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978-1-107-61953-1 - Culture Media, Solutions, and Systems in Human ART
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Section 1 Culture media and solutions

Chapter 1

Overview

Patrick Quinn

Introduction

In this book we have attempted to review current formulations of human assisted reproductive technology (ART) media and solutions, where they came from, and possible contentious areas such as deficiencies and the like that need further investigation, for example the epigenetic effects of media on offspring [1–3]. Most of these topics are contained in the first section of the book. In the second section, which we have entitled "Culture systems," not only the liquid products but also the devices used for in vitro culture of gametes and embryos and how they are used, for example incubators, time-lapse photography and other procedures proposed for determining the selection of the best quality embryos are the subjects of discussion for it does no good to have a good medium if it is not used effectively. Certain aspects of the ART laboratory environment have not been included but should still be considered to optimize the results from any ART program and here I am referring to the macroenvironment in the laboratory or general geographic locale. In particular, things such as toxins, volatile organic compounds, insecticides, building materials, and the like if not considered or recognized as potential detrimental factors could have a very negative impact on outcomes [4, 5].

As we are reminded by Biggers [6], the accomplishments of in vitro fertilization (IVF) and embryo transfer (ET) in humans and other mammals are not something that happened through the work of only a few but were achieved through the cumulative efforts of many workers in the field, past and present, and no doubt will continue in the future. One of the main aims of this work is to summarize these efforts, highlight some of the critical aspects that have been studied, and hopefully provide some inspiration to those who have just entered the field or are considering a career in the area. I can personally say that I am grateful that I have had the opportunity to work in the field of preimplantation mammalian embryology for over four decades, the culmination of which was the birth of our grandson who was conceived and developed for several days in media I had designed.

Progress in media development

When I first entered the field in the late 1960s, the ability to culture mouse embryos in a relatively well-defined medium was possible [7]. One of the primary breakthroughs was the use of Krebs–Ringer solution as the base medium for mouse embryo culture in vitro [8, 9]. This approach was justified in further studies that showed the benefit of using what was

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considered to be a close chemical imitation of the contents of the female reproductive tract fluids when formulating culture media for the embryos of various species [10–12]. Such a strategy has been called the “back to nature” approach. Another strategy is where every possible compound that the embryo may need over the whole preimplantation period is added to the medium and this is called the “let the embryo choose” approach [13]. A more detailed discussion of these two concepts is presented in Chapter 2. There are many reviews that detail how ART media have evolved and the results that have been obtained with them [14–16] and the reader is directed to these references for further information on this topic. Some of the more pertinent aspects of culture media formulation, how they are used, and their performance are the topics I wish to discuss in more detail and is the primary emphasis of the next chapter and the whole book overall.

Summary

The aim of this work is to describe the history, current status, and significance of culture media and solutions and the culture systems in which they are used for human ART. Various chapters describe culture media and solutions used in human ART, how they have been developed for in vitro human preimplantation embryo development, the function and importance of the various components in these media and solutions, and how systems and equipment in which the media and solutions are used can influence the outcomes obtained in human ART. Additionally, oocyte maturation in vitro, oocyte and embryo cryopreservation, and regulatory matters are discussed. We hope this book will be of interest to students, embryologists, physicians, patients, and other personnel involved in or with an interest in the fluid products used in human ART for the culture and handling of gametes, embryos, and reproductive tissues.

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Section 1 Culture media and solutions

Chapter 2 Media and embryo interactions

Patrick Quinn

Introduction

Over the past several decades the success rates of in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) assisted reproductive technology (ART) procedures using fresh non-donor oocytes with or without ICSI have increased. Quinn reported a linear increase in viable rates for IVF/ICSI procedures in Australia and New Zealand from 1992 to 2001 [1]; rates more than doubled from 10% to 24% per transfer (Figure 2.1). Pregnancy rates in gamete intrafallopian transfer (GIFT) remained constant at 21% over the same period, indicating that the primary reason for the increase in the IVF/ICSI patients during this time was due to improvements in the culture systems being used. Similar increases over the same time span were reported for US data [2]. These increases in success rates in the USA have continued with live births per embryo transfer for fresh embryos from non-donor eggs significantly increasing from 35.8% to 36.4% to 38.5% for the years 2002 to 2006 to 2010, respectively, with the average number of embryos transferred decreasing from 3.0 to 2.6 to 2.4 over this period [3]. These data taken as a whole can be interpreted to indicate that improvements in the laboratory system are responsible for the majority of the increased success rates. Various aspects of these improvements, both in the culture media and in the important interacting aspects of the culture systems are discussed below.

