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Modulating oxidative stress and epigenetic homeostasis in preimplantation IVF embryos

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Review Article

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Summary

Assisted reproductive technology is today considered a safe and reliable medical intervention, with healthy live births a reality for many IVF and ICSI treatment cycles. However, there are increasing numbers of published reports describing epigenetic/imprinting anomalies in children born as a result of these procedures. These anomalies have been attributed to methylation errors in embryo chromatin remodelling during *in vitro* culture. Here we re-visit three concepts: (1) the so-called '*in vitro* toxicity' of 'essential amino acids' before the maternal to zygotic transition period; (2) the effect of hyperstimulation (controlled ovarian hyperstimulation) on homocysteine in the oocyte environment and the effect on methylation in the absence of essential amino acids; and (3) the fact/postulate that during the early stages of development the embryo undergoes a 'global' demethylation. Methylation processes require efficient protection against oxidative stress, which jeopardizes the correct acquisition of methylation marks as well as subsequent methylation maintenance. The universal precursor of methylation [by *S*-adenosyl methionine (SAM)], methionine, 'an essential amino acid', should be present in the culture. Polyamines, regulators of methylation, require SAM and arginine for their syntheses. Cystine, another 'semi-essential amino acid', is the precursor of the universal protective antioxidant molecule: glutathione. It protects methylation marks against some undue DNA demethylation processes through ten-eleven translocation (TET), after formation of hydroxymethyl cytosine. Early embryos are unable to convert homocysteine to cysteine as the cystathionine β-synthase pathway is not active. In this way, cysteine is a 'real essential amino acid'. Most IVF culture medium do not maintain methylation/epigenetic processes, even in mouse assays. Essential amino acids should be present in human IVF medium to maintain adequate epigenetic marking in preimplantation embryos. Furthermore, morphological and morphometric data need to be re-evaluated, taking into account the basic biochemical processes involved in early life.

Introduction

Assisted reproductive technology is now considered a safe and reliable medical intervention, applied globally, and the outcome of a healthy live birth is a reality for the majority of successful IVF and ICSI treatment cycles. Human IVF/ICSI culture conditions and manipulations generate reactive oxygen species (ROS) that can jeopardize the integrity of DNA, potentially creating abasic sites [apurinic/apyrimidinic (AP)], mutagenic DNA lesions that interfere with DNA replication and transcription (Pabon *et al.*, 1989; Nasr-Esfahani *et al.*, 1990). Human oocytes and early embryos are equipped to combat damage induced by maternal as well as sperm-borne ROS, but these defences are finite and decrease with maternal age (Hamatani *et al.*, 2004). Current scientific literature increasingly describes epigenetic/imprinting anomalies in children born as a result of IVF/ICSI procedures, and these have been attributed to methylation errors affecting the embryonic genome during *in vitro* culture. There is a strong correlation between oxidative stress and DNA methylation, through influence on the one-carbon cycle. Oxidative stress can induce inappropriate gene expression, i.e. methylation anomalies and oxidative stress represent two sides of the same coin (Menezo *et al.*, 2016). The regulation of epigenesis and imprinting relies mainly on histones and DNA methylation. RNA interference also plays a role as an actor/regulator, but elucidating the mode of action is so far limited/difficult, moreover a potential mechanism for manipulating this process is therefore very inaccessible. It is possible that RNAi intervenes as a regulator of methylation as well as in the protection against oxidative stress and its significant effects on methylation/epigenetic marking. RG Edwards had previously expressed this concern about *in vitro* culture (Edwards and Ludwig, 2003), highlighting the fact that the *in vitro* zygote may lack a mechanism for methylation maintenance. Data based on methylation studies carried out on *in vitro* cultured human embryos has led to misleading observations (Smith *et al.*, 2014): early stage *in vitro* culture leads to anomalies of methylation, with global detrimental effects that are observed only at a later time. A high incidence of four major imprinting disorders in Japanese babies born after assisted reproductive technology (ART) has

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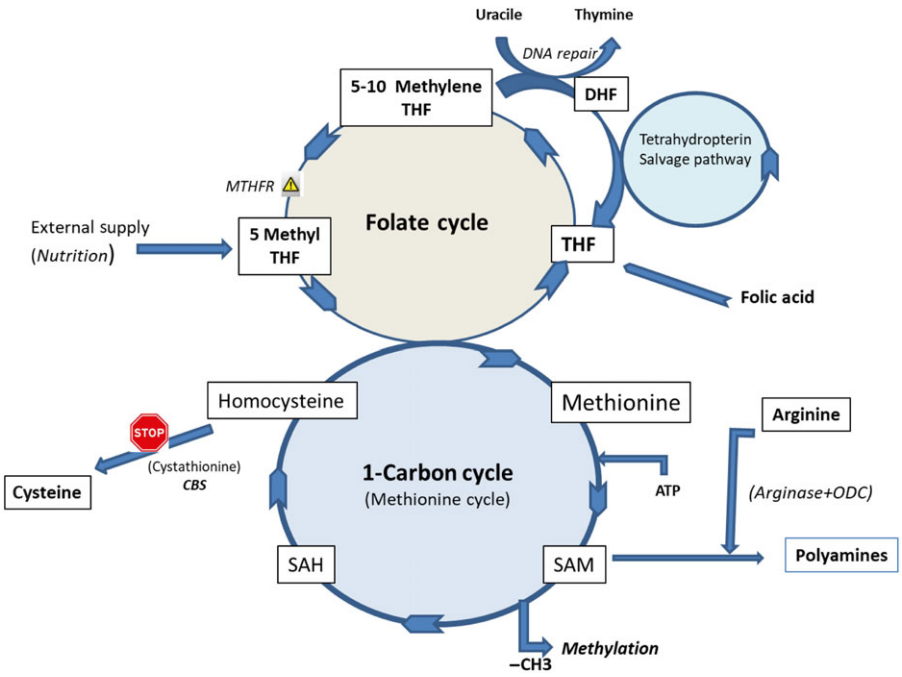

