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ORIGINAL ARTICLE *Embryology*

Ammonium accumulation in commercially available embryo culture media and protein supplements during storage at 2–8°C and during incubation at 37°C

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STUDY QUESTION: Does ammonium accumulate in commercially available culture media and protein supplements used for *in vitro* development of human pre-implantation embryos during storage and incubation?

SUMMARY ANSWER: Ammonium accumulates in ready-to-use *in vitro* fertilization (IVF) culture media during storage at 2–8°C and in ready-to-use IVF culture media and protein supplements during incubation at 37°C.

WHAT IS KNOWN ALREADY: Both animal and human studies have shown that the presence of ammonium in culture medium has detrimental effects on embryonic development and pregnancy rate. It is, therefore, important to assess the amount of ammonium accumulation in ready-to-use IVF culture media under conditions that are common in daily practice.

STUDY DESIGN, SIZE, DURATION: Ammonium accumulation was investigated in 15 ready-to-use media, 11 protein-free media and 8 protein supplements.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Ammonium was measured by the use of an enzymatic method with glutamate dehydrogenase. To simulate the storage and incubation conditions during IVF treatments, ammonium concentrations were measured at different time-points during storage at 2–8°C for 6 weeks and during incubation at 37°C for 4 days.

MAIN RESULTS AND THE ROLE OF CHANCE: All ready-to-use, i.e. protein supplemented, culture media showed ammonium accumulation during storage for 6 weeks (ranging from 9.2 to 99.8 μM) and during incubation for 4 days (ranging from 8.4 to 138.6 μM), resulting in levels that might affect embryo development. The protein supplements also showed ammonium accumulation, while the culture media without protein supplementation did not. The main sources of ammonium buildup in ready-to-use culture media were unstable glutamine and the protein supplements. No additional ammonium buildup was found during incubation when using an oil overlay or with the presence of an embryo in the culture droplet.

LIMITATIONS, REASONS FOR CAUTION: In addition to the unstable glutamine and the protein supplements, other free amino acids might contribute to the ammonium buildup. We did not investigate the deterioration of other components in the media.

WIDER IMPLICATIONS OF THE FINDINGS: Break-down of components into ammonium is more pronounced during incubation at 37°C, however, it is not negligible during storage at 2–8°C. This results in increasing ammonium levels in culture media over time that may affect embryo development. Therefore, it is important that the use of free L-glutamine in human embryo culture media is stopped and that the use of protein supplements is thoroughly evaluated.

STUDY FUNDING/COMPETING INTEREST(S): No funding or no competing interests declared.

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Key words: ammonium / culture medium / IVF/ICSI / glutamine / protein

Introduction

Commercially available embryo culture media are routinely used to culture human pre-implantation embryos for 2–6 days during an *in vitro* fertilization (IVF) treatment. Culture media are considered to be an important factor in IVF, as they can affect the live birth rate, pregnancy rate, implantation rate, fertilization rate and the number of good quality embryos (Mantikou *et al.*, 2013). Amino acids have been added to most embryo culture media, as it has been shown that they have a beneficial effect on pre-implantation embryo development in several species (Bavister, 1995; Gardner and Lane, 1997; Biggers and Summers, 2008). However, it is known that amino acids, especially free L-glutamine, spontaneously break-down into ammonium during incubation at 37°C (Gardner and Lane, 1993).

Studies with animal models have shown that the addition of exogenous ammonium to embryo culture media has detrimental effects on embryonic development, pregnancy rates and fetal development (Lane and Gardner, 1994,2003; Hammon *et al.*, 2000; Sinawat *et al.*, 2003; Zander *et al.*, 2006; Gardner *et al.*, 2013). Especially during the cleavage stage, embryos are vulnerable to ammonium in culture media (Zander *et al.*, 2006; Gardner *et al.*, 2013). In human studies, it was found that elevated ammonium levels in culture media negatively affect blastocyst formation (Virant-Klun *et al.*, 2006; Hashimoto *et al.*, 2008) and result in the altered expression of 390 genes involved in metabolism, cell growth and/or maintenance, transcription, cell communication, transport, development and regulation of transcription (Gardner *et al.*, 2013).

To reduce the ammonium accumulation in embryo culture media, the unstable L-glutamine has been replaced with a dipeptide form, e.g. alanyl-glutamine or glycyl-glutamine, in most embryo culture media. However, in these media, a slight ammonium accumulation has been observed (Gilbert *et al.*, 2012; Li *et al.*, 2013). Apart from amino acids, other factors, such as the protein supplementation, might contribute to the ammonium accumulation during culture (Meintjes, 2012). The aim of the present study was to assess ammonium accumulation in currently commercially available culture media with and without protein supplementation and in separate protein supplements used for *in vitro* development of human pre-implantation embryos.

