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Review Article

Protein Supplementation of Human IVF Culture