Figure 1. Two separate but interacting pathways that are crucial to methylation processes revolve around methionine and folate, respectively. The outputs from these two cycles are important in the metabolism of nucleotides, proteins and lipids, providing substrates for methylation reactions as well as generating reducing power to maintain appropriate redox potential. Homocysteine recycling is an important feature of these pathways. The tetrahydropterin salvage pathway, involved in the regulation of the biopterins synthesis, is grafted onto the folate cycle; it is not directly involved in the regulation of methylation/epigenesis, but it interferes with the folate endogenous pool available.

recently been documented (Hattori *et al.*, 2019). Culture conditions have been shown to affect epigenetic chromatin remodelling during preimplantation development both in human and in animal species (Lafontaine *et al.*, 2020), On a global scale, babies born as a result of IVF procedures show different patterns of DNA methylation compared with those conceived naturally (Katari *et al.*, 2009; Hiura *et al.*, 2012; Song *et al.*, 2015; Choux *et al.*, 2018). These problems are thought to arise from the technology itself rather than the aetiology of male and/or female infertility (Song *et al.*, 2015). If we consider the technologies used in IVF, anomalies can potentially be introduced by defects arising during two separate steps: controlled ovarian hyperstimulation (COH) and embryo culture. We will describe the influence of COH, the mechanisms through which current *in vitro* culture conditions during the period prior to genomic activation can create oxidative stress that may aggravate epigenetic/imprinting problems associated with methylation, and review the crucial effects of the folate and one-carbon cycles (Figure 1). Homocysteine is a cause and a consequence of oxidative stress (Hoffman, 2011) and is at the epicentre of impairments to the methylation process.

Effects of controlled ovarian hyperstimulation

The effect of COH *per se* is difficult to estimate, as it is generally followed by *in vitro* culture of the oocytes retrieved after stimulation. However, animal studies in monovulatory animals such as the bovine demonstrate that transfer of the resultant embryos to recipients have a good prognosis with respect to development to term. In the bovine model, COH does not apparently impair embryo quality, but *in vitro* culture does affect imprinting (Lafontaine *et al.*, 2020), leading to large offspring syndrome (LOS) (Young *et al.*, 1998). Epigenetic/imprinting problems observed following human ART may represent a parallel situation to LOS seen in ruminants (Chen *et al.*, 2013). Animal models generally follow superovulation with embryo culture (Huffman *et al.*, 2015), so that it is impossible to distinguish between the effects of hyperstimulation and those that may be due to embryo culture. In some models, *in vitro* maturation adds further uncertainty, as oocyte methylation patterns may be re-set during the final stages of *in vivo* maturation in the ovary. The same dilemma occurs in humans: most embryos undergo *in vitro* culture after ovarian hyperstimulation. A study by Sato *et al.* (2007) describes methylation anomalies following COH, but the GV and MI oocytes collected for study were matured *in vitro*.

Any direct contribution by superovulation to perturbations in embryonic imprinting/DNA methylation is probably only marginal (Denomme *et al.*, 2011), at least in mono-ovulatory animals. However, the influence of estradiol is an important parameter that has significant consequences for the embryo. Estradiol triggers DNA/histone methylation (Kovács *et al.*, 2020), initiating methylation of CpG islands as well as the histone 3 lysine 4 (H3K4) methylation process that triggers gene transcription (Greer and Shi, 2012). Oestrogen receptors are important partners in remodelling chromatin structure by methylation. In human oocytes, oestrogen receptor beta is expressed at 13× the basic signal level, and oestrogen receptor binding protein is expressed at a level of >200×. Ovarian hyperstimulation significantly raises both circulating and follicular estradiol. During oocyte maturation, there is an initial requirement for methyl groups at several oocyte targets, and oocytes express DNA methyltransferases 3A and 3B at levels that are 100× the background signal. The process of methylation generates homocysteine (Hcy), which accumulates in follicular fluid, but not in serum (Boxmeer *et al.*, 2008, 2009; Berker *et al.*, 2009; Ocal *et al.*, 2012). Homocysteine and methionine share and compete for the same transporter (Menezo *et al.*, 1989). An environment that is rich in Hcy restricts methionine transport and uptake, and this will modify the intracellular Hcy/Met balance, creating a transitory elevation of Hcy and a decreased endogenous pool of methionine within the oocyte (see Figures 2 and 3). Folates are required to support regeneration of methionine from Hcy. There is a clear association between low follicular fluid (FF) Hcy concentrations and oocyte maturation/embryo quality (Szymański and Kazdepka-Ziemińska, 2003; Ocal *et al.*, 2012) and lower FF Hcy concentrations are observed in younger women.