Materials and Methods

Culture media

Ammonium buildup was investigated in protein supplements and both ready-to-use (protein supplemented) and protein-free culture media from different manufacturers.

The ready-to-use culture media were Cook Sydney IVF fertilization medium (K-SIFM-20), Cook Sydney IVF cleavage medium (K-SICM-20), Cook Sydney IVF blastocyst medium (K-SIBM-20), Sage Quinn's advantage protein plus fertilization medium (ART-1520), Sage Quinn's advantage protein plus cleavage medium (ART-1526), Sage Quinn's advantage protein plus blastocyst medium (ART-1529), Irvine Complete Early Cleavage Medium with dextran serum supplement (DSS) (90 142), Irvine Complete MultiBlast medium with DSS (90 143), Irvine Continuous Single Culture Complete (CSC) medium (90 165), Global total for fertilization (LGTF-050), Global total medium (LGGT-030), Origio ISM1 culture medium (1150), Vitrolife G-IVF PLUS v5 (10 136), Vitrolife G-1 PLUS v5 (10 128) and Vitrolife G-2 PLUS v5 (10 132) (Supplementary data, Table SI).

The protein-free culture media were Sage Quinn's advantage fertilization medium (ART-1020), Sage Quinn's advantage cleavage medium (ART-1026), Sage Quinn's advantage blastocyst medium (ART-1029), Irvine Early Cleavage Medium (90 138), Irvine MultiBlast medium (90 139), Global for fertilization (LGGF-020), Global medium (LGGG-020), Lonza human tubal fluid (HTF) medium (BE02-036F), Vitrolife G-IVF (10 135), Vitrolife G-1 (10 127) and Vitrolife G-2 (10 131) (Supplementary data, Table SII).

The protein supplements were Sage human serum albumin (HSA) (ART-3001), Sage serum protein substitute (SPS; ART-3011), Irvine HSA (9988), Irvine serum substitute supplement (SSS; 99 193), Global HSA (GHSA-125), Sanquin Albuman (21–136), Vitrolife HSA (10 064) and Vitrolife G-MM (10 038) (Supplementary data, Table SIII). Furthermore, two freshly made glutamine solutions were tested: 1 mM of L-glutamine (Sigma-Aldrich, St Louis, USA; G8540-25G) and 1 mM of glycyl-glutamine (Sigma-Aldrich, St Louis, USA; G5149-1G) in the HTF medium (Lonza, Verviers, Belgium; BE02-036F). Glutamine is frequently used at a concentration of 0.5–1 mM in embryo culture (Gardner, 2007) and HTF is a medium devoid of amino acids and protein.

Immediately upon arrival, ordered culture media and protein supplements were stored in a refrigerator (2–8°C) connected to a temperature real-time monitoring and alerting system XiltriX (IKS International, Torphins, UK).

Ammonium measurements

To simulate the storage and incubation conditions during IVF treatments, ammonium concentrations were measured at different time-points during storage at 2–8°C and during incubation at 37°C (90% relative humidity, 6% CO_2, 5% O_2). Incubation was performed without gametes or embryos for 2 days in case of fertilization medium and for 4 days in case of the cleavage and blastocyst medium, i.e. the number of days the medium would be in the incubator during a normal IVF treatment, taking into account that the medium is placed in the incubator a day before use to allow equilibration. At the time of ammonium analysis, samples were collected and transported on ice to the Central Diagnostic Laboratory of our hospital, where the samples were measured immediately.

Ammonium was measured by an enzymatic method with glutamate dehydrogenase (GLDH). This method is based on the chemical reaction in which glutamate and $NADP^+$ are formed by the reductive amination of 2-oxoglutarate with NH_4^+ and NADPH, which is catalyzed by GLDH. The ammonium concentration is directly proportional to the concentration of the formed $NADP^+$, which is determined by measuring the decrease in absorbance by using the Cobas 6000 Analyzer Series (Roche Diagnostics, Basel, Switzerland). As the Cobas 6000 analyzer has been calibrated for analysis of plasma, the method has been validated for the analysis of embryo culture media. A serum control sample (218.2 μM NH_4^+) and an embryo culture medium sample (46.3 μM NH_4^+) have been used to create a dilution series (1:1, 1:3, 1:6 and 1:9). Measured concentrations were similar to the theoretical concentrations, with a deviation ranging from −0.2 to 3.6% (average 1.3%). This indicates that there is no matrix effect. Precision was determined at two different concentrations (99.4 μM NH_4^+ and 203.1 μM NH_4^+), CV (within-run) values were 2.1 and 3.1%, CV (between-day) values were 1.3 and 1.7% and the total imprecision was 3.7 and 2.1%, respectively. The minimal detection threshold was 10 μM.

