?
 - Objectives?
 - Validated design?
 - ?

- The ART lab needs to be totally redesigned:
 - Purpose built
 - Validated
 - No touch – closed systems
 - Automatic
 - Traceability
 - QC/QA

Epigenetic effect of in vitro culture?

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Cl+
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Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
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Glutamate
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- Epigenetic effects:
 - A change in expression pattern of genes and thereby the protein content in a cell without a change in the DNA sequence.
 - i.e. control over transcription, translation and posttranslational modification
- Genomic Imprinting
 - Epigenetic modification of certain regions in gametes and embryos to ensure uni-parental expression of some important genes

Epigenetic effect of in vitro culture?

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- Epigenetic modification of gene expression is a normal mechanism for cell differentiation and adaptation to the environment.
 - It is a frequent event in a cell
- It would perhaps be more surprising if ART didn't have an epigenetic effect than if it does have one....
- Epigenetic effects may be mediated through chemical modification of
 - Histones
 - Open/close regions of DNA for transcription
 - DNA in promotor regions
 - Open/close a gene for transcription

What about assisted reproduction in animals?

- **IVF culture in cattle and sheep results in wrong imprinting**
 - Khosla et al. HRU 7, 419, 2001
- **ART in mice results in aberrant imprinting of the *Igf2-H19* region and a range of other imprinted genes**
 - Rivera et al., Hum Mol Genet. 17 1, 2008
- **Different culture media have different imprinting effects.**
 - Doherty et al Biol. Reprod.62, 1526, 2000
- **Presence of insulin in the culture media will change imprinting pattern in the *Igf2-H19* region**
 - Shao et al, Comparative Med, 57, 482, 2007

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What about assisted reproduction in humans ?

- Been looking for possible imprinting defects in ART offspring
- Primarily looking for known and very rare imprinting disorders
 - Angelmann
 - Prader-Willi
 - Beckwith-Wiedemann
- ART may be associated with a small increase in imprinting related disorders
 - Difficult to know for certain.. Low frequency events
- BUT....what about Large Offspring Syndrome (LOS)??

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H₂CO₃⁻
Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
Glutamine
Glutamate
Alanine
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What about assisted reproduction?

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The Journal of Clinical Endocrinology & Metabolism 92(9):3441-3445
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doi: 10.1210/jc.2006-2465