ART media formulations

This topic is one of the oldest, most studied and discussed in preimplantation mammalian embryology so the discussion below will be more of a summary of the situation as seen by this author. There are many reviews on the topic that provide further details [4–10] and, of course, several chapters in this book.

Mammalian ovulated oocytes, spermatozoa, and preimplantation embryos are very amenable to collection and culture in vitro. The gametes and embryos of most mammals carry very few endogenous nutrients and they have to gain their nutrients from the reproductive tract fluid. They will not survive for very long when placed into an isotonic solution devoid of nutrients. If we have some idea of the nutrients and other solutes required to sustain the embryo during the preimplantation period, we can attempt to imitate the in vivo environment in the reproductive tract in the in vitro situation. This has been the basis of the so-called imitative principle, more popularly called the "back to nature" strategy [11], for formulating tissue culture media in general and IVF media in particular.

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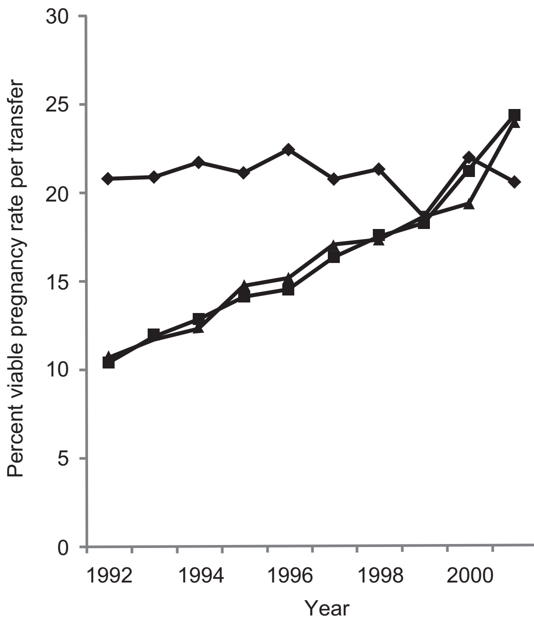

Figure 2.1 Reported viable pregnancy rates per completed procedure for IVF, ICSI, and GIFT in Australia and New Zealand from 1992 to 2001. Diamonds = GIFT; squares = IVF; triangles = ICSI. Reproduced from Quinn [1] with permission.

ART media need to provide hydration, ions, and nutrients and be able to dilute waste products.

Here is a list of potential ingredients:

1. Water
2. Inorganic ions: cations and anions – Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, $SO_4{}^{2-}$, $PO_4{}^{3-}$, $HCO_3{}^-$
3. Energy substrates – glucose, lactate, pyruvate, amino acids
4. Amino acids – non-essential amino acids, essential amino acids
5. Vitamins
6. Fatty acids or precursors
7. Nucleic acid precursors
8. Chelators – ethylenediaminetetraacetic acid (EDTA)
9. Antioxidants
10. Proteins or macromolecules
11. Polypeptide growth factors/hormones
12. Buffering system
13. Antibiotics.

A minimal mix of components would include a mix of electrolytes (cations and anions), energy substrates, amino acids, probably vitamins, oxygen (O_2) and carbon dioxide (CO_2), and a macromolecule such as albumin.

In addition the following aspects form an integral part of how culture media perform:

1. Oil overlay
2. Gas phase
3. Incubation chamber

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4. Embryo density – embryo number per volume of medium
5. Contact supplies – culture dishes/microfluidic device, flasks, pipettes
6. Quality control (QC)
7. Quality assurance/management.