All ammonium concentrations are given as mean values of duplicate measurements and for all media two or three different batches were used to investigate the average ammonium accumulation, except for Sage SPS, which was discontinued in the Netherlands during this study by the manufacturer due to the absence of Conformité Européenne marking. For the construction of the ammonium buildup curves, the measurement at the first time-point of each medium or solution was set to zero and all measurements at subsequent time-points were related to this initial measurement. This was done to allow a reliable comparison in ammonium buildup between the different culture media, as age of the medium (from the time point of manufacture) upon arrival at our department was unknown for some of the media. First measurements started within 2 weeks of the arrival of the medium at the laboratory and all measurements were performed before the expiry date.

Statistical analysis

Differences in ammonium buildup between the two glutamine solutions and between culture media were tested by use of Student's *t*-test and by the use of one-way analysis of variance followed by Scheffe's *post hoc* multiple comparisons test, respectively.

Results

Ammonium production in ready-to-use culture media

The ammonium buildup during storage in the refrigerator at 2–8°C in several ready-to-use culture media, which are already supplemented with protein, are presented in Fig. 1. The average accumulation during storage was lowest in the fertilization medium from Global (10.0 ± 1.2 μM), Irvine (14.0 ± 2.1 μM), Vitrolife (15.8 ± 2.9 μM), Irvine CSC (22.5 ± 0.5 μM) and Cook (24.8 ± 3.7 μM). The ammonium buildup was significantly higher in the Sage medium (38.8 ± 1.0 μM) when compared with the Global, Irvine, Vitrolife or Irvine CSC medium, however, it was highest in the Origio ISM1 medium, which reached a level of 99.8 ± 3.5 μM in 6 weeks (Fig. 1A). In the cleavage media (Fig. 1B), the ammonium buildup during storage was not significantly different between Global (9.2 ± 0.7 μM), Irvine (14.0 ± 2.1 μM), Vitrolife (20.5 ± 2.8 μM), Irvine CSC (22.5 ± 0.5), Sage (28.2 ± 6.2 μM) and Cook (28.8 ± 1.1 μM), while the ammonium buildup was significantly higher in the Origio ISM1 medium (99.8 ± 3.5 μM). In the blastocyst media (Fig. 1C), the average ammonium buildup during storage was not significantly different between Global (9.2 ± 0.7 μM), Sage (13.3 ± 0.7 μM), Irvine (22.0 ± 3.1 μM), Cook (22.1 ± 3.1 μM), Irvine CSC (22.5 ± 0.5 μM) and Vitrolife (24.2 ± 4.8 μM).

The ammonium buildup in several ready-to-use culture media during incubation at 37°C are presented in Fig. 2. The average accumulation during incubation was lowest in fertilization medium from Vitrolife (8.4 ± 1.6 μM), Irvine (8.9 ± 2.0 μM), Global (11.2 ± 0.6 μM), Cook (13.5 ± 0.9 μM) and Irvine CSC (14.2 ± 1.8). The ammonium buildup was higher in the Sage medium (22.6 ± 2.9 μM) when compared with the Vitrolife or Irvine medium, however, it was highest in the Origio ISM1 medium, which reached a level of 85.4 ± 3.0 μM in 2 days (Fig. 2A). In cleavage media (Fig. 2B), the ammonium buildup during incubation was not significantly different between the media from Sage (10.6 ± 1.0 μM), Irvine (13.7 ± 2.8 μM), Vitrolife (15.3 ± 2.0 μM) and Global (17.8 ± 0.9 μM). The ammonium buildup in Cook (24.8 ± 1.6 μM) was only significantly higher when compared with Sage, while Irvine CSC (26.3 ± 0.8 μM) was significantly higher when compared with the Sage, Irvine or Vitrolife medium and ammonium buildup was highest in the Origio ISM1 medium (138.6 ± 1.9 μM). In blastocyst media (Fig. 2C), the average ammonium buildup during incubation was significantly lower in the Sage medium (6.6 ± 1.1 μM) when compared with Global (17.8 ± 0.9 μM), Irvine (19.5 ± 1.1 μM), Vitrolife (23.4 ± 2.4 μM), Cook (25.8 ± 3.6 μM) and Irvine CSC (26.3 ± 0.8 μM).

