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13 ABSTRACT

14 Measuring the metabolism of early embryos has the potential to be used as a
15 prospective marker for post-transfer development, either alone or in conjunction with
16 other embryo quality assessment tools. This is necessary to maximise the opportunity
17 of couples to have a healthy child from assisted reproduction (ART) and for livestock
18 breeders to efficiently improve the genetics of their animals. Nevertheless, although
19 many promising candidate substrates (such as glucose uptake) and methods (such as
20 metabolomics utilizing different spectroscopic techniques) have been promoted as
21 viability markers, none have yet been widely used clinically or in livestock
22 production. Here we review the major techniques that have been reported; these are
23 divided into indirect techniques, where measurements are made from the embryo's
24 immediate micro-environment, or direct techniques that measure intracellular
25 metabolic activity. Both have strengths and weaknesses, the latter ruling out some
26 from contention for use in human ART, but not necessarily for use in livestock
27 embryo assessment. We also introduce a new method, multi- (or hyper-) spectral
28 analysis, which measures naturally occurring autofluorescence. Several metabolically
29 important molecules have fluorescent properties, which we are pursuing in
30 conjunction with improved image analysis as a viable embryo quality assessment
31 methodology.

32

33

34 INTRODUCTION

35 Over several decades, metabolic determination of oocyte and embryo quality
36 has been promoted as an adjunct, if not primary, method for predicting subsequent
37 development. The ability to predict development following embryo transfer is
38 enormously attractive to both human clinical laboratories and cattle embryo
39 production laboratories (the two largest applications of embryo production
40 technology). For IVF clinics, selecting the embryo with the highest implantation
41 potential enables single embryo transfer, alleviating the health complications arising
42 from multiple births for both mother and infants (Gardner and Sakkas 2003). In cattle
43 embryo production, minimising recipient returns to oestrus following transfer is an
44 economic advantage. Yet today, neither clinical ART units nor cattle veterinarians
45 routinely perform embryo metabolic assessment prior to transfer. In contrast, other
46 techniques (Figure 1), such as morphology grading are routinely applied (e.g.
47 (Gardner *et al.* 2000), albeit highly reliant on the skills and experience of the
48 embryologist. The development of "Time lapse" systems (Meseguer *et al.* 2011;
49 Herrero and Meseguer 2013) has taken morphokinetics to a greater predictive
50 capacity, and has confirmed that the timely progression of cellular division is
51 indicative of embryo competence. In addition, pre-implantation genetic screening of
52 human embryos (Figure 1) has emerged from a criticised clinical technique of
53 assessment, due to the poor predictability of ploidy status by early methods
54 (especially fluorescence in situ hybridisation, FISH) and the high degree of ploidy
55 errors within individual blastomeres (Vanneste *et al.* 2009; Harper and Sengupta
56 2012), to a more robust predictive method using comparative genomic hybridisation,
57 especially when applied to blastocyst stage embryos combined with vitrified cycles
58 (Schoolcraft and Katz-Jaffe 2013).

As highlighted in several recent reviews (Krisher and Prather 2012; Leese 2012; Lonergan and Fair 2014; Gardner and Harvey 2015; Krisher *et al.* 2015b), metabolic studies have been fundamental in the grounding behind embryo culture media formulations and provide valuable insights into what aspects of metabolism are associated with embryo quality, or more so, what aspects are associated with failed development or embryo stress. So where does this leave the measurement of metabolism as a prospective embryo quality assessment technology? Do current techniques have the scope to be used routinely? Has the need for determining metabolic markers of quality been overtaken? In this paper we assess the state of the field and provide a view of where the field should head.

METHODS FOR MEASURING METABOLISM OF EMBRYOS

Indirect Measures of Metabolism

Indirect measures of embryo metabolism rely on a change in substrate concentration in the immediate micro-environment surrounding the embryo. Typically this is the media surrounding the embryo, often referred to as 'spent' culture media (Figure 2). The benefit of such techniques is that theoretically, there is no impact to the embryo, thus regarded as non-invasive.

