Non-invasive Profiling of Embryos

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Australia
With the move to single embryo transfers the need for methods to quantify viability is paramount.
For 30 years Morphology has been the only criterion available upon which to base the decision of which embryo to transfer.
Timelapse
Timelapse = Morphology
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            Time
With the development of commercial Time-Lapse systems we can now be more definitive about when key Morphological events occur.
With the development of commercial Time-Lapse systems we can now be more definitive about when key Morphological events occur. This will facilitate the creation of algorithms for successful embryo selection.
Although Time-Lapse systems increase the amount of information available about an embryo, it cannot assess its physiology.
Beyond Morphology
Uptake

- Glucose
- Pyruvate
- Amino Acids
- Other Sugars
- Oxygen

Production

- Lactate
- Ammonium
- Amino Acids
- Enzymes, eg LDH
- sHLA-G
- HOXA10 regulator
- PAF
- Other Peptides & Factors

µl drop of defined culture medium

Metabolomics / Proteomics

Metabolism
Metabolism
So Much More than ATP!
Metabolomics and Metabolism

- Metabolomics
  Systematic study of the unique chemical fingerprints that specific cellular processes leave behind

- Targeted Metabolomics (Metabolic activity)
  Analysis of specific metabolic pathways, their relative activities and their regulation
Metabolomics

“Systematic study of the unique chemical footprints that specific cellular processes leave behind”
Metabolomics: Driftnet fishing in a sea of metabolites
NIR and Raman Spectroscopy

- IR, NIR and Raman are the most common vibrational spectroscopies for assessing molecular motion and fingerprinting species.
- NIR is based on absorption by vibrational mode overtones in molecules.
- RAMAN is based on inelastic scattering of monochromatic excitation source.

Brison (2007) RBM Online 15, 296-302
What is Measured?

- **Thesis**
  - A viable embryo has a different metabolome than a non-viable embryo and this can be evaluated in the culture media the embryo grows in.

- **Clinically**
  - How the embryo modifies its environment

- **Biologically**
  - Changes in concentrations of:

<table>
<thead>
<tr>
<th>Functional Groups</th>
<th>Constituents</th>
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<tbody>
<tr>
<td>• CH</td>
<td>• Albumin</td>
</tr>
<tr>
<td>• NH</td>
<td>• Lactate</td>
</tr>
<tr>
<td>• OH</td>
<td>• Pyruvate</td>
</tr>
<tr>
<td>• SH</td>
<td>• Glutamate</td>
</tr>
<tr>
<td>• C=C</td>
<td>• Glucose</td>
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</table>

Advantages of Metabolomics by NIR

- Non-invasive
- Quick
- Analyse whole sample matrix; i.e. a lot of individual molecules

Disadvantages of Metabolomics

- Do not determine the identity or quantity of a specific nutrient / metabolite
- Requires generation of specific algorithms
- To date this approach has not been validated
Targeted Metabolomics
Targeted Metabolomics: After one fish …..
Targeted Metabolomics: After one fish …….. and you will know how big it is!!
With this approach one can measure nearly any nutrient by linking it to a coupled reaction with a fluorescent co-factor or label. This method can be used to analyze fluids in the nano (10⁻⁹) and pico (10⁻¹²) litre range. Consequently it can be used to quantitate nutrient utilization by a single cell in real time, over just a few hours.

With this approach one can measure all free amino acids. This method can be used to analyze fluids in the microlitre range. It can be used to quantitate nutrient utilization by a single cell in real time, over just a few hours.

Both methods are quantitative and completely non-invasive.
Understanding Embryos
Energy is fundamental to the survival and development of any cell.

The metabolism of the preimplantation embryo changes during development and differentiation.

Loss of metabolic regulation is associated with poor development and a loss of viability.