Ammonium production in glutamine solutions

The Origio ISM1 medium was the only medium in this study that contained the relatively unstable free L-glutamine, which might be the cause of the high-ammonium buildup. Two glutamine solutions were prepared, one with the unstable free L-glutamine and one with a stable dipeptide of glutamine, to verify the difference in ammonium buildup.

The average ammonium buildup during storage at 2–8°C and during incubation at 37°C in solutions with L-glutamine or with the dipeptide glycyl-glutamine are presented in Table I. There was no detectable ammonium buildup during storage for 6 weeks and little buildup during incubation for 4 days in the glycyl-glutamine solution. There was significantly more ammonium production in the L-glutamine solution, both during storage for 6 weeks and incubation for 4 days (230.6 ± 2.5 μM and 208.0 ± 3.5 μM, respectively).

Ammonium production in protein-free culture media and protein supplements

To investigate the role of the protein supplements in the ammonium accumulation, ammonium buildup was first evaluated in protein-free culture media. Both during storage at 2–8°C for 6 weeks and incubation at 37°C for 4 days, there was no detectable ammonium buildup in these culture media (data not shown). This indicates that the protein supplement might be another source for the ammonium buildup. Next, the average ammonium buildup in protein supplements was measured (Fig. 3). All protein supplements showed ammonium buildup during incubation for 4 days, ranging from 18.9 μM in Vitrolife G-MM up to 144.7 μM in Irvine SSS. No statistical comparisons between groups were performed, since the protein supplements were used undiluted and the concentrations of protein were not the same in all solutions.

Ammonium production during embryo culture and with oil overlay

In order to assess the effect of the presence of an embryo in the culture medium, we collected 26 pooled droplets (15 μl) of Vitrolife G-1 PLUS v5 incubated with an embryo (originating from 2PN zygotes) and 26 pooled droplets (15 μl) of Vitrolife G-1 PLUS v5 incubated without an embryo from the same culture dish, cultured in a 5% O_2, 6% CO_2 and 89% N_2 environment. From both pools, 200 μl was taken to compare ammonium levels and no additional ammonium buildup was detected by the presence of an embryo in the medium droplet at Day 3 after ovum retrieval. Furthermore, the effect of a mineral oil overlay was evaluated by comparing the ammonium levels of Vitrolife G-1 PLUS v5 with and without a mineral oil overlay (Irvine Scientific, 9305), which resulted in similar ammonium buildup after 4 days of incubation at 37°C.