Water

The basic foundation of any culture medium is high quality water. When one looks over the past 50 years at the processing of water used to make culture media for embryos, a progression in the treatment to improve water quality is evident. In 1963, Brinster used deionized water [12]; in 1971, Whitten used water which was distilled from deionized tap water [13]. When I started my own mouse embryo lab in 1974 I followed the procedure I had learned in Wes Whitten's laboratory and used triple glass distilled water to make culture medium. Glassware was washed in 1% 7X detergent and glass tubes to be used for culture were then siliconized with 1% soluble silicone for at least 5 seconds before being rinsed 10 times in deionized water, five times in double glass distilled water, and then sterilized by dry heat [14]. When I started human IVF in the early 1980s, we initially used rainwater which was collected on a clean plastic sheet placed on the roof of the laboratory. The collected water was redistilled six times in glass [15]. Finally we used water that had been processed through a Millipore reverse osmosis unit and Milliq treatment system [16]. Most commercial ART companies now either purchase or have their own water purification system. The water must meet strict specifications, is USP/EP certified sterile, and is water for injection quality water. The specifications for such water can be found on line [17]. The water, together with all other components being used, needs to be QC tested prior to use in a clinical or research setting. Protocols for the mouse embryo assay (MEA) and human sperm motility assay are given in Quinn *et al.* [15].

Inorganic ions

This topic has been recently reviewed by Baltz [18] who has given a good historical perspective of the topic. The milestones for culture media for mammalian preimplantation embryo culture were the media formulated by Whitten [19] and Brinster [12] both of which were based on Krebs–Ringer Bicarbonate (KRB). KRB contains Na^+, K^+, Mg^{2+}, Ca^{2+}, Cl^-, SO_4^{2-}, PO_4^{3-}, and HCO_3^-. This was the basis for the inorganic salts in embryo culture media up until the mid 1980s. When I formulated my Human Tubal Fluid (HTF) medium [16] I did find that an excessive ratio of Na^+ to K^+, as in Tyrode's T6 medium that was being used for IVF at that time, inhibited mouse embryo development which was overcome when the K^+ concentration was increased in HTF to levels closer to Whitten's medium. A similar strategy was used during the development of KSOM medium [20]

By the late 1980s the phenomenon of the 2-cell block in hamster and mouse embryos and at other stages in other mammalian species was being studied [21] and it was found that phosphate and/or glucose was involved in the hamster, and that removal of phosphate in particular overcame this block. Removal of phosphate in human ART media resulted in good IVF outcomes [22]. This was probably due to the excessive stimulation of glycolysis by the high levels of glucose and phosphate in the media, as proposed by Schini and Bavister [21]. The addition of amino acids to media have negated this effect and most commercial ART media now contain between 0.25 and 0.35 mEq/L of phosphate ions.

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Another modulation of inorganic ion content in media is with Ca^{2+} and Mg^{2+}. Both of these ions are required by embryos as shown by the reversal of compaction when embryos are placed in Ca/Mg-free medium prior to biopsy. However, it has been shown that in 1- and 2-cell hamster embryos an excessive uptake of Ca^{2+} occurred due to inappropriate handling, metabolic perturbations, and just by the act of collecting the embryos and placing them in culture, which reduced further development in vitro [23]. The increase in intracellular Ca^{2+} came from intracellular storage sites and also by influx from the medium through L-gated calcium channels but it could be lessened by increasing Mg^{2+} concentration in the medium. This strategy has been adopted by several commercial ART media companies. It must be remembered however that there is a Ca^{2+} spike in spermatozoa during the fertilization process so a lower Mg^{2+} concentration is required in fertilization medium [24]

The final inorganic ion component in media is HCO_3^-, which will be discussed further in the section on buffering systems.

Energy substrates

This is a huge and diverse topic that has been studied over the past 50+ years so the comments made will be brief and of a summary type. For readers who want more details I highly recommend recent reviews by Gardner [5], Gardner and Wale [25], and Leese [7]. For reference, see Figures 2.2 and 2.3, which are from Gardner and Wale [25] and show the major metabolic pathways and the compounds involved at the zygote and blastocyst stage, respectively.