NAD(P)H-based assays for carbohydrates and carboxylic acids

Inspired by the work of Oliver Lowry, Henry Leese devised fluorometric assays for measuring ATP, glucose and lactate from tissues (Leese and Bronk 1972), based on the oxidation and/or reduction of nicotinamide adenine dinucleotides (NAD(P)H) and their fluorescent properties. Indeed, these assays are used routinely in many automated substrate analysis systems today, due to their high sensitivity and

84 capability to measure from small volumes. These assays were based on the discovery
85 of Oliver Lowry that the reduced forms of NAD(P)H were fluorescent molecules
86 (emission maxima 460 nm) under UV excitation wavelengths (330-350 nm), whereas
87 the oxidised forms of both ($NAD(P)^+$) were not, described in an account of his work
88 (Lowry 1990). Lowry recognised that as these were co-factors required for
89 dehydrogenase enzymes; by harnessing this property, he could measure the activity of
90 these enzymes. Leese built on this concept and with John Biggers and colleagues,
91 scaled down the assay system to measure fluorescence from nanolitre and picolitre
92 samples, enabling the ability to measure the metabolite turnover of a single COC and
93 embryo (Leese *et al.* 1984). As dehydrogenases metabolise carbohydrates (with the
94 primary interest focussed on glucose) and carboxylic acids (pyruvate and lactate, via
95 lactic acid dehydrogenase), substrate appearance or disappearance from the embryo
96 culture medium is measurable over time, enabling estimates of metabolic activity;
97 examples include: (Gardner and Leese 1988; Leese *et al.* 1994; Thompson *et al.*
98 1996a; Butcher *et al.* 1998).
99
100 *Spectrophotometric techniques*
101 Metabolomics is the term generally used to describe the identification and
102 quantification of multiple metabolites in a single analysis. Measurement of a broad
103 range of substrates and metabolites allows not only measurement of substrate turnover
104 but also provides a better estimation of changes in metabolic pathway activity and
105 downstream targets such as redox control and proliferation, and as such is a much
106 more powerful discovery technique than targeted substrate analysis (Krisher,
107 Heuberger *et al.*, 2015). With this definition in mind, metabolomics combines two
108 technologies; firstly the separation (gas chromatography, high performance liquid

109 chromatography (HPLC)) and then the detection (mass spectrometry, near infrared,
110 nuclear magnetic resonance, Raman spectrometry) of larger numbers of metabolites
111 within 'spent' culture media compared to other analytical methods. Both quantitative
112 and/or qualitative measurements can be performed (depending on the technology
113 used), with quantitative measures requiring standards, which may reduce the number
114 of substrates to be measured with accuracy. Application of one spectrometry platform
115 (near infrared spectrometry, NIR) for spent human embryo culture media analysis was
116 initially favourable (Sakkas 2014). Nevertheless, several randomised control trials
117 could not support initial results (Vergouw *et al.* 2014) and for now the application of
118 NIR has been abandoned, until technology refinements or alternatives are developed.
119 Indeed, metabolomics of spent culture media is still actively pursued using alternative
120 platforms (mass spectrometry; (Krisher *et al.* 2015a).

121

122 Amino acid analysis within 'spent' medium has shown promise as a predictive
123 tool for subsequent embryo quality. Most amino acid analyses have utilised HPLC
124 separation following a fluorescent tagging method that enables detection following
125 separation (Lamb and Leese 1994). Subsequent reports have identified that amino
126 acid appearance and disappearance from 'spent' medium can predict sex, ploidy
127 status, embryo development and post-implantation survival (Houghton *et al.* 2002;
128 Brison *et al.* 2004; Picton *et al.* 2010; Sturmey *et al.* 2010).

129

130 *Polarographic electrodes*

131 Polarographic scanning electrodes quantify the concentration of a single
132 molecular species, dependent on their sensing mechanism. For example,
133 measurement of ions usually requires a specific ionophore (Trimarchi *et al.* 2000b).