Uptake

Glucose

Production

Lactate
One can compare this pattern of metabolic activity to embryos developed in vivo.
Rate and Fate Hypothesis
Amino acids and vitamins prevent culture-induced metabolic perturbations and associated loss of viability of mouse blastocysts

Michelle Lane¹ and David K.Gardner²

Institute of Reproduction and Development, Monash University, Clayton, Victoria, 3168, Australia
Long-term Effects of Short-term exposure to conditions that induce Metabolic Perturbations

Blastocysts were developed in vivo, flushed and their metabolism measured immediately OR after 6h incubation in the presence or absence of amino acids and vitamins

*; significantly different compared to other treatments (P<0.05)

a; like letters significantly different (P<0.05)

**Long-term Effects of Short-term exposure to conditions that induce Metabolic Perturbations**

**Table IV.** Effect of short-term culture for 6 h on mouse blastocyst viability after transfer

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Implantation sites/blastocyst transferred (%)</th>
<th>Fetuses/blastocyst transferred (%)</th>
<th>Fetuses/implantation site (%)</th>
<th>Fetal weight (mg) (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>In vivo</em></td>
<td>88</td>
<td>70</td>
<td>80</td>
<td>306.3 ± 7.5</td>
</tr>
<tr>
<td>mMTF</td>
<td>74*</td>
<td>66</td>
<td>89</td>
<td>248.7 ± 6.4*</td>
</tr>
<tr>
<td>mMTF + AA + VIT</td>
<td>81</td>
<td>75</td>
<td>93</td>
<td>277.4 ± 5.9</td>
</tr>
</tbody>
</table>

*n = 48 in-vivo blastocysts transferred; n = 54 blastocysts transferred after culture in modified mouse tubal fluid (mMTF) medium and mMTF + AA + VIT.
AA + VIT: mMTF supplemented with Eagle’s 20 amino acids and vitamins.
All recipient females became pregnant.
*Significantly different to in-vivo-developed blastocysts (P < 0.05).*

Oxygen
In vitro preimplantation embryo culture: Oxygen

- historically tissue culture in atmospheric oxygen (~20%)
  - most tissues ~ 5% O$_2$
  - oviduct and uterus (in vivo) O$_2$ levels between 2 – 8% (Fischer and Bavister, 1993)

- studies have reported superior embryonic development with reduced oxygen (5 – 7%):
  - sheep (Tervit, et al. 1972; Thompson, et al., 1990)
  - mouse (Quinn & Harlow, 1978, Harlow & Quinn, 1979, Gardner & Lane, 1996)
  - pig (Wright, et al. 1977; Berthelot & Terqui, 1996)
  - cow (Thompson, et al., 1990)
  - goat (Batt, et al., 1991)
  - and human (Catt & Henman, 2000, Meintjes, et al., 2009)
All stages of preimplantation embryo development are vulnerable to atmospheric oxygen

- 0.5 hour delay by 1st cleavage (2 cell)
- 0.8 hour delay by 2nd cleavage (4 cell)
- 3 hour delay by 3rd cleavage (8 cell)

- Detrimental effects of atmospheric oxygen on early embryo development are irreversible

- Decreased blastocyst development

- Reduction cell numbers

- Culminate in loss of viability

Wale and Gardner, RBM Online (2010) 21, 402 - 410
Atmospheric oxygen alters embryo gene expression

- culture in 5% $O_2$ is associated with fewer perturbations in the global pattern of gene expression
- more closely resembles that of the in vivo control embryo (Gardner and Lane, 2005; Rinaudo et al., 2006)

Atmospheric oxygen alters the embryonic proteome

- protein profile from embryos which were cultured at 5% $O_2$ more closely resembled in vivo developed embryo profiles (Katz-Jaffe et al., 2005)
Regulation of Embryo Metabolism and Viability by Oxygen
Oxygen Regulates Amino Acid Turnover and Carbohydrate Uptake During the Preimplantation Period of Mouse Embryo Development

Petra L. Wale and David K. Gardner

Department of Zoology, University of Melbourne, Parkville, Victoria, Australia
Box-plots: cleavage stage individual amino acid utilisation

![Box-plot diagram showing amino acid utilisation.](image)