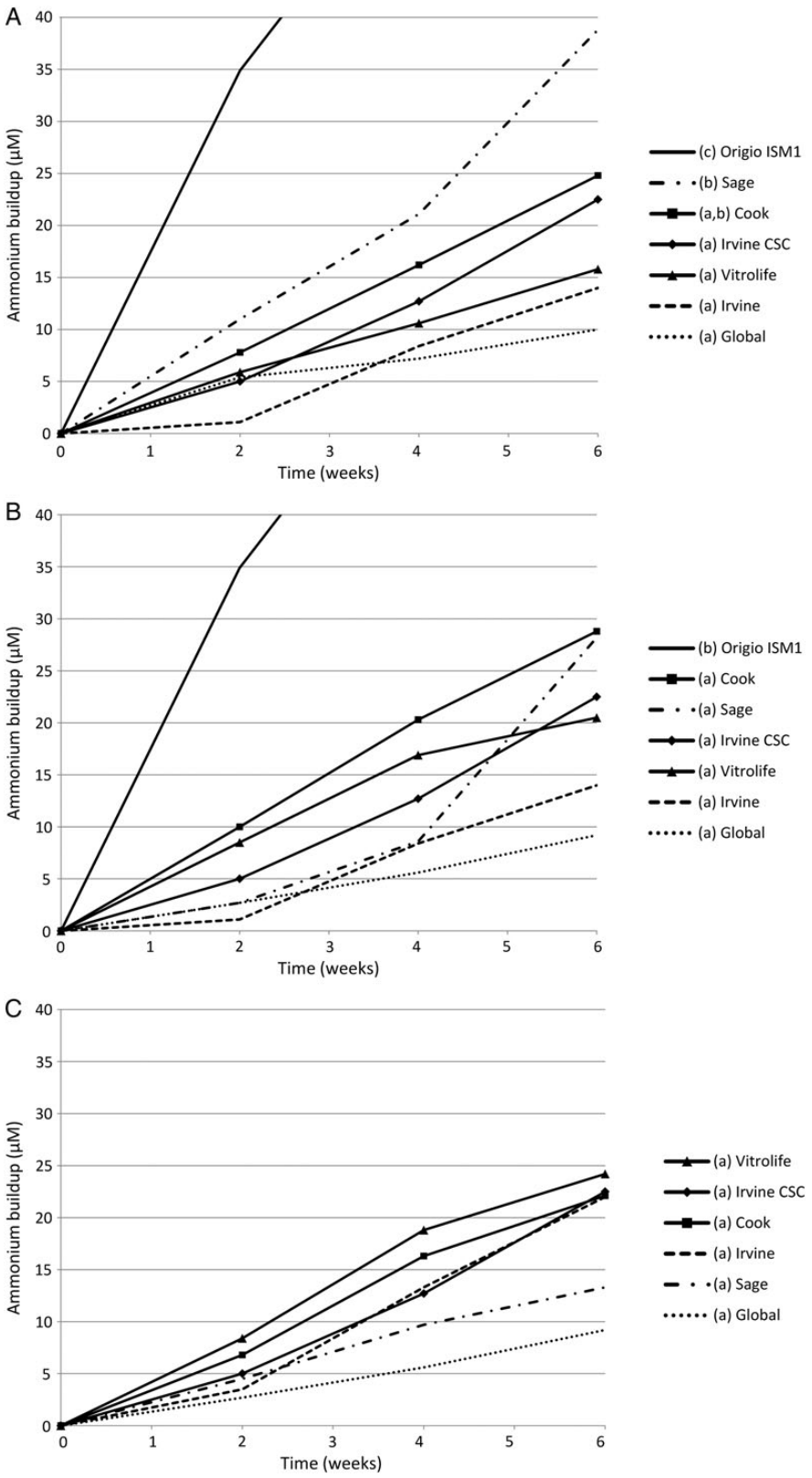

Figure 1 Average ammonium buildup in ready-to-use, protein supplemented, media during storage at 2–8°C. The fertilization media (**A**) and cleavage media (**B**) from Cook, Global, Irvine, Origio, Sage and Vitrolife. The average ammonium buildup of the Origio ISM1 culture medium, which contains the mono-peptide L-glutamine, was 66.1 µM in 4 weeks and 99.8 µM in 6 weeks (not displayed in figures A and B). The blastocyst media (**C**) from Cook, Global, Irvine, Sage and Vitrolife. Different letters in the legend indicate significant differences ($P < 0.05$) in ammonium buildup at 6 weeks. SEMs are given in the main text.