134 Undoubtedly the widest application is for the measurement of dissolved O_2, especially
135 in relation to embryo metabolism (Trimarchi *et al.* 2000a; Shiku *et al.* 2001; Lopes *et*
136 *al.* 2007). Oxygen consumption by embryos has been proposed as an obvious
137 candidate for determining embryo viability, as oxidative phosphorylation is critical
138 for development (Houghton *et al.* 1996). Oxygen consumption should accurately
139 reflect the rate of ATP production via oxidative phosphorylation and therefore the
140 energy demand within an embryo. Several studies demonstrated that O_2 demand in
141 mouse and bovine embryos increases with the onset of compaction and blastulation
142 (Houghton *et al.* 1996; Thompson *et al.* 1996b). In a retrospective study of O_2
143 consumption in cattle embryos followed by embryo transfer, Lopes and colleagues
144 (Lopes *et al.* 2007) found that blastocysts with the highest implantation success were
145 in the 'mid-range' of consumption measurements, supporting the 'Quiet embryo
146 hypothesis' (Leese 2002) (see section below). Nevertheless, day 3 human embryos
147 may be selected on their O_2 consumption rate, as a retrospective analysis of
148 implanting embryos had a higher average consumption than non-implanting embryos
149 (Tejera *et al.* 2012). Polarographic O_2 electrodes coupled with Time lapse
150 morphokinetics for embryo assessment was prototyped by the Danish company,
151 "Unisense Pty Ltd". However, they abandoned the O_2 sensing aspect as it became
152 clear that replacing probes between patients would be mandatory and therefore
153 technically challenging and commercially unviable, especially as Time-lapse
154 microscopy alone was proving a better predictor of embryo quality than other
155 morphometry methods. Apart from their wide use of O_2 measurements in a variety of
156 applications, polarographic electrodes are also capable of measuring other gases such
157 as NO and CO_2 in addition to both cations and anions.
158

159 **Intracellular Measurements**

160 Intracellular measurements by their definition must involve measuring
161 metabolic activity within the embryo itself, and therefore cannot be regarded as non-
162 invasive (Figure 2). The challenge is therefore to determine the extent of impact on
163 the embryo whilst measuring metabolism. This poses potential regulatory safety
164 issues, especially on long-term outcomes following transfer, for this technology to be
165 clinically useful.

166

167 *Non-toxic colorimetric and fluorometric dyes*

168 Brilliant Cresyl Blue (BCB) is an supravital stain (oxazine family) which has
169 been successfully used to segregate fully grown germinal vesicle stage oocytes from
170 more immature oocytes, with subsequent embryo transfers proving this assay is non-
171 toxic (Opiela and Katska-Ksiazkiewicz 2013). The assay is dependent on the activity
172 of the X-linked enzyme, glucose-6-phosphate dehydrogenase (G6PDH), whereby
173 fully grown oocytes exposed to BCB remain blue (low enzyme activity), whereas
174 growing oocytes metabolise the stain and become clear. G6PDH activity reduces
175 during development to the blastocyst stage in the mouse (Brinster 1966; De Schepper
176 *et al.* 1993), with levels much lower than in the oocyte. Other than measuring activity
177 in oocytes, there has been no attempt to measure G6PDH in embryos for viability
178 determination. BCB staining has been assessed for determining the sex of blastocyst
179 stage embryos (Williams 1986), and in doing so demonstrating there is little toxicity
180 with this procedure. However, other sexing technologies (FACS –separated sperm
181 and embryo biopsy-DNA analysis) have surpassed its relatively weak capacity for sex
182 selection. Furthermore, such assays are certain to fall foul of national regulatory
183 authorities, especially for human embryo application. Nevertheless, it is quite feasible

184 that non-toxic dyes sensitive to metabolic activity can still have application in other
185 species, such as domesticated ruminants.
186 Most fluorescent probes are unusable for determining metabolic activity for
187 viability assessment, as many will either have an inherent toxicity, or become toxic
188 due to the chemical interaction that creates the fluorescent capacity of the probe.
189 Thus probes such as the mitochondrial respiratory dyes JC-1 (5,5',6,6'-tetrachloro-
190 1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) and the Mitotracker probes
191 (carboryanine or rosamine-based probes) are not practical measures of viability for
192 post-transfer work, but remain proven research tools. On the other hand, non-
193 metabolised probes may have a role in relating to viability post-transfer. For
194 example, glucose uptake into an embryo can be measured using 6-(*N*-(7-nitrobenz-2-
195 oxa-1,3-diazol-4-yl)amino)-6-deoxyglucose (6-NBDG), a fluorescent glucose
196 analogue that is not metabolised (Zander *et al.* 2006), and is brightly (green)
197 fluorescent at relatively low concentrations. As yet, as far as we know, there has been
198 no attempt to determine post-transfer viability with this particular probe.
199
200 *Radiolabel isotopes*
201 Most of the early studies on embryo metabolism were conducted utilising radio-
202 labelled substrates, in particular glucose and pyruvate. Depending on which
203 carbon/hydrogen atom was labelled, the production of $^{14}CO_2$ or $^{3}H_2O$ indicated the
204 activity of different metabolic pathways. For example, the production of $^{14}CO_2$ from
205 [1-^{14}C] glucose measured activity through the pentose phosphate pathway (PPP) and
206 tricarboxylic acid (TCA) cycle. Likewise, the production of $^{3}H_2O$ from [5-^{3}H]
207 glucose is indicative of glycolytic activity (Rieger and Guay 1988; Rieger and
208 Loskutoff 1994). These measurements primarily utilised a 'Hanging Drop' assay,