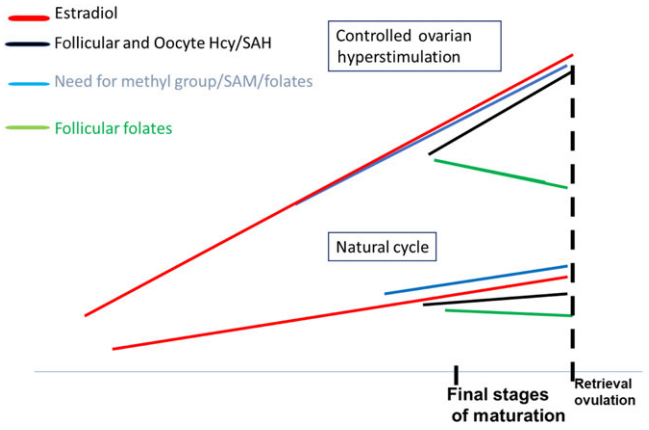

Figure 2. Evolution of ovarian and oocyte parameters that affect the generation of methyl groups during follicular growth in stimulated and unstimulated cycles.

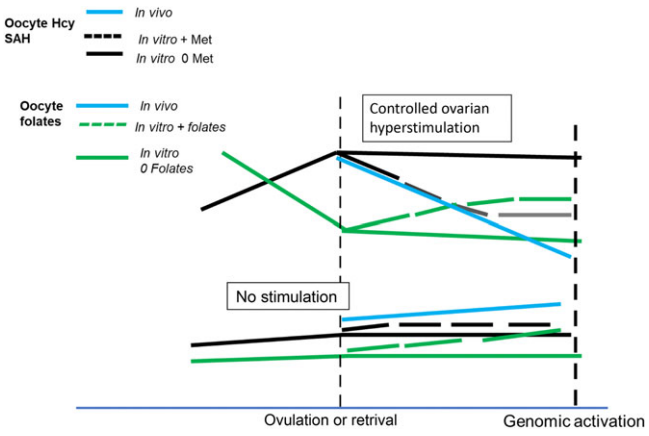

Figure 3. Trajectory of compounds related to methylation in oocytes/early embryos in controlled ovarian hyperstimulation (COH) or unstimulated cycles, cultured in media with or without *in vitro* folate support.

It is also of note that Hcy accumulation will have a negative effect on the fertility of women who carry methylene tetrahydrofolate reductase single nucleotide polymorphisms (MTHFR SNPs) (Altmäe *et al.*, 2010; Servy *et al.*, 2018). This mutation interferes with the folate cycle by limiting the conversion of folic acid to active 5-methyl tetrahydrofolate (see Figure 1); suboptimal methylation in these patients leads to DNA instability, and this is associated with anomalies in the chromosomal status of the embryo (Enciso *et al.*, 2016).

The observation that FF Hcy concentrations remain low in natural (unstimulated) cycles/ovaries (Szymański and Kazdepka-Ziemińska, 2003) provides further confirmation of the biochemistry outlined above. *In vivo*, the tubal environment provides adequate levels of methionine and folates, whereas Hcy levels are low. This allows a dynamic decrease in the ratio of Hcy to Met, so that the appropriate intracellular Met pool is maintained. Active transport of folates also facilitates Hcy recycling. In a natural cycle, oocyte Hcy levels remain low through recycling, and methionine can enter the embryo either from tubal fluids *in vivo* (Menezo and Laviolette, 1972; Aguilar and Reyley, 2005), or from culture medium if this essential amino acid is present. This biochemical feature is of major importance for further embryo culture: a medium that contains methionine and folate can mimic the *in vivo* mechanism for Hcy recycling. In the absence of methionine, an intracellular excess of Hcy can inhibit methylation during early embryonic stages prior to maternal to zygotic transition/zygotic genomic activation (MZT/ZGA), and a lack of folate prevents Hcy recycling. In the bovine, the quality of embryos obtained by superovulation followed by *in vivo* insemination can be explained by the capacity for Hcy/Met exchange in tubal fluid, which has an estimated 50 micromolar concentration of methionine (Menezo and Laviolette, 1972; Aguilar and Reyley, 2005). Similarly, the high incidence of multiple gestations sometimes seen after COH combined with artificial insemination in humans may reflect biochemical *in vivo* compensation, with exchange and recovery from elevated Hcy levels by the milieu of the tubal environment.

Preventing oxidative stress in the embryo

The negative influence of oxidative stress (OS) on embryo quality, and the mechanisms of defence have been previously described (El-Mouatassim *et al.*, 1999; Guérin *et al.*, 2001) and should be emphasized when examining mechanisms for biochemical protection against OS during IVF/ICSI.

The oocyte and early embryo contain a group of enzymes that are involved in the destruction of ROS, and there is a good correlation between specific mRNA expression and the activity of enzymes that cannot be synthesized before maternal to zygotic transition, such as superoxide dismutases (Cu, Zn and Mn SODs) and glutathione peroxidase (El-Mouatassim *et al.*, 1999). Upregulation of the pentose phosphate pathway (PPP) immediately post fertilization allows the production of NADPH, necessary for synthesis of reducing compounds such as glutathione (GSH). GSH has a certain capacity for crossing any type of membrane, in many cells. However, in most cases, the nature of these transporters remains unclear. GSH has never been detected in tubal fluid; adding glutathione to culture medium can potentially offer only a questionable protection of embryonic membranes. Cysteine sulfinate decarboxylase (CSD), which decarboxylates cysteine sulfinic acid (CSA) to form hypotaurine, another reducing compound, is absent; hypotaurine cannot be synthesized by oocytes or embryos and is acquired from tubal fluid *in vivo* (Guérin and Ménézo, 1995). Tubal fluid is rich in transferrin and ceruloplasmin (Menezo and Laviolette, 1972); these proteins reduce/remove free copper and iron divalent cations to minimize the risk of a Fenton reaction that will generate free radicals in the embryonic environment. Free radicals as an entity were first recognized by Fenton in 1894. Fe^{2+} and Cu^{2+} gave similar results:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$$