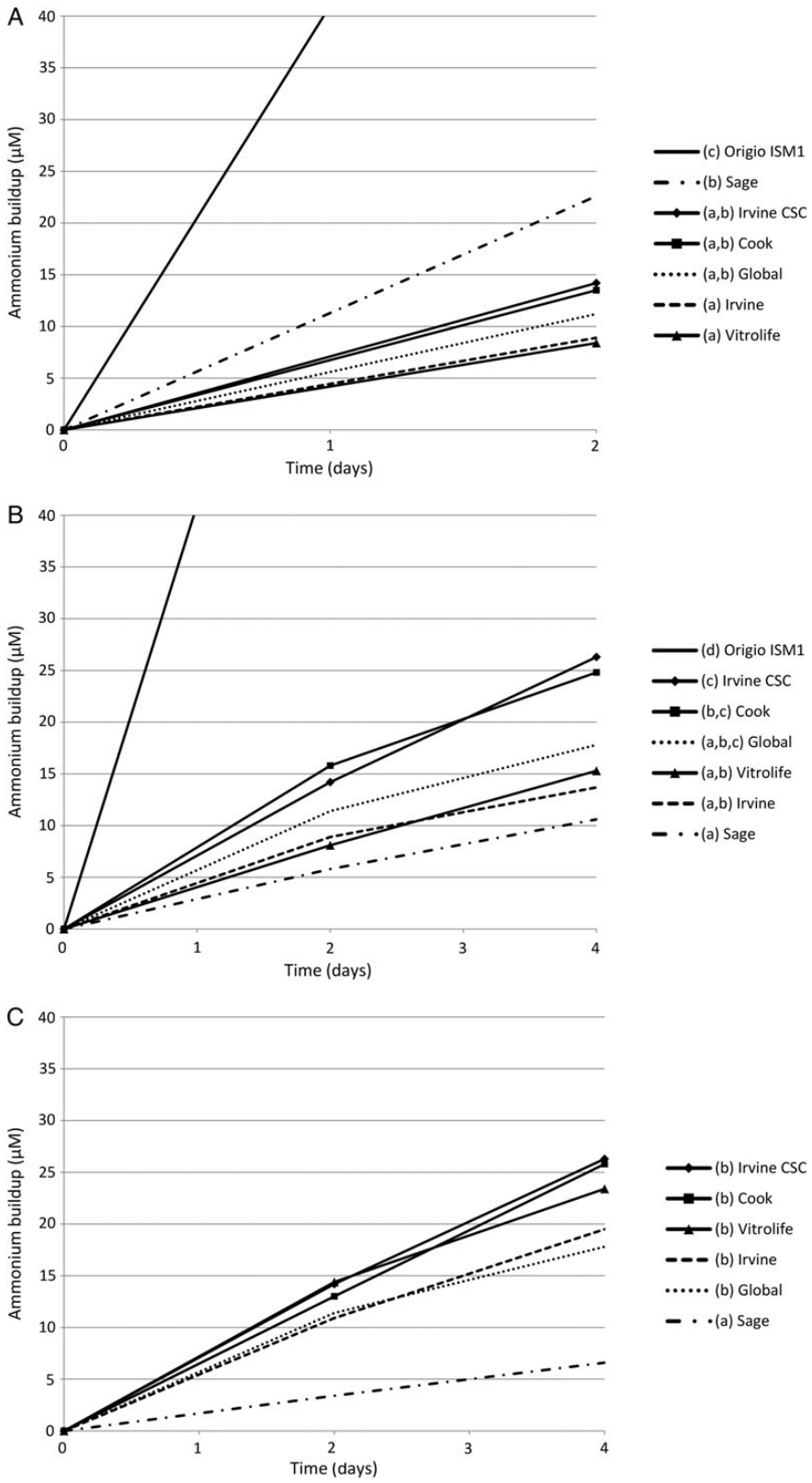

Figure 2 Average ammonium buildup in ready-to-use, protein supplemented, media during incubation at 37°C. The fertilization media (**A**) and cleavage media (**B**) from Cook, Global, Irvine, Origio, Sage and Vitrolife. The average ammonium buildup of the Origio ISM1 culture medium, which contains the mono-peptide L-glutamine, was 85.4 µM in 2 days and 138.6 µM in 4 days (not displayed in figures A and B). The blastocyst media (**C**) from Cook, Global, Irvine, Sage and Vitrolife. Different letters in the legend indicate significant differences ($P < 0.05$) in ammonium buildup at Day 2 (A) or Day 4 (B and C). SEMs are given in the main text.

Table I Average ammonium buildup (μM) in solutions of L-glutamine (1 mM) and glycyl-glutamine (1 mM) during storage at 2–8°C and during incubation at 37°C.

Condition	Time-point	L-glutamine Ammonium buildup (μM)	Glycyl-glutamine Ammonium buildup (μM)	*P*-values
Storage	Week 2	58.0 ± 0.9	0.0 ± 0.0	<0.001
Storage	Week 4	130.5 ± 4.1	0.0 ± 0.0	0.001
Storage	Week 6	230.6 ± 2.5	0.0 ± 0.0	<0.001
Incubation	Day 2	103.6 ± 3.4	0.0 ± 0.0	0.001
Incubation	Day 4	208.0 ± 3.5	7.5 ± 1.3	<0.001

Data presented as mean ± SEM.