209 where oocytes or embryos were incubated in ~3 μl of culture media containing the
210 radiolabelled substrates in the lid of a centrifuge tube. The drop was then suspended
211 by capping the lid over a reservoir containing solutions of NaOH or $NaHCO_3$, which
212 acts as a metabolite "trap" (O'Fallon and Wright 1987). Following the principle of
213 mass transfer meant that greater than 95% of the metabolised label was trapped over a
214 3-4 h period of time (Rieger and Guay 1988). Because of the use of radioisotopes, the
215 technique is very sensitive, capable of measuring pathway activity in single embryos
216 (O'Fallon and Wright 1986; Rieger and Guay 1988; Thompson *et al.* 1991; Rieger and
217 Loskutoff 1994; Downs and Utecht 1999). Radioisotope-labelled substrates have
218 never been utilised for embryo transfer and post-natal development assessment, due to
219 the radioactivity involved, even though in reality the levels are relatively harmless, as
220 only β-emitters are normally utilised. Furthermore, there has never been an
221 assessment of whether these cause mitochondrial or DNA damage to the embryo.
222 These assays still remain useful for research, so their demise as a routine method to
223 investigate metabolism is most likely related to institutional and ethical reluctance to
224 support radioisotope-based tools and embryo transfer.
225
226 *Autofluorescence*
227 Researchers utilising fluorescence microscopy will be familiar with
228 autofluorescence within specimens. However, most will view it as a nuisance as
229 autofluorescence is the cause of background fluorescence that may decrease the
230 contrast in fluorescence with a specific fluoroprobe. However, there is a diversity of
231 endogenous molecules that are fluorescent (Table 1, (Ramanujam 2000)).
232 Significantly fluorescent molecules are NAD(P)H (as previously discussed), flavin
233 adenine dinucleotide (FAD), collagen and porphyrins (Table 1). Because of their

234 fluorescent properties and roles in metabolism, NAD(P)H and FAD are widely used
235 together, especially as the ratio can be regarded as a *de facto* measure of the
236 intracellular redox state. The majority of NAD(P)H is represented by NADH, and just
237 as significantly, FAD fluorescence is associated with mitochondrial activity, as the
238 vast majority of FAD/$FADH_2$ is localised there (Heikal 2010). A drawback is that the
239 excitation and emission spectrum of NADH and NADPH are very similar. Use of
240 these fluorophores as measures of metabolic activity within embryos was pioneered
241 by Dumollard and colleagues (Dumollard *et al.* 2007; Dumollard *et al.* 2009), who
242 successfully measured changes in metabolism during the process of fertilisation and
243 subsequent embryo development over periods of time, particularly investigating the
244 influence of substrate changes in the medium on FAD and NAD(P)H fluorescence.
245 The power of this approach was subsequently demonstrated by Banrezes and
246 colleagues (Banrezes *et al.* 2011), whereby changing the levels of pyruvate and lactate
247 in the pronuclear embryo medium and observing the ensuing redox alterations, they
248 observed altered fetal growth related to the redox state at this early stage. Not only
249 did this study demonstrate a new developmental regulatory insight that is energy
250 sensitive at the pronuclear stage, but also that the measurement of autofluorescence
251 has seemingly no consequences to viability and can be utilized with subsequent
252 embryo transfer. However, one cannot rule out a biological impact of laser exposure,
253 and will be dependent on laser energy utilised and length of exposure and frequency.
254 Accompanying the development of fluorescence microscopy, textural image
255 analysis has also evolved to measure different pixel attributes, such as distribution,
256 co-localisation and patterning, in addition to pixel intensity. This can improve the
257 quality of information from microscopic images, whether they are fluorescent or not.
258 Ultrasound sonography, dermatology and cancer research are fields that routinely use