Oocyte and early cleavage stage embryos do not utilize glucose but have low levels of oxidation of pyruvate. Hence the ATP:ADP ratio is high as there is low biosynthesis and limited cell division. ATP:ADP ratios fall as embryo development proceeds and there is an increase in energy demand. This change in ATP:ADP ratio with development was first reported by Quinn and Wales [26, 27] and subsequently confirmed by Leese *et al.* [28]. Quinn and Wales [27] also proposed that the lowering of ATP:ADP ratio at later stages of development would increase the utilization of glucose by glycolysis via an increase in the activity of the glycolytic enzyme phosphofructokinase, which is inhibited by high ATP levels, and also an increase in the tricarboxylic acid (TCA) cycle activity, which is also limited by high levels of ATP. Increased TCA cycle activity would then increase the rate of oxidative phosphorylation to provide greater amounts of ATP for biosynthesis and blastocoel cavity formation during the later stages of preimplantation development.

A more erudite summary of what happens in metabolism during preimplanataion development is given by Leese [7] and is worthy of quoting:

> The Krebs cycle [also called the TCA cycle] and oxidative phosphorylation provide the main source of energy throughout the preimplantation period. Pyruvate is the central energy substrate during the first cleavage in those species in which energy source requirements of the embryo have been examined, although it is not obligatory for all species (e.g., porcine). Other substrates, notably, amino acids, lactate and endogenous fatty acids derived from triglyceride, combine with pyruvate to provide embryos with a range of potential energy sources through to, and including the blastocyst stage. These nutrients have numerous, overlapping, metabolic roles. Prior to the morula stage, glucose consumption and metabolism is low, although some glucose is necessary for intracellular signaling purposes. With blastocyst formation, large increases in O_2 consumption and the uptake and incorporation of carbon occur and there is a sharp increase in glycolysis, at least in

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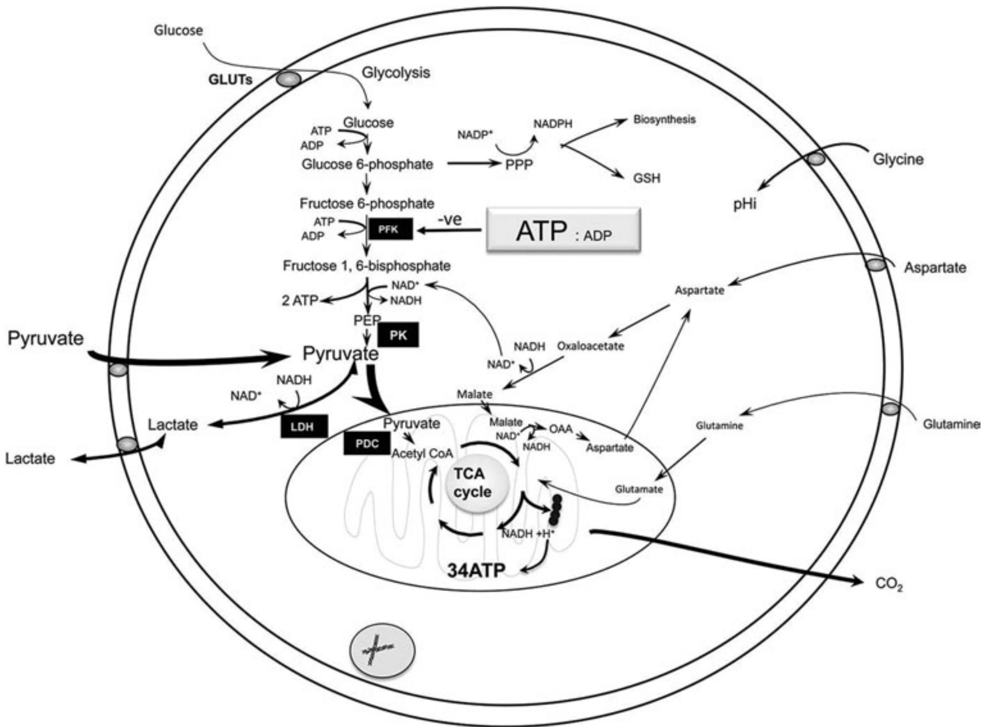

Figure 2.2 Metabolism of the pronucleate oocyte and cleavage stage embryo. The thickness of the lines in the figure represents the relative flux of metabolites through that pathway. GLUTs = glucose transporters; GSH = reduced glutathione; OAA = oxaloacetate; PDC = pyruvate dehydrogenase complex; PK = pyruvate kinase; PPP = pentose phosphate pathway. Reproduced from Gardner and Wale [25] with permission.