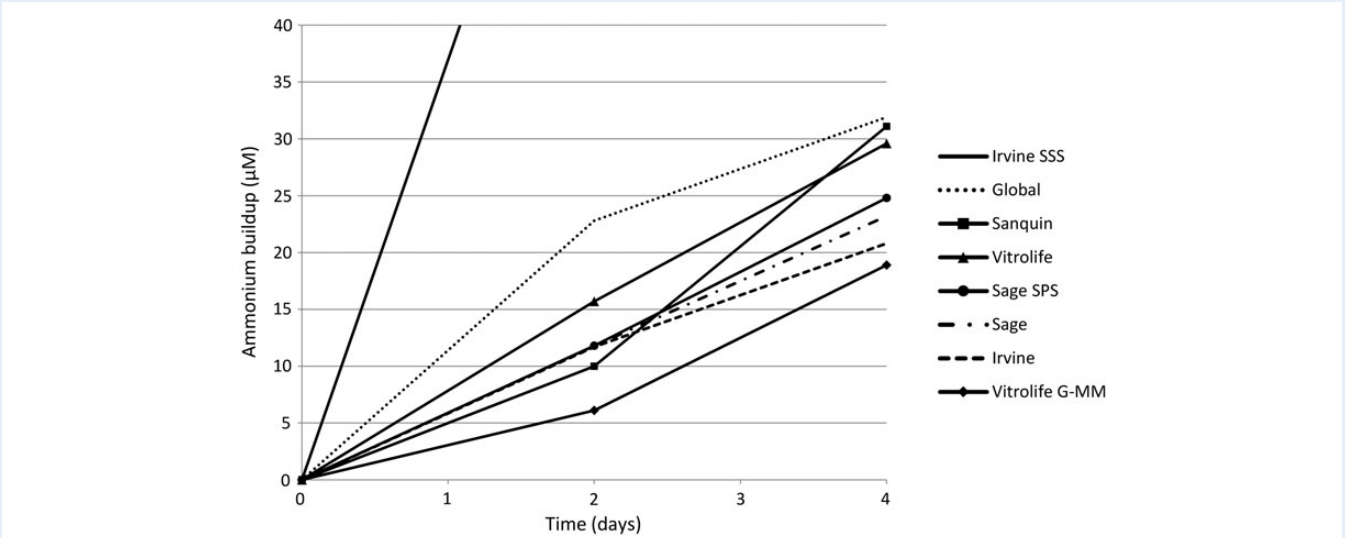

Figure 3 Average ammonium buildup in undiluted protein supplements from Global (100 mg/ml HSA), Irvine (100 mg/ml HSA), Irvine SSS (50 mg/ml HSA + 10 mg/ml α- and β-globulins), Sage (100 mg/ml HSA), Sage SPS (44 mg/ml HSA + 6 mg/ml α- and β-globulins), Sanquin (40 mg/ml HSA), Vitrolife (100 mg/ml HSA) and Vitrolife G-MM (50 mg/ml recombinant HSA) during incubation at 37°C. The average ammonium buildup of Irvine SSS was 73.9 μM in 2 days and 144.7 μM in 4 days (not displayed). No statistical comparisons between groups were performed.

Discussion

This study shows that ammonium accumulates in ready-to-use IVF culture media during storage at 2–8°C and during incubation at 37°C. The main sources are unstable glutamine and the protein supplement.

In the early days of IVF, embryo culture media consisted of simple salt solutions (Lane and Gardner, 2007). Nowadays, most commercially available culture media consist of a mixture including salts, energy sources, proteins, antibiotics, amino acids and vitamins. The different components have been added to the media to support embryo development and implantation rates (Smith *et al.*, 2012). However, it is known that amino acids may spontaneously break-down into ammonium, which is toxic for the embryo (Gardner and Lane, 1993).

It was reported that free L-glutamine leads to more ammonium production compared with a dipeptide form of glutamine during incubation (Lane and Gardner, 2003; Tareq *et al.*, 2007; Hashimoto *et al.*, 2008). This study confirms that indeed a solution with L-glutamine results in more ammonium production than a glycyl-glutamine solution during incubation and storage. This might explain the significantly higher level of ammonium buildup in the Origio ISM1 medium, since this was the only medium in the present study that contained the unstable L-glutamine at a concentration of 778 μM according to Morbeck *et al.* (2014a). The replacement of L-glutamine with a dipeptide form of glutamine might enhance embryo development (Biggers *et al.*, 2004b; Summers *et al.*, 2005; Hashimoto *et al.*, 2008; Meintjes *et al.*, 2009), although, this was not found by others (Gray *et al.*, 1992).

The commercially available culture media that have been measured in the present study were ready-to-use media, which were already supplemented with protein, and protein-free media, which need to be supplemented with protein before use. We found that there was no detectable ammonium buildup in the protein-free media, while their ready-to-use counterparts showed ammonium buildup. Furthermore, all protein supplements, which can be used to supplement the protein-free media, showed ammonium buildup during incubation. This indicates that the ammonium buildup in the ready-to-use protein supplemented culture media might not only be a result of amino acid degradation, but also

the degradation of proteins or of contaminants in the protein supplement (Gray et al., 1992; Chan et al., 1995; Meintjes et al., 2009; Meintjes, 2012; Xiao and Isaacs, 2012; Dyrlund et al., 2014; Morbeck et al., 2014b). Contamination of the protein supplement might play an important role. In a recent study by Morbeck et al. (2014b), it was shown that Irvine SSS contained high levels of both essential and non-essential amino acids, including glutamine, while other protein supplements such as HSA and Sage SPS contained no or very low levels of amino acids. In our study, ammonium buildup during incubation at 37°C for 4 days was relatively high in Irvine SSS (144.7 μM) when compared with the other protein supplements (ranging from 18.9 to 31.9 μM).

Apart from the unstable glutamine and protein supplements, also other free amino acids might contribute to the ammonium buildup (Moravek et al., 2012). We did not investigate the deterioration of other components in the media.