The chain reaction known as the Haber–Weiss reaction was described by Haber and Willstätter (1931), then by Haber and Weiss (1932) and then adopted by Weiss and Humphrey (1949):

$$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + OH^- + O_2.$$

Glutathione (GSH), γ-L-glutamyl-L-cysteinyl glycine and GSH/cysteine balance

GSH deserves special attention as the universal antioxidant molecule. First of all, reduced glutathione is necessary for sperm head swelling, and must be present at a significant concentration during fertilization. It is also used in the reduction of ribonucleotides to

produce deoxyribonucleotides by ribonucleotide reductase (expressed in the oocyte at 120× background signal). As mentioned previously, the oocyte/early embryo is equipped with key enzymes necessary for glutathione synthesis. GSH is synthesized from cysteine in the cytosol, by glutamate cysteine ligase (GCL), which has two subunits, catalytic and modifier; both are expressed in the oocyte/early embryo. Glutathione synthetase (GS), the enzyme that catalyzes condensation of gamma-glutamylcysteine and glycine to form glutathione is also highly expressed. These enzymes are ATP dependent.

Glutamic acid and glycine are easily synthesized by the embryo, but uptake is also possible as these two amino acids are present at high levels in tubal fluid (glycine: 2–3 mM, glutamic acid + glutamine: 0.5 mM). Glutathione-disulfide reductase (GSR) reduces oxidized glutathione to reduced glutathione, and GSR is expressed in the early embryo at 25× the background signal. This reaction requires NADPH, which is provided by upregulation of PPP (Comizzoli *et al.*, 2003); a transaldolase enzyme that allows some PPP metabolites to enter glycolysis is expressed at 1400× the background signal.

Cysteine is a true rate-limiting compound; cysteine/cystine is incorporated into the embryo by alanine/serine/cysteine transporter 2 solute carrier family 1 member 5 (SLC1A5), which is highly expressed both in the oocyte (30× the background signal) and in the preimplantation embryo up to the time of genomic activation. However, availability of the enzyme is dependent on the endogenous store accumulated during oocyte maturation. DNA/histone methylation releases homocysteine, and this cannot be recycled to form cystathionine, then cysteine, via the cystathionine β-synthase (CBS) pathway, which is not expressed in the human oocyte (Benkhalifa *et al.*, 2010; Ménézo *et al.*, 2013), another factor in the potentially toxic accumulation of Hcy. In these conditions, cysteine becomes an essential amino acid and the embryo is completely dependent on cysteine from the external compartment. *In vivo*, cysteine is provided by tubal fluid; its transport is activated by solute carrier family 3 member 1 (SLC3A1; transports cystine and dibasic and neutral amino acids), which is also highly expressed in the oocyte (35–40× background signal). Elevated SLC3A1 expression accelerates cysteine uptake, with regulated accumulation of reduced glutathione (GSH) (Ménézo and Elder, 2020).

GSH is the 'control tower' of embryonic redox status and homeostasis, and therefore activity of the enzymes involved in GSH metabolism is highly regulated at the levels of transcription, translation and post translation. Negative feedback control mechanism of gamma-glutamylcysteine synthetase by glutathione (Michaelis–Menten effect) is an important regulatory mechanism: both a deficit and an excess of GSH are deleterious. GSH is synthesized only in the cytosol and is transported into intracellular organelles.

Glutaredoxins are thiol-disulfide oxidoreductase enzymes, 'light proteins' that use glutathione as a cofactor; they are highly expressed in oocytes at levels between 25× and 50× background signal. Glutaredoxins are oxidized by oxidized substrates and are non-enzymatically reduced by reduced glutathione that is then regenerated to its reduced form by glutathione reductase (GSR), in the presence of NADPH obtained from the PPP. Glutaredoxins protect methylation processes by converting oxidized methionine sulfone to methionine. Based on intrinsic embryonic biochemical machinery alone, methionine inter-conversion is impossible. Methionine can be recycled from Hcy by methionine synthase activity, but this pathway cannot lead to the formation of cysteine. In a recent paper (Truong and Gardner, 2017), an improvement in embryo development was observed after adding acetyl cysteine and acetyl carnitine to the culture medium, even in the presence of 20% O_2. The authors attributed the benefit to the 'antioxidant' effect of these compounds. However, acetyl cysteine is a stable precursor of glutathione and does not have intrinsic antioxidant properties. Acetyl carnitine is not an antioxidant, it is a 'catalyzer' of lipid beta oxidation, and FF provides a source of acetyl carnitine *in vivo* (Montjean *et al.*, 2012).