259 advanced imaging matrices to assess variations in patterns of pixel characteristics,
260 described in a textural context, such as wrinkles, smoothness, uniformity and entropy
261 of images (Murata *et al.* 2001; Castellano *et al.* 2004; Alvarenga *et al.* 2007). In
262 comparison, image analysis within the pre-implantation research field is largely
263 limited to measurements of fluorescence intensity. We have begun to assess textural
264 analyses of early cleavage stage embryos to gain further information other than
265 intensity, an example of which has been applied to examining oocytes following
266 different cumulus-oocyte complex treatments (Sutton-McDowall *et al.* 2015a).
267

268 MEASURING EMBRYO METABOLISM – WHAT ARE WE MEASURING?

269 ***In situ* vs. *ex vivo* embryo metabolism?**

270 Pre-implantation stage embryos survive in the reproductive tract and are
271 dependent on a histotrophic substrate and protein supply, where some of these are
272 oviduct specific proteins (Killian 2004). It is widely accepted, yet not demonstrated,
273 that the microenvironment of substrates in the luminal fluid of the maternal tract (in
274 particular the oviduct) surrounding the early embryo is not constant but in a state of
275 flux. It is very likely the reproductive tract environment has a high degree of
276 sensitivity to maternal signals. Supporting this are the elegant observations by Leese
277 and colleagues (Dickens *et al.* 1993; Cox and Leese 1995) who measured rapid
278 changes in secretory behaviour of cultured oviduct epithelial cells when treated with
279 stimulatory ATP. Furthermore, the volume of oviductal fluid relative to luminal
280 surface area is small and cumulus-oocyte complexes (COCs) and embryos are in very
281 close proximity to the oviductal wall (for an excellent *ex vivo* visualisation of this,
282 view the videos found in (Kolle *et al.* 2009). No doubt this facilitates sperm-oocyte
283 collision, but changes in local luminal fluid composition are likely to occur as well.

284 Like cumulus cells (Aardema *et al.* 2013; Lolicato *et al.* 2015), a function of the zona
285 pellucida surrounding the embryonic cells is possibly to buffer the oocyte and
286 subsequent blastomeres from sudden shifts in substrate (and soluble gas)
287 concentrations, in addition to its other protective and sperm-binding, capacitation and
288 fertilization roles. Nevertheless, metabolic activity of embryos *in situ* could feasibly
289 be more dynamic than what occurs within a drop in a petri dish. Perhaps this is why
290 measurement of several metabolic parameters such as glucose, carboxylic acids,
291 amino acids and oxygen uptake has such a broad range of values when assayed
292 immediately following collection (Leese 2012). Embryos are thought of as
293 'developmentally plastic', an awkward term commonly used to describe the tolerance,
294 or adaptation (with variable success), to different media formulations during *in vitro*
295 culture. In actual fact, it appears that adaptability is an inherent feature of early
296 embryo development (Leese 2012). Here then is the conundrum for all past and
297 present work on embryo metabolism – we speculate on what 'normal' metabolism *in*
298 *situ* really means. Our best attempts to measure this metabolism is restricted to
299 immediate measures within an *ex vivo* environment following collection, where we
300 know that within 3 h, the metabolic pattern between freshly flushed mouse embryos
301 and cultured embryos can be markedly different (Lane and Gardner 1998). The
302 assumption made is that this reflects the metabolic profile *in situ*. Until we develop
303 such assays that allow us to track metabolism *in situ* we should speculate with caution
304 on the relationship of what we are measuring *in vitro* and what occurs *in situ*. Perhaps
305 in the future, the application of photonic fibres and nanoparticles will provide better
306 access to embryos to measure their metabolism *in situ*.
307
308 **Changes with stage of development**