***In Vitro* Fertilization Improves Childhood Growth and Metabolism**

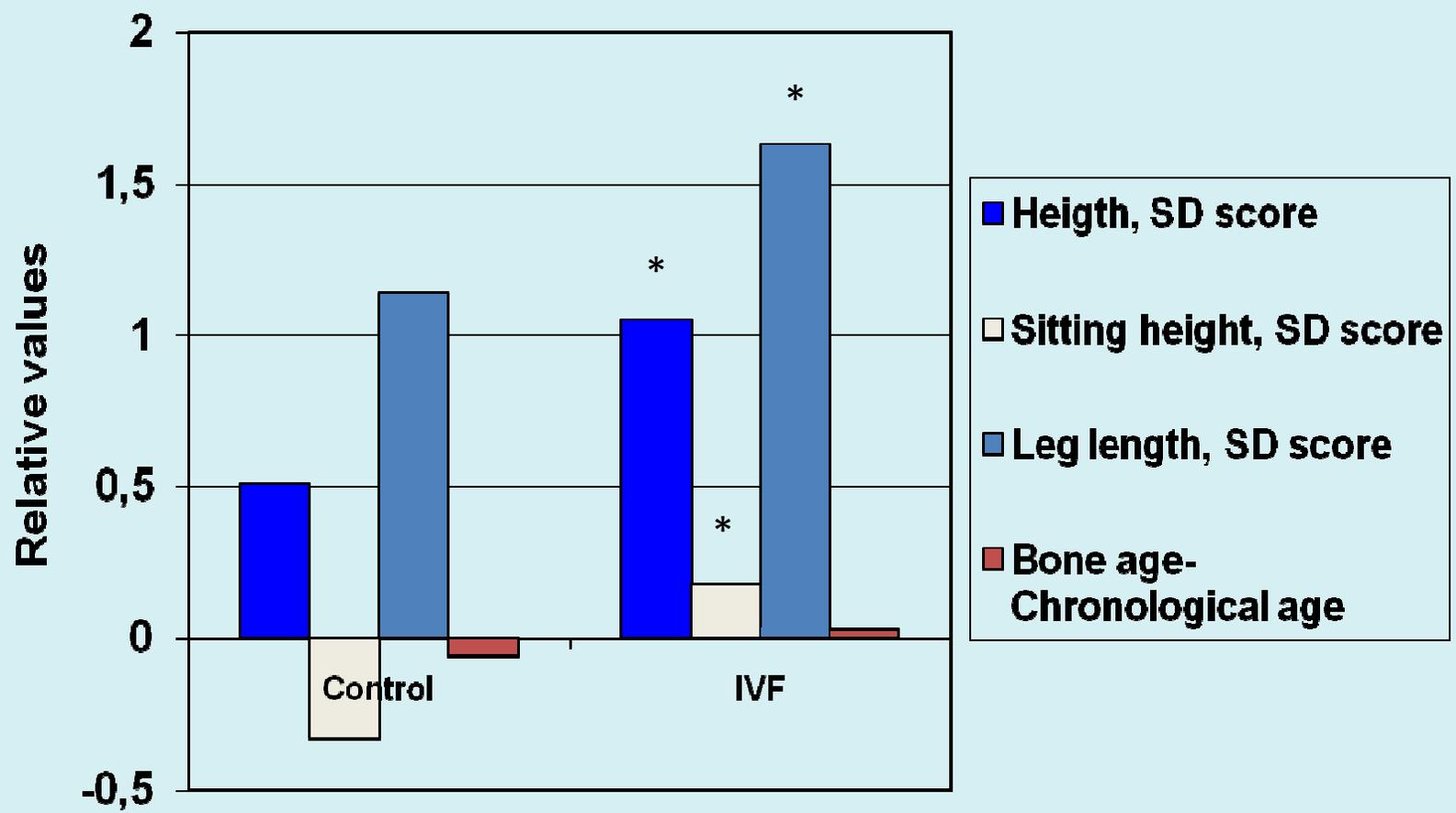
Harriet L. Miles, Paul L. Hofman, John Peek, Mark Harris, Dyanne Wilson, Elizabeth M. Robinson, Peter D. Gluckman, and Wayne S. Cutfield

The National Research Centre for Growth and Development and Liggins Institute (H.L.M., P.L.H., M.H., D.W., P.D.G., W.S.C.) and Department of Community Health (E.M.R.), University of Auckland, Auckland 1010, New Zealand; and Fertility Associates (J.P.), Auckland 1051, New Zealand