$n = 330$ (33 replicates, 10 embryos per sample) per treatment

* $p<0.05$, ** $p<0.01$, *** $p<0.001$
Increased total amino acid turnover for cleavage stage embryo cultured atmospheric O\textsubscript{2}

mean ± SEM, n = 330 (33 replicates, 10 embryos per sample) per treatment

*** p<0.001
Box-plot: post-compaction individual amino acid utilisation

$n = 75$ (25 replicates, 3 embryos per sample) per treatment

* $p<0.05$, ** $p<0.01$
Decreased amino acid turnover from post-compaction embryos cultured in atmospheric O$_2$

mean ± SEM, n = 75 (25 replicates, 3 embryos per sample) per treatment

* p<0.05
Effect of Oxygen on Mouse Blastocyst Glucose Metabolism

Biomarkers for Embryo Selection
Tests of Embryo Viability need to be:

- Non-invasive
- Quantitative
- Quick
- Affordable
Metabolic Activity & Normality
Attempts to Relate Embryo Viability and Metabolism

- Glucose consumption by day 10 bovine blastocysts

Bovine blastocysts which had a glucose uptake of > 5µg/h developed better in vitro and in utero than embryos with an uptake below this value.

This was achieved using spectrophotometric methods. Although suitable for day-10 bovine blastocysts, with a diameter of ~1000µm, such technology could not be used for earlier stages or smaller blastocysts.

Attempts to Relate Embryo Viability and Metabolism

- Glucose consumption by day 4 mouse blastocyst
  
  In order to measure the nutrient utilisation by day 4 mouse blastocysts (diameter of ~100µm, i.e. 10 times less than a day 10 bovine blastocyst), ultramicrofluorescence was employed.

Attempts to Relate Embryo Viability and Metabolism (Retrospective)

Mouse blastocysts that were deemed viable through embryo transfer exhibited a significantly higher glucose uptake than non-viable embryos.

Females took up 13% more glucose than males.

Attempts to Relate Embryo Viability and Metabolism (Prospective)

Attempts to Relate Embryo Viability and Metabolism (Prospective)

Attempts to Relate Embryo Viability and Metabolism (Prospective)

Assessment of Human Embryo Metabolism
Metabolism of Human Blastocysts with the Same Score (4AA) from the Same Patient

Glucose consumption of single post-compaction human embryos is predictive of embryo sex and live birth outcome

David K. Gardner¹,* , Petra L Wale¹, Rebecca Collins², and Michelle Lane²

¹Department of Zoology, University of Melbourne, Melbourne, Australia ²Repromed, Dulwich, Adelaide, Australia
Glucose Uptake on day 4 of development and pregnancy outcome

Glucose uptake (pmols/embryo/h)

Pregnant  Non-pregnant

**
Glucose Uptake on day 4 of development and relation to embryo sex

**27% increase by females**
Glucose Uptake on day 5 of development and pregnancy outcome

Glucose uptake (pmols/embryo/h)

Pregnant  Non-pregnant

**
Glucose Uptake on day 5 of development and relation to embryo sex

19% increase by females
Blastocyst Quality and Metabolism

Glucose uptake (pmols/embryo/h)

- 3AA or >
- < 3AA
- M

Pregnant
Not Pregnant
When Morphokinetics Meets Metabolism

Lisa Lee and David K Gardner (2014), unpublished
Methods: Embryo development and analysis

IVF F1 mice CBAxC57BL/6

Culture media to be collected for amino acid analysis

“Faster”

“Slower”

Assessment of embryo transfers

Stained for cell numbers or transferred to a recipient mouse

Day 14

Day 14

Day 5

Day 4

Day 3

Day 2

Culture media to be collected for amino acid analysis

Carbohydrate metabolism analysis
Time of First cleavage division

Frequency distribution of 2-cell cleavage times

**Frequency distribution of 2-cell cleavage times**

Number of embryos

Hours post fertilisation (h)

Faster  Slower

n= 183 embryos. 5 replicates
Meseguer et al. (2011) Human Reprod, 26(10) 2658-2671
First cleavage division

Cell numbers with respect to quartiles

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<tr>
<th>Quartiles</th>
<th>Cell numbers</th>
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<tbody>
<tr>
<td>1</td>
<td>80</td>
</tr>
<tr>
<td>2</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>120</td>
</tr>
<tr>
<td>4</td>
<td>40</td>
</tr>
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Faster embryos have a **higher total cell number**

n= >43 embryos per group. 5 replicates
p<0.01**, p<0.001***
Day 5 Carbohydrate Metabolism