309 The widely accepted pattern of embryonic metabolism (measured under *in vitro*
310 conditions, Figure 3) for most species examined, including human and cow, is that
311 pre-compaction (early cleavage) stages of development are dependent on oxidative
312 phosphorylation (Thomson 1967; Leese 1995; Thompson *et al.* 2000). Then as
313 compaction and blastulation occur, glycolysis increases (Figure 3), even in the
314 presence of O_2. This is not to say that post-compaction development does not require
315 oxidative phosphorylation. Indeed, it is clear that within the blastocyst stage of most
316 species examined, trophectoderm cells are reliant on oxidative phosphorylation for
317 their higher energy demands, whereas the inner cell mass cells are highly glycolytic.
318 Some have likened this metabolic profile to the 'Warburg effect' (Krisher and Prather
319 2012) observed in some tumour cells, where despite the availability of O_2, significant
320 lactate production occurs relative to the uptake of glucose, rather than glucose
321 oxidation via the TCA cycle and oxidative phosphorylation (Krisher and Prather
322 2012). Fatty acid metabolism contributing to oxidative phosphorylation is now
323 recognised as a fundamental requirement in several species (Paczkowski *et al.* 2013),
324 most likely meeting the oxidative phosphorylation requirement. Exceptions to this
325 picture are the rat embryo, where blastocysts were produced in the presence of
326 oxidative phosphorylation inhibitors (Brison and Leese 1991), and the rabbit embryo,
327 where the reliance for oxidative phosphorylation from fatty acid oxidation is
328 continuous from the 1-cell stage, most likely to enable the substantial proliferation
329 that occurs within the embryo (Kane 1979). Although we have a picture of major
330 changes in metabolism for several species, it is clear that the degree of substrate
331 uptake and metabolic pathway preference throughout development is variable
332 amongst such species, as recently summarised for mouse, cow and pig in the review
333 by Krisher and Prather (Krisher and Prather 2012). As embryos of other species are

334 investigated, further departures from what is regarded as the 'characteristic pattern' of
335 mammalian embryo metabolism will no doubt emerge.
336

337 **In vitro composition of medium and influence of physical parameters, such as**
338 **embryo density and gas composition.**

339 The metabolism of the preimplantation stage embryo is also significantly
340 influenced by the culture environment. This can be divided into 1) the culture media
341 formulation, especially the energy substrate availability, supplemental protein
342 concentration and influence of anti-apoptotic/mitogenic growth factors; 2) the
343 influence of intrinsic factors during culture (e.g. the impact of autocrine and paracrine
344 growth factors, or the presence (deliberate or otherwise) of somatic cells, to create a
345 co-culture system; 3) the impact of extrinsic factors, such as gas composition, most
346 notably the partial pressure of oxygen used for culture, but also CO_2.

347 Arguably, one of the least understood aspects of *in vitro* culture is the influence
348 of the embryo itself on the culture environment, even if it is being deliberately
349 measured. Often described as a "static" culture system, the culture media
350 composition itself within the near-universally applied microdrop under mineral oil, is
351 continuously changing. In particular, the smaller the culture drop, or the density of
352 embryos per unit volume of media, the more change to media composition will occur
353 over a period of time. Indeed, this is the whole basis for assays that measure the
354 temporal change in substrate content as a proxy measure for substrate uptake. With
355 specific reference to metabolomics, changes to substrates and metabolites reflect both
356 the initial concentration and the substrate movement into or from the embryo, with a
357 broad range in differences in concentration observable over time; some being
358 undetectable (which will also depend on sensitivity of detection systems) and others

359 possibly at a point of significant depletion that may impact metabolic pathway
360 activity. Recently, Krisher and colleagues (Krisher *et al.* 2015a) argued that as long
361 as sufficient substrate levels were “available” to embryos, and the difference in
362 concentration of substrates was small relative to their appearance or disappearance
363 from the medium, then issues of significant depletion during the measurement period
364 would be avoided. Culture in larger volumes or as single embryos (Lane and Gardner
365 1992; Keefer *et al.* 1994) will impact both developmental potential and metabolism
366 and is thought to increase embryo stress, due to the waning influence of autocrine and
367 paracrine growth factors. As Krisher and colleagues (Krisher *et al.* 2015a) concluded,
368 “*Metabolic measurements should occur in optimal volumes to best reflect metabolism*
369 *of a viable embryo, as well as to be clinically relevant*”. As such, the metabolic
370 profile of an embryo is uniquely dependent on media formulation and volume,
371 causing difficulties if extrapolating from one culture system to another (Sakkas 2014).
372 The most influential extrinsic factor that varies significantly in measurement of
373 metabolism is gas composition. Systematic reviews of the literature addressing the
374 influence of O_2 conclude that a low O_2 atmosphere (5-7%) has a positive impact on
375 developmental consequences, especially post-compaction development. Yet much of
376 the work conducted *measuring* metabolism has been performed in air-based
377 atmospheres (Wale and Gardner 2013). Atmospheric O_2 levels are associated with
378 oxidative stress and altered gene expression profiles in blastocysts compared with low
379 O_2 embryo culture (Harvey 2007; Amin *et al.* 2014). In particular, low O_2 levels will
380 increase hypoxia inducible factor activity (HIFs), especially post-compaction
381 (Thompson and Kind, unpublished observations;(Harvey 2007), which then work to
382 adapt the metabolism of cells to enable growth under such conditions.
383