Under conditions of cysteine restriction, the resulting imbalance may lead to autophagy by activation of glucose uptake, mediated by mitogen activated protein kinase, with PPP upregulation to generate NADPH to counteract ROS (Aquilano *et al.*, 2014). Thioredoxins (Trxs) are also important low-molecular-weight oxido-reductases whose active sites contain cysteine; Trx mutations rapidly lead to cell death. Cytoplasmic Trx1 and mitochondrial Trx2, as well as their reductases, are highly expressed in the human oocyte/early embryo, both present at 100× background signal. Trx reductases again require NADPH to reactivate their reducing activity. APEX/Ref-1 (strongly expressed), a multifunctional protein that is a major effector of DNA repair in human embryos (El-Mouatassim *et al.*, 2007) is associated with thioredoxin (Trx) in upregulation of redox potential (Hedley *et al.*, 2004). Elevated levels of both APEX/Ref-1 and Trx increase cell growth and resistance to programmed cell death (Powis *et al.*, 2000). APEX/Ref-1 controls the redox status of transcription factors such as Fos and Jun (both expressed in the oocyte), keeping them in an active reduced state (Kelley and Parsons, 2001).

A lack of reduced thiols and thiol redox imbalance upregulates glucose uptake and the PPP, which has already been activated during fertilization to increase the supply of NADPH. A risk of NADPH shortage is significant. For this reason, the supply of glucose for preimplantation embryos must not be severely reduced on the grounds of potential toxicity (Chatot *et al.*, 1989; Quinn, 1995; Quinn *et al.*, 1995) reviewed by Summers and Biggers (2003) by metabolic generation of ROS (Aquilano *et al.*, 2014).

Methionine (Met) and *S*-adenosyl methionine (SAM)

If we consider glutathione to be the control tower of embryo redox homeostasis, methionine is the mastermind behind methylation, imprinting and epigenesis. SAM is the universal cofactor of all methylation processes. Methionine uptake is very active in oocytes and preimplantation embryos; uptake is higher in humans than in the mouse, even after consideration of the difference in size (Menezo *et al.*, 1989). Methionine that is present in follicular and tubal fluids is sensitive to oxidation, generating methionine sulfone and methionine sulfoxide; this oxidation can be reversed, mainly by glutaredoxins; methionine sulfoxide reductases A and B are expressed, but are less active. All of the enzymatic steps necessary for SAM synthesis and for the one-carbon cycle are present in mouse, bovine and human embryos (Ménézo *et al.*, 2013) expressed at high levels (Benkhalifa *et al.*, 2010). Conversion to SAM is marginally higher in human than in mouse embryos and is regulated, rapidly reaching a plateau in the presence of increasing external Met concentrations. *S*-Adenosylmethionine acts as a switch between remethylation and transsulfuration through allosteric inhibition of methylene-tetrahydrofolate reductase and activation of cystathionine β-synthase (Fowler, 2005), but the CBS pathway is inactive

Table 1. Transport of methionine into the oocyte/early embryo. Expression of the transporters in the early embryo (microarrays; Menezo *et al.*, 2007; Benkhalifa *et al.*, 2010)

System	Name	Expression/background signal (BS)
A		Absent
ASC	SLC1A5	30×
L	SLC7A5	40×
	SLC7A8	60×
γ + L	SLC7A7	200×

System A: Alanine-preferring amino acid transporters, important in regulation of cell growth. These transporters are sodium-dependent active transporters that are able to transport amino acids against their concentration gradients.
System L: A major nutrient amino acid transport system that is responsible for Na^+-independent transport of neutral amino acids, including several essential amino acids.
System γ: Gamma transporter, a process that binds the AA with glutamic acid to form a gamma-glutamyl peptide for transport: usually a slow process.
Abbreviations: A, subfamily; ASC, alanine/serine/cysteine transporters subfamily (Na^+-dependent exchange of small neutral amino acids); L: corresponds to the amino acid form (D- or L-); SLC: SoLute Carrier.

before genomic activation. Methionine transporters are multiple and redundant (Table 1).

Methylation processes in gametes and embryos

Methylation of DNA/histone targets results in release of homocysteine (Hcy); this must be recycled to methionine by methionine synthase (MS), a system that is supported by the folate cycle. 5-Methyltetrahydrofolate-homocysteine transferase (MTR) and methyltetrahydrofolate-homocysteine methyltransferase reductase (MTRR) are highly expressed and active in early embryos. As the CBS pathway leading to the formation of cysteine and homoserine is not expressed, there is an absolute requirement for the MS system, and 'active folate', 5MTHF, must be available (see Figure 1). DNA methylation stabilizes the genome and for this reason carriers of MTHFR SNPs are susceptible to anomalies in DNA methylation that can lead to embryos with high rates of chromosomal aberrations (Enciso *et al.*, 2016; Servy *et al.*, 2018) leading to implantation failures and repeat miscarriages (Tara *et al.*, 2015). Methylation also participates in DNA repair by synthesis of thymine; DNA repair is partially processed at the expense of methylation. Thymidylate synthase is highly expressed in the early embryo (200× background signal) during the period when mRNA synthesis is weak or absent, prior to ZGA. Thymine synthesis also requires serine hydroxymethyl transferase and dihydrofolate reductase (DHFR), both also highly expressed before genomic activation. Folates are clearly at the epicentre of numerous biochemical pathways during this period before maternal to zygotic transition; oocytes express high levels of folate receptor 1 and folate transporter 1 solute carrier family 19 member 1 (SLC19A1), as well as all of the enzymes involved in the folate and one-carbon cycles (Ménézo *et al.*, 2013). Global rapid demethylation has been postulated to occur immediately post fertilization in human IVF embryos (Smith *et al.*, 2014), however there are increasingly further indications that the situation is far more complex. The paternal genome is rapidly demethylated first, followed by passive demethylation of maternal genes during the subsequent cell cycle. Paternal demethylated DNA appears to be immediately re-methylated (Park *et al.*, 2007). Methylation anomalies at these early stages will affect placental development (Georgiades *et al.*, 2001). Experiments in the mouse suggest that