In this study, no additional ammonium buildup during incubation at 37°C was detected by the presence of an embryo in the medium droplet at Day 3 after ovum retrieval. This seems to be in contrast with a study by Gardner et al. (2001), in which they found an ammonium production up to 30 pmol per embryo per hour for human blastocysts. However, it is not unlikely that the ammonium production of a cleavage stage embryo (consisting of ~8 cells) is negligible compared with that of a blastocyst.

Several studies showed that ammonium has a negative effect on embryo development, pregnancy rates and fetal development in animals (Gardner and Lane, 1993; Lane and Gardner, 1994, 2003; Hammon et al., 2000; Sinawat et al., 2003; Zander et al., 2006; Tareq et al., 2007; Yuan and Krisher, 2010; Gardner et al., 2013) and in humans (Virant-Klun et al., 2006; Hashimoto et al., 2008; Gardner et al., 2013; Li et al., 2013). Most of these studies reported relatively high levels of ammonium in the culture media or added high levels of exogenous ammonium (up to 600 μM). The addition of high levels of exogenous ammonium to the culture medium might provide a biased model as the embryos might encounter an artificially high-stressful environment (Biggers et al., 2004a). However, in a study by Lane and Gardner (2003), it was found that low levels of exogenous ammonium addition, i.e. 18.8 and 75 μM, resulted in perturbed metabolism during preimplantation embryo development, lower ICM/total cells ratio and more apoptotic cells in blastocysts and abnormal fetuses. Gardner et al. (2013) also showed in human blastocysts that a gradient of ammonium in the culture medium can result in the differential expression of 390 genes involved in metabolism, cell growth and/or maintenance, transcription, cell communication, transport, development and regulation of transcription. The ammonium levels of the ready-to-use embryo culture media from the present study can reach those relatively low levels of ammonium after incubation, especially when they have been stored for several weeks before use.

In line with this, we recently found an inverse association between the age of the embryo culture medium (G-1 PLUS v5) and birthweight of singletons born after fresh embryo transfer (Kleijkers et al., 2015). It can be deduced that embryos faced a less than optimal environment when cultured in older medium, which might be a result of the ammonium accumulation in the embryo culture media during storage.

With the introduction of time-lapse imaging, new culture media have been developed to support uninterrupted culture of pre-implantation embryos (Conaghan, 2014). One of the disadvantages of those continuous single-step media might be the accumulation of toxins, including ammonium (Biggers and Summers, 2008). In the present study, we included Irvine CSC medium and measured comparable levels of ammonium buildup during incubation at 37°C as reported by Gilbert et al. (2012). We found no significant differences in ammonium buildup during incubation at 37°C between Irvine CSC and the other media from this study, except for some of the cleavage stage media. Recently, in a study comparing the single-step time-lapse medium G-TL and the sequential medium G5 PLUS (G-1 PLUS/G-2 PLUS), low levels (<35 μM) of ammonium buildup were measured in both G-TL and G5 PLUS (Hardarson et al., 2015). We did not investigate the ammonium accumulation in G-TL in our study, however, our findings for G-1 PLUS and G-2 PLUS were comparable with those from the study by Hardarson et al. (2015).

We have reported that media used for culturing human IVF embryos significantly affected embryo development, pregnancy rates, prenatal and postnatal growth (Dumoulin et al., 2010; Nelissen et al., 2012,2013; Kleijkers et al., 2014). One notable difference, according to the product inserts of the media used during the study period (2003–2006), was the amino acid glutamine; Cook medium contained the free L-glutamine, while Vitrolife medium contained the more stable dipeptide alanyl-glutamine. From the results of the present study, it can be deduced that embryos that were cultured in the Cook medium (with the free L-glutamine), were exposed to higher ammonium levels. It is possible that this resulted in the lower birthweight and post-natal weight of children in the Cook group when compared with children in the Vitrolife group (Dumoulin et al., 2010; Nelissen et al., 2012; Kleijkers et al., 2014).

In conclusion, the main sources of ammonium buildup in ready-to-use culture media are unstable glutamine and the protein supplement. The break-down of these components into ammonium is more pronounced during incubation at 37°C, however, it is not negligible during storage at 2–8°C. This results in increasing ammonium levels in these media over time. Therefore, it is important that the use of free L-glutamine in human embryo culture media is stopped and that the use of protein supplements is thoroughly evaluated.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Authors' roles

J.C.M.D. and S.H.M.K. initiated and designed the study. J.C.M.D. and S.H.M.K. coordinated the data collection and quality control of data. S.H.M.K. analyzed the data. All authors interpreted the data. S.H.M.K. wrote the report with input from the other authors.

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Conflict of interest

None declared.

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