Rate of Glucose consumption and Lactate production

Faster embryos consume more glucose and produce more lactate

n = >31 embryos per group. 12 replicates
*p<0.05, p<0.001***
Day 5 Carbohydrate Metabolism

Faster embryos have a lower glycolytic rate

n = >31 embryos per group. 12 replicates
*p<0.05
Dual stain of blastocysts measured for carbohydrate metabolism

Faster embryos have significantly more inner cell mass cells and subsequently, a higher total cell number.

n= >23 embryos per group. 12 replicates

***p<0.001
Dual stain of blastocysts measured for carbohydrate metabolism

Faster embryos have significantly higher ICM:TE ratio

n= > 23 embryos per group, 12 replicates

**p<0.01
Amino acid metabolism

Aspartate (Faster embryos *consume more* aspartate)
- Rate-limiting factor in activity of Malate-Aspartate Shuttle, which regulates carbohydrate metabolism

Glutamate (Slower embryos *produce* glutamate)
- Known to play a role in ammonium sequestration via its conversion to glutamine

Embryo transfers

n= 125 embryos per group
25 replicates
*p<0.05, p<0.001***
Summary

Faster embryos
- ↑ Blastocyst cell numbers
- ↑ Fetal development per implantation

Developmental kinetics

Carbohydrate metabolism
- ↑ ICM
- ↑ Glucose consumption
- ↓ Lactate production
- ↓ Glycolytic rate

Amino acid metabolism
- ↑ Aspartate consumption
- Glutamate consumption instead of production

• Aspartate has been shown to regulate carbohydrate metabolism
The Future of IVF?
Medium Introduced:
Changing metabolite pool, introduction of stage specific factors etc.

Medium Expelled:
Removal of toxins such as ammonium

Channel or microchamber created in biologically compatible, gas permeable and transparent material, such as PDMS

Embryos cultured individually or in groups in volumes ranging from nano to microlitres

Sequential Embryo Assessment

- **106-108 hours post insemination / ICSI**: Blastocyst >3AA
- **66-68 hours**: Top quality 8-cell
- **42-44 hours**: Top quality 4-cell
- **25-26 hours**: Early 2-cell
- **16-19 hours**: Pronuclear embryo assessment

**Analysis of genomics, proteomics and metabolomics is now feasible and can be used to augment morphological grading systems.**

Conclusions

- Non-invasive targeted analysis of nutrient utilisation can be used to further understand the relationship between metabolism and embryonic development and viability.
- Even a brief perturbation in embryonic metabolism can have significant downstream effects on fetal development.
- Atmospheric oxygen has a profound (negative) effect on embryo metabolism.
- There is a significant correlation between glucose utilisation on day 4 and day 5 in the human and subsequent pregnancy outcome, and metabolic activity appears independent of human blastocyst grade.
- The relationship between cleavage timings and subsequent embryo metabolism is under further examination.
- Microfluidic devices hold great promise for rapid, and multiple, metabolic analyses of embryo culture media samples.
# Reproductive Biology & ART Research Laboratories

<table>
<thead>
<tr>
<th>Fellow</th>
<th>PhD Student</th>
<th>Collaborator</th>
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<tbody>
<tr>
<td>Dr Alex Harvey</td>
<td>Natalie Binder</td>
<td>Dr Petra Wale</td>
</tr>
<tr>
<td>Dr John Sheedy</td>
<td>Rebecca Kelley</td>
<td>Melbourne IVF</td>
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<tr>
<td></td>
<td><strong>Lisa Lee</strong></td>
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<tr>
<td>Research Assistants</td>
<td>Jarmon Lees</td>
<td>Basak Balaban</td>
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<tr>
<td>Dr Yu May So</td>
<td>Cynthia Martin</td>
<td>American Hospital, Istanbul</td>
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<tr>
<td>Mai Troung</td>
<td>Nic Tan</td>
<td>Dr Denny Sakkas</td>
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