> vitro. The embryo goes from a relatively inactive metabolic tissue at ovulation to a rapid metabolism at implantation. Mitochondria play a pivotal role during early development, as well as providing a cellular focus for metabolic events. We are almost totally ignorant of the metabolism of preimplantation embryos *in situ* (in the oviduct and uterus) and understanding of signal transduction within embryos is in its infancy as is the molecular dialog between embryos in culture and with the maternal tract in vivo.

A possible practical application of metabolic analysis of human embryos for selection of those more likely to implant and progress to live birth has been reported [29]. It was reported that by measuring glucose consumption of mouse blastocysts it was possible to determine prospectively which embryos were more likely to implant and develop. The embryos chosen for transfer were those with a glycolytic activity similar to that of in vivo-derived embryos [29]. Similar results were observed of the glucose uptake by human embryos on day 4 of development and it was also found that female embryos on day 4 had a significantly greater glucose uptake [30]. A prospective trial using this technology for the selection of viable human embryos for transfer is awaited.

On a practical note, care is needed with the storage of sodium pyruvate raw material. Pyruvate is a free radical scavenger but has limited aqueous stability [31], converting to parapyruvate, which is a metabolic inhibitor. Wales and Whittingham [32] found that the

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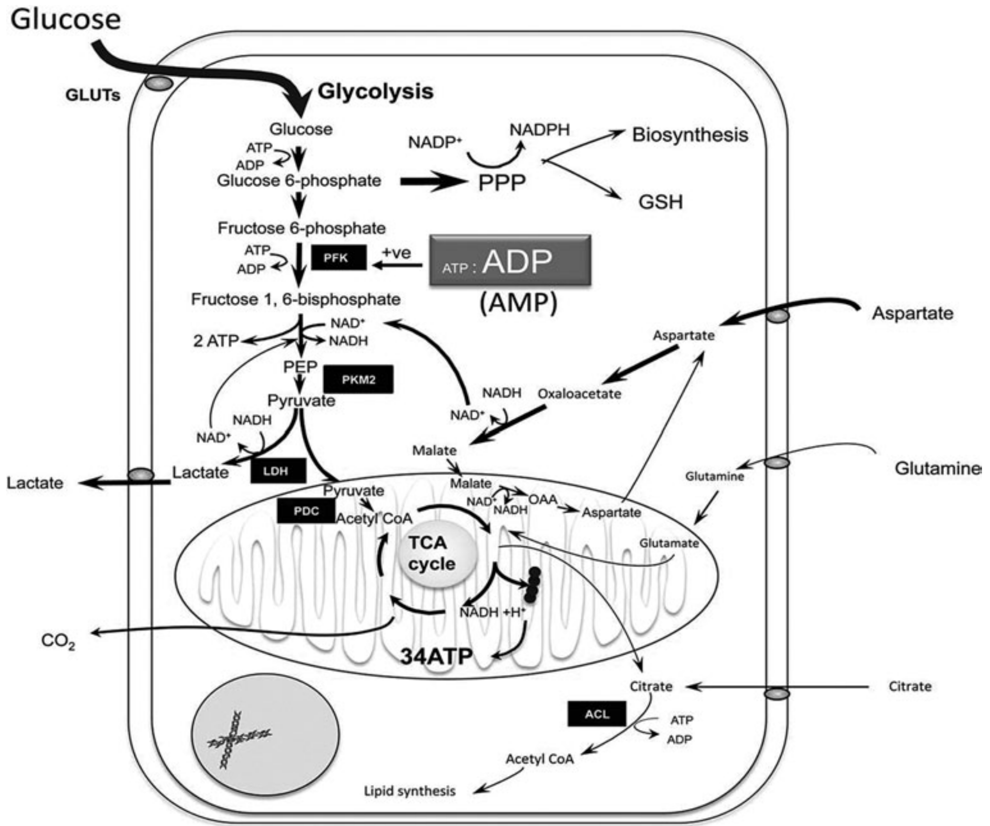

Figure 2.3 Metabolism of the blastocyst. The thickness of the lines in the figure represents the relative flux of metabolites through that pathway. ACL = acetyl-citrate lyase; GLUTs = glucose transporters; LDH = lactate dehydrogenase; OAA = oxaloacetate; PDC = pyruvate dehydrogenase complex. Reproduced from Gardner and Wale [25] with permission.

stability of pyruvate was improved and its embryotoxicity was reduced when it was stored below −40 °C. Swain and Pool [33] reported that ethyl pyruvate and, to a lesser extent, methyl pyruvate, more stable esterified forms of pyruvate, improved mouse embryo development in vitro. These esterified forms of pyruvate are also more membrane permeable and could therefore more easily enter mitochondria and stimulate NADH/NADPH production, thereby maintaining metabolism at physiological levels and reducing stress on the embryo.