Improves... 😊

Children conceived after IVF vs. natural conception at age 6-7

- Na+
- Cl+
- H₂CO₃⁻
- Ca²⁺
- K+
- Mn
- Mg
- PO₄
- Glucose
- Pyruvate
- Lactate
- Citrate
- Glutamine
- Glutamate
- Alanine
- Leucine
- Proline
- Isoleucine
- Tryptophan
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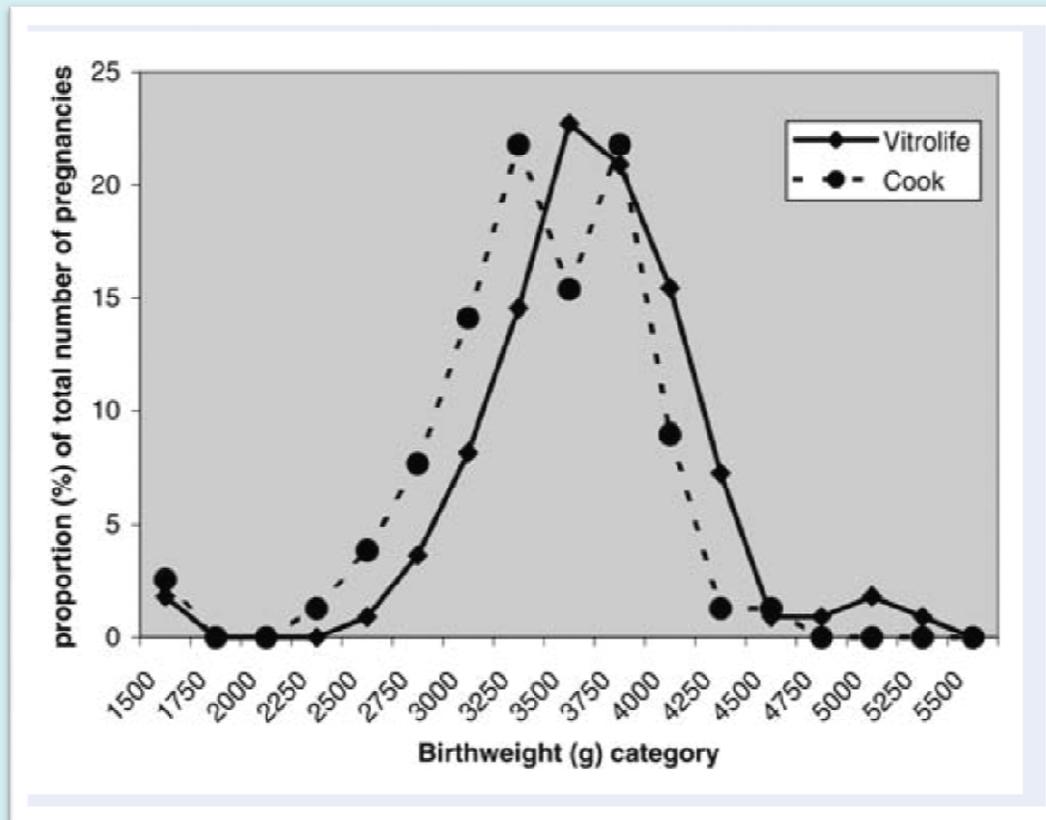
Miles at al. J. Clin. Endocr Metab 92, 3441-45, 2007

Standard score = $\frac{\chi - \mu}{\sigma}$

Effect of culture media on birth weight!!!

This is most likely mediated by epigenetic effects

Na+
Cl+
H₂CO₃⁻
Ca²⁺
K+
Mn
Mg
PO₄
Glucose
Pyruvate
Lactate
Citrate
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Glutamate
Alanine
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SillyF
Cosmetin
Hoax-II



Mean weight:

Vitrolife: 3453g

COOK: 3208g

p < 0,003

Dumoulin et al., Effect of in vitro culture of human embryos on birthweight of newborns ,
Hum. Reprod. 25, 605–612, 2010,

Imprinting effects of ART in humans?

- Growing evidence that ART may lead to differences in
 - Growth, bone length,
 - Fat distribution
 - Cardiometabolites
 - Lipid metabolism
 - Carbohydrate metabolism
 - Systolic blood pressure
- Between adolescents born after ART and spontaneous conception
- This may be caused by aberrant imprinting
 - Ceelen et al., Fertil Steril, 90, 1662, 2008
 - Ceelen et al., Human Reprod, 23, 2791, 2008
 - Ceelen et al., J Clin Endocrinol Metab 93, 1682, 2008
 - Ceelen et al., J Clin Endocrinol Metab 92, 3417, 2007
 - Ceelen et al., Hum Reprod, Advance Access published August 1, 2008

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PO₄
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IGF-1
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Summary: Culture media

- We have to demand more openness from the manufacturers.
- We must be offered science based formulations.
- We should be informed about the rationale behind formulations and ingredients.

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Alanine
Leucine
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Summary:

New criteria to evaluate embryo culture?

- Traditionally we have only focused on efficiency
 - i.e. pregnancy/implantation rates
 - We should focus more on risks like epigenetic effects of:
 - Culture conditions and Culture media
 - Utensils and consumables
 - Gamete and embryo Manipulations
 - We should be proactive now..not reactive later..

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Glucose
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