Table 2. Expression of the enzymes involved in polyamine synthetic pathways (× times the background signal) in human oocytes/early embryos (microarrays; Menezo *et al.*, 2007; Benkhalifa *et al.*, 2010)

Enzymes	Expression signal (×)
Arginase II	200
SAM decarboxylase	200
Ornithine decarboxylase (ODC)	1300
Spermidine synthase	12
Spermine synthase	100

levels of methylation are stable up to and immediately after genomic activation (Croteau and Menezo, 1994; Fulka *et al.*, 2004; Okamoto *et al.*, 2016); embryonic DNA methyl cytosine content remains stable during this period. In human oocytes, DNA methyltransferase 1 (DNMT1), the enzyme that is responsible for maintenance of DNA methylation, is highly expressed. Its polyA mRNA is one of the most abundant transcripts found. DNMT3, responsible for *de novo* DNA methylation is also expressed, but at a much lower level. Active *de novo* methylation has been observed in ruminants (Park *et al.*, 2007) and in mouse (Croteau and Menezo, 1994) and may also occur in humans. Methylation maintenance and, to a lesser extent, *de novo* methylation persist in human embryos until, and immediately after, MZT and attenuating the exclusion of imprinted genes during the global demethylation that takes place during preimplantation embryo development. This means that methionine, the 'basic fuel' necessary for methylation, is a crucial requirement at this time. In addition, methionine restriction increases mitochondrial OS (Lu *et al.*, 2003) and methionine must also be protected from oxidation. Preimplantation embryos must maintain a balance between active and passive demethylation, with co-existing demethylation and maintenance of DNA methylation (Inoue and Zhang, 2011; Wang *et al.*, 2014). Methylation processes must be efficiently protected against OS that jeopardizes correct acquisition of methylation marks, as well as subsequent methylation maintenance. *In vivo*, this regulatory protection against OS is provided by endogenous glutathione and by hypotaurine in tubal cells (Guérin and Ménézo, 1995; Guérin *et al.*, 2001).

Polyamines and the one-carbon cycle (Figure 5 and Table 2)

SAM is a precursor for polyamine synthesis. These molecules are recognized as a major influence throughout the reproductive process (Lefèvre *et al.*, 2011), particularly in spermatogenesis. Their role in early embryogenesis is less clear, but has been demonstrated in the mouse (Pendeville *et al.*, 2001; Nishimura *et al.*, 2002). The two main enzymes involved in polyamine biosynthesis are highly expressed in human oocytes: ornithine decarboxylase (ODC) (1000× background signal), and arginase (100× background, see Figure 4). Members of the solute carrier (SLC)7 γ+ family of cationic amino acid transporters that are responsible for polyamine transport are expressed in oocytes and early embryos. Polyamines, and spermidine in particular, are regulators of early embryo methylation and decreased polyamine concentrations in general have been associated with aberrant methylation of the entire genome (Soda, 2018). Spermine protects against decreased DNMT activity and aberrant DNA methylation (Soda, 2018), and spermine synthase is

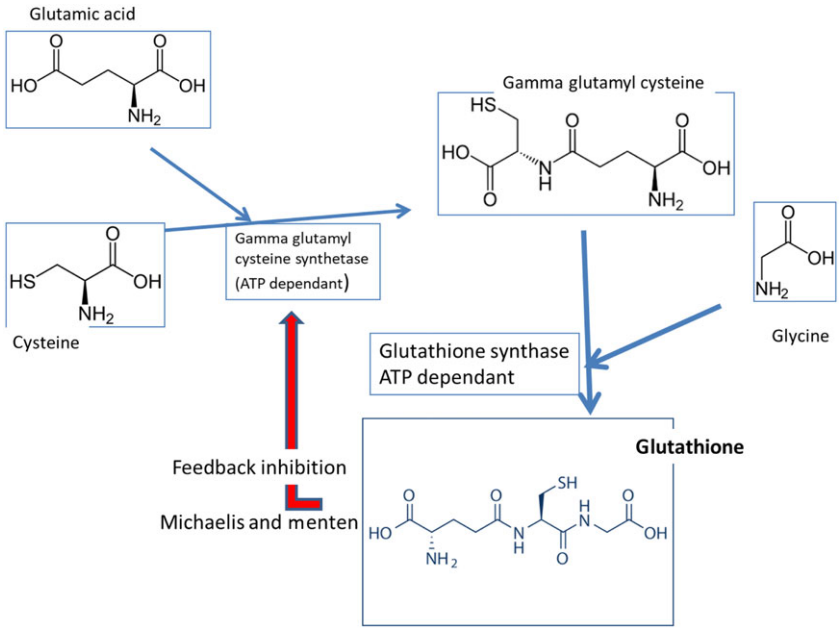

Figure 4. Glutathione synthesis from glycine and glutamine; 'retro-control' by Michaelis–Menten negative feedback by glutathione on gamma-glutamylcysteine synthetase.