Amino acids

This topic has been concisely reviewed by Gardner and Lane [5, 34]. Amino acids have varied and important roles during early mammalian embryo development which range from biosynthetic precursors and energy sources to osmolytes, intracellular pH buffers, antioxidants, chelators, and regulators of differentiation [5]. The addition of amino acids to culture media makes the development of embryos closer to that of in vivo rates [34]. As

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amino acids are present in reproductive tract fluids [35] it makes sense to include them in embryo culture media even though mouse embryos can develop to expanded blastocysts in vitro in the absence of amino acids. Steptoe *et al.* [36] used Ham's F10 medium, which contains amino acids, in some of their early work but as human IVF became more utilized in the 1980s, media without amino acids such as Tyrode's T6 and HTF became widely used. The works of Gardner and Lane (reviewed in [5]) brought the focus back on amino acids and all commercial ART media for human IVF contain amino acids. Not only do amino acids alleviate stress on embryos and oocytes during culture, their presence prevents efflux of endogenous amino acids from the embryo during handling procedures such as oocyte collection, micromanipulation, cryopreservation, and embryo transfer [37].

The concentration and necessity for all amino acids during all of the preimplantation period have been debated. The initial recommendation by Gardner and Lane based on mouse studies [38] was to use amino acids at the same concentration as that recommended in Eagle's medium and adding the non-essential group for the first 48 hours of culture and then including the essential along with the non-essential amino acids for the second 48 hours, that is, from day 3 until day 5/6 for human embryos. The authors also reported that exposure of mouse zygotes to essential amino acids impaired their development. We have also substantiated this negative effect of essential amino acids on mouse zygotes but not on 2-cell embryos (P. Quinn, unpublished observations, 2005). In 2000, Biggers *et al.* [39] reported that non-essential and essential amino acids at half the concentration in Eagle's medium gave improved development rates of mouse embryos and his laboratory have retained that concentration in their KSOM^{AA} medium [40]. On the other hand, Lane *et al.* reduced the concentration of only essential amino acids by one half [41]. This reduction in essential amino acids to one half of the previous concentration did significantly increase blastocyst cell number in mouse blastocysts, as well as the number of cells in the inner cell mass (ICM) and the proportion of total cells that were in the ICM. It should be noted that Gardner and Lane incorrectly reported a 10-fold higher concentration of tryptophan in their medium in several of their papers [41]. The correct concentration is 0.05 mM in Eagle's medium, as stated in the article by Lane et al. [41]. There have been few reports on the effects of individual amino acids on human IVF. One exception is the abstract from Mortimer *et al.* in 1998 [42] in which they found that after removal of isoleucine and phenylalanine from medium used for culture from the zygote to 4/8 cells, entry into the first cleavage division was faster and positive pregnancy and implantation rates were significantly higher.

The major negative effect of amino acids is their deamination, which releases ammonium most of which comes from spontaneous deamination and a smaller proportion from the metabolism of amino acids by embryos. See the paper of Lane *et al.* [41] to see an example of the differences in ammonium released by chemical deamination alone and by the addition of embryos to the same medium. The buildup of ammonium in vitro causes embryo retardation and fetal defects after implantation [43]. The problems with ammonium accumulation in medium can be lessened by renewing the medium at least every 48 hours and also substituting glutamine with the stable dipeptide alanyl-L-glutamine or glycyl-L-glutamine, which are stable at 37 °C. Eagle [44] showed that human and mouse cells in culture are able to substitute and use dipeptides for a missing essential amino acid. Despite the suggestion that glycyl-L-glutamine may be a better source of stable glutamine for mouse embryos [45], there is a report in which human IVF was performed using KSOM^{AA} containing either alanyl-L-glutamine or glycyl-L-glutamine and the

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