384 **The Quiet embryo hypothesis**

385 Is a higher metabolism better for embryo health? If the question is directed to
386 ATP turnover alone, then the answer appears to be 'yes' (Van Blerkom 2011;
387 Fragouli *et al.* 2015). But ATP turnover is derived from the sum of glycolytic and
388 oxidative phosphorylation activity and the demand for cellular energy, and this
389 turnover is in the order of tens of seconds in embryos (Leese 1991), revealing that a
390 simple measure of ATP content alone at a single point in time does not measure rate
391 of turnover. A central constituent to this important energy equation is how
392 mitochondria behave, or put another way, their efficiency to generate ATP during *in*
393 *vitro* culture in the face of demand, which is a major determinant of embryo health
394 (Fragouli *et al.* 2015). It was Henry Leese and colleagues (Leese 2002; Leese *et al.*
395 2007; Leese *et al.* 2008) who noted that the most viable embryos were neither
396 associated with the highest, nor lowest metabolic readout(s), when measuring key
397 metabolic parameters such as glucose uptake, net amino acid uptake and O_2 uptake.
398 The 'Quiet embryo hypothesis' was drawn from metabolic profiles measured between
399 *in vivo* derived and *in vitro* produced embryos or from retrospective analysis of
400 metabolic parameters measured prior to embryo transfer. Leese concluded that
401 embryos with a high probability of further development have an efficient metabolism,
402 therefore an efficient utilisation of substrates, particularly within mitochondria. The
403 juxtaposition is that embryos with very high metabolic levels do so as they are
404 stressed, and likely to generate higher levels of reactive oxygen species (free radicals)
405 from mitochondria, thus setting the embryo on a self-destructive course. This
406 hypothesis is both supported and argued against in the ensuing literature. The major
407 criticism (Gardner and Wale 2013) is that many of the founding studies analysed to
408 develop the hypothesis utilized sub-optimal incubation conditions during the analysis

409 period, particularly the use of atmospheric O_2 levels. Under such conditions, the
410 levels of glucose uptake, particularly post-compaction, correlate with subsequent
411 viability post-transfer in mice and human embryos, thereby demonstrating that the
412 metabolic assessment environment is fundamental to the capacity of metabolism to be
413 considered as an indicator of subsequent development. One common element of the
414 arguments for and against the 'Quiet embryo hypothesis' is that *in vitro* cultured
415 embryos are more stressed than their *in vivo* derived counterparts. Several stress
416 activated signalling pathways operate within embryos, including sirtuins, AMP-
417 dependent kinase (AMPK), Hypoxia Inducible Factors (HIFs) and Stress Activated
418 Protein Kinases (SAPK, or JNK), and such have the capacity to rapidly modify
419 metabolism; this is comprehensively reviewed by Puscheck and colleagues (Puscheck
420 *et al.* 2015). It is feasible that with increasing and also different types of stress,
421 metabolic relationships with competence change in non-linear patterns, thereby
422 adding to the confusion about what is predictive of competence. Perhaps the real
423 implication of the current debate is that our ability to accurately measure embryonic
424 stress by metabolic measures with current capabilities remains unsatisfactory. A new
425 hypothesis is helping to shed light on this (Brison *et al.* 2014), in that embryonic
426 stress is associated with heterogeneity in metabolic profiles between individual
427 blastomeres, with the ability for further development related to not only synchrony in
428 division but synchrony and homogeneity of metabolic change during development.
429 This is particularly so for pre-compaction stages, as post-compaction gap-junction
430 formation enables cell-cell communication and therefore at least there is capacity for
431 attaining some metabolic homogeneity (Brison *et al.* 2014). This attractive
432 hypothesis is being actively researched and points to the need for more intracellular
433 metabolic readouts that can be compared between blastomeres of each embryo, as

434 these may be more powerful than an 'averaged' readout examined within 'spent'

435 medium.

436

437 A NEW APPROACH - MULTISPECTRAL ANALYSIS

438 Multi- (or hyper-) spectral imaging has been widely used in food quality

439 monitoring (Huang *et al.* 2014). Its application to cellular biology has only been

440 recent, as at a research level, there is a requirement for significant computing input,

441 statistical data management and hardware. At a cellular level, spectral analysis is an

442 alternative metabolomics approach using the spectral properties of the endogenous

443 fluorophores within cells, with the capacity to measure differences within and

444 between individual cells (and therefore an embryo) (Table 1). The application of

445 multiple excitation wavelengths, whether by generation with a tuneable laser over a

446 wide range of wavelengths, or by using multiple excitation diodes (up to 18 different

447 excitation wavelengths), enables a broad spectral pattern to be generated, which then

448 requires analysis. It can be used either to identify a naturally fluorescent substrate or

449 product, such as NADH (either in a free- or protein bound-state), or provide a picture

450 of the degree of spectral shifts associated with variation in cellular metabolism. We

451 are currently assessing the technology for embryo quality predictive capacity during

452 development of early embryos, with our partners (Sutton-McDowall *et al.* 2015b).

453

454 CONCLUSIONS

455 Our understanding of embryo metabolism has grown considerably over the

456 past two decades. There is unambiguous evidence that embryo viability and embryo

457 metabolism are closely interrelated at the experimental level. Significant new insights

458 into the importance of metabolic sensing pathways in regulating metabolism and

459 viability are rapidly emerging, such as sirtuins, AMPK and HIFs, giving a clearer
460 picture as to how flexible embryos are at adapting to different conditions.
461 Nevertheless, differences in media composition from various laboratories and
462 manufacturers (where mostly the formulation is not available, apart from a list of
463 constituents) provide barriers for 'spent' media metabolomics to provide a predictive
464 assessment of viability. However, some success with measuring glucose plus lactate
465 level changes in media under low O_2 atmospheres and amino acid
466 appearance/disappearance have been identified as predictive of further development.
467 Alternative approaches that have developed with the advent of advancing microscope
468 and imaging technology and computing power, such as spectral analysis of multiple
469 endogenous fluorophores during the development period, holds great promise for
470 determining intracellular metabolic activity. When this is coupled with Time-lapse
471 morphokinetics, and possibly in conjunction with extracellular metabolomics, then
472 current limitations should be resolved and this poses the best hope for accurately
473 assessing embryonic developmental potential.
474

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795 FIGURE LEGENDS

796

797 Figure 1. Images representing the three major technique groups for assessing quality

798 of embryos prior to transfer: Morphometry techniques; Metabolic techniques; Biopsy

799 techniques.

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801

802 Figure 2. Techniques for determining metabolic activity in embryos under *in vitro*

803 conditions, which can feasibly be used to determine embryonic health prior to embryo

804 transfer.

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806

807 Figure 3. Major changes in metabolism (glycolysis and oxidative phosphorylation),

808 RNA and protein synthesis in a generalised mammalian embryo. Adapted from

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813

814 Table 1. Excitation and emission maxima of endogenous fluorophores.
815

Endogenous Fluorophores	**Excitation maxima (nm)**	**Emission maxima (nm)**
Amino acids		
Tryptophan	280	350
Tyrosine	275	300
Phenylalanine	260	280
Structural Proteins		
Collagen	325, 360	400, 405
Elastin	290, 325	340, 400
Enzymes and coenzymes		
FAD, Flavins	450	535
NADH	290, 351	440, 460
NADPH	336	464
Vitamins		
Vitamin A	327	510
Vitamin K	335	480
Vitamin D	390	480
Vitamin B_6 compounds		
Pyridoxine	332, 340	400
Pyridoxamine	335	400
Pyridoxal	330	385
Pyridoxic acid	315	425
Pyridoxal 5'-phosphate	330	400
Vitamin B_{12}	275	305
Lipids		
Phospholipids	436	540, 560
Lipofuscin	340 – 395	540, 430 – 460
Ceroid	340 – 395	430 – 460, 540
Porphyrins	400 – 450	630, 690

816 *NADH,* reduced nicotinamide dinucleotide; *NAD(P)H,* reduced nicotinamide
817 dinucleotide phosphate; *FAD,* flavin adenine dinucleotide. Taken from Ramanujam,
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