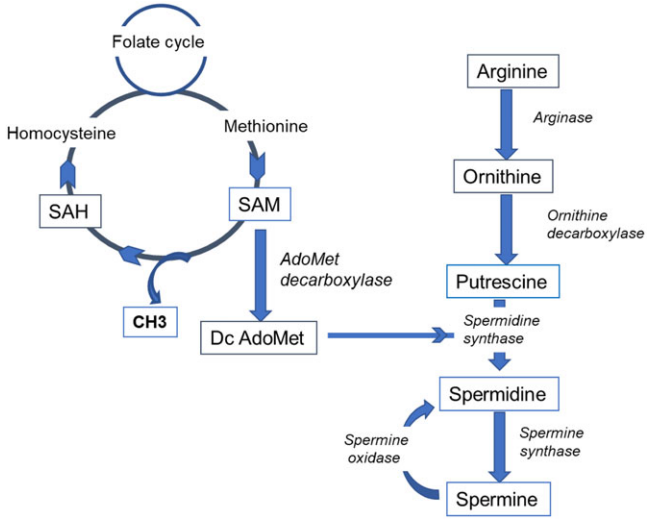

Figure 5. Polyamine synthesis and its connection with SAM and the folates and one-carbon cycles.

expressed at >100× background signal (BS); spermidine synthase has lower expression at >10× BS. Ornithine is also present in tubal fluid (Menezo and Laviolette, 1972); one of its transporters, solute carrier family 25 member 15 (SLC25A15) is not expressed in the embryo, but a second transporter, solute carrier family 25 member 29 (SLC25A29), is expressed at 30× basic signal. This means that during preimplantation stages the active synthesis of polyamines in the embryo is mainly dependent upon endogenous arginine. This is established in the oocyte during follicular growth and then depends on uptake from the tubal fluid environment after fertilization, in which it is present at ±200 µM during the embryonic free-floating stages (Aguilar and Reyley, 2005). The arginine transporter solute carrier family 7 member 7 (SLC7A7) is expressed at 220× BS in embryos (Closs *et al.*, 2004).

If ornithine decarboxylase (ODC) initiates the polyamine biosynthetic pathway and is 'the control tower', (Pegg, 2006) this does not seem to be the case in the early embryo where the expression of ODC is very high. Ornithine decarboxylase antizyme 1 and the antizyme inhibitor 1 are very strongly expressed at 600× and 30× BS respectively, confirming a strong trafficking activity around these molecules.

Methylation and oxidative stress *in vitro*

The processes involved in imprinting/epigenetic marking are crucial to normal development; conditions to allow their correct establishment *in vitro* must be provided and protected from OS.

Acquisition of methyl marks

Methylation requires SAM, the universal methylation cofactor. Demethylation immediately after fertilization has been described in human IVF embryos (Smith *et al.*, 2014), and methylation anomalies in babies conceived by ART have been described, associated with imprinting defects in some cases (Song *et al.*, 2015; Hattori *et al.*, 2019). Methylation processes cannot be correctly maintained without the appropriate substrates, and yet, with one exception, most IVF culture media that are commercially available do not contain methyl donors such as folates. Moreover, methionine is absent in three out of six first phase culture media; another contains 4 µM, which is insufficient to allow active uptake (Morbeck *et al.*, 2014). This omission apparently arose from a suggestion, based on mouse embryo culture, that some essential amino acids are toxic to early preimplantation embryos in sequential media (Lane and Gardner, 1997a, 1997b; Lane *et al.*, 2001). This concept was challenged by several authors, for several species (Ho *et al.*, 1994, 1995; Leese, 1998; Biggers and Summers, 2008; Herrick *et al.*, 2018). According to Leese (1998):

> 'This requirement (high turnover of some amino acids including methionine) would obviously not be fulfilled by culture media which included only nonessential amino acids during the early preimplantation phase. Our data led us to favour including all 20 amino acids in human embryo culture', cited by Biggers and Summers (2008).

Studies by Market-Velker *et al.* (2010, 2012) unambiguously confirm that current IVF culture media do not allow correct embryonic methylation status to be established, even in mouse embryos, the model that is used to evaluate media for human IVF culture (MEA, mouse embryo assay). Therefore, IVF embryos cultured in currently available culture media are an inappropriate model for evaluating the regulation of methylation in early stage embryos. Moreover, a suggestion that essential amino acids decrease the rate of first cleavage divisions in the mouse (Lane and Gardner, 1997a) was used as a further basis for removing essential amino acids from IVF culture media. This observation should be interpreted in the context of methylation studies by Market-Velker *et al.* (2010, 2012): epigenetic marking requires a certain amount of time, which decreases the speed of development so that rapid cleavage results in loss of imprinting. Developmental speed *in vitro* should be treated with caution, and with respect for the timing of essential processes that are crucial to normal development.

COH results in accumulation of Hcy, and its release from the oocyte after COH is an important feature for consideration. If methionine is absent from culture medium used prior to MZT, Hcy cannot be exchanged for Met (Menezo *et al.*, 1989), creating an abnormally high Hcy/Met in the embryonic environment. This imbalance inhibits methylation by at least two mechanisms: lack of methionine for synthesis of SAM, and inhibition due to the accumulation of SAH and Hcy in the embryo. SAH binds to the catalytic region of most SAM-dependent methyltransferases with high affinity (Hoffman *et al.*, 1980) and is a potent inhibitor of DNMT(s) (Cohen *et al.*, 2005).

Protecting methylation against OS

A clear correlation has been described between OS and methylation anomalies (Ménézo *et al.*, 2013). This is a significant issue *in vitro*, as IVF culture media spontaneously generate free radicals during incubation (Martín-Romero *et al.*, 2008) and there is no antioxidant protection: embryos have limited protection against oxidative insults and are not able to synthesize glutathione, if the precursor cysteine is lacking. Most of the endogenous oocyte glutathione has been used for sperm swelling. An excess of oxygenated free radicals can lead to the formation of hydroxymethyl cytosine, which may precipitate undue and abnormal demethylation processes. Oxidation of methylcytosine (MeC) can cause active demethylation of some CpG sites, which are known to be crucially related to imprinting mechanisms (Menezo *et al.*, 2019a). Decreased oxygen tension can partially compensate for lack of some types of antioxidant protection, with the exception of hypotaurine, which is present in the natural embryonic environment. *In vivo*, the embryo can generate glutathione from cysteine by GCL or GS as endogenous protection against OS. However, as the CBS pathway is not active, Hcy cannot be converted to cystine, and cysteine/cystine must be provided by the culture medium. Most (three out of six) of 'first phase' sequential culture media did not contain cystine, and one contained 2 µM. Fertilization media (when information is available) do not contain cystine.

Arginine and regulation of methylation

Arginine, which is also considered to be an essential amino acid, is essential for polyamine synthesis: this feature should be taken into consideration in the composition of *in vitro* culture media, as the embryo has appropriate arginine transport capacity. A lack of arginine removes another level of the regulatory processes involved in methylation, again contributing to genomic instability as a result of altered methylation status (Soda, 2018).

Conclusion

Recent observations have highlighted a risk of aberrant epigenetic/imprinting events associated with ART, and IVF/ICSI in particular. There is a consensus opinion that these risks originate during the period of *in vitro* culture, and may be due specifically to the composition of culture media (Edwards and Ludwig, 2003; Menezo *et al.*, 2019b; Ménézo and Elder, 2020). Sunde *et al.* (2016) have correctly observed that it is 'Time to take human embryo culture seriously'. The literature surrounding human IVF culture media is replete with confounding and unclear statements, many without real scientific foundation. In relation to biochemical aspects of methylation and its association with OS, the suggestion that essential amino acids are toxic during early stages of development (Lane and Gardner, 1997a, 1997b; Lane *et al.*, 2001) must be questioned. Early embryos in culture need methionine, cystine/cysteine, arginine and a certain amount of glucose to generate NADPH and ribose-5-phosphate required for nucleotide synthesis. NADPH is a key effector/regulator of the majority of the anabolic processes. Methionine is also required to re-establish the correct oocyte/embryo Met/Hcy ratio after COH. Under appropriate conditions, the embryo uses methionine to produce SAM, arginine to produce polyamines and cystine/cysteine for glutathione synthesis. Glutathione provides protection against ROS and preserves methylation markers from inappropriate demethylation due to oxidized methyl cytosine and demethylation by the TET system. Addition of folates merits attention, not only for their role in embryo metabolism, but also with respect to the genomic instability observed in embryos originating from women carrying the two MTHFR SNPs C677T and A1298C (Enciso *et al.*, 2016).

Interestingly, it was recently demonstrated that addition of *N*-acetyl cysteine to culture media alleviates the need for reduced oxygen tension (Truong and Gardner, 2017): this strongly suggests that embryos might best be protected against OS by allowing them to manufacture their own protection, by glutathione and the associated light proteins, thioredoxins and glutaredoxins. This fits with the 'back to nature' concept (Tervit *et al.*, 1972; Leese, 1998; Houghton *et al.*, 2002), which should also involve closer inspection of the biochemistry associated with major metabolic processes of the embryo. Addition of external antioxidants is a complicated issue as some antioxidants, such as vitamins E and C, can rapidly switch to pro-oxidant action in the absence of antioxidant enzymes such as superoxide dismutases.

Large numbers of sequential media formulations do not contain essential amino acids, and therefore major effectors/regulators/protectors of crucial methylation processes are missing. Similarly, a certain amount of glucose is required to activate the pentose pathway, which generates the NADPH necessary for glutathione synthesis and anabolic processes in general. This role of glucose should not be overlooked in the design of culture media.

Sophisticated developments in analytic technologies over the past decade have provided elegant and precise tools for studying the molecular biology of human gametes and embryos at the single-cell level, allowing metabolic pathways and general metabolism to be probed in detail. This has particular significance with respect to epigenetic regulation by DNA and histone methylation, as well as the hazardous influence of OS. Aspects of modern lifestyles now add a further level of concern, with environmental

pollution by endocrine disruptor compounds that generate high levels of OS and strongly affect methylation processes (Skinner *et al.*, 2010; Manikkam *et al.*, 2013; Nahar *et al.*, 2015; Montrose *et al.*, 2018; Menezo *et al.*, 2019b). Technological advancement in microscopy and culture systems have also accumulated vast amounts of data surrounding morphologic and morphometric parameters of embryo development. Revisiting these types of data, such as speed of development/methylation process/embryo quality (Bos-Mikich *et al.*, 2001) in the perspective of biochemical/molecular biology observations reviewed here (Market-Velker *et al.*, 2012) can lead to a deeper understanding of the fundamental processes involved in the early stages of life. There needs to be understanding based upon a scientific approach of the basic principles involved in providing safe and appropriate culture conditions for *in vitro* culture of human gametes and embryos. It is time to leave the 'mouse model' behind as part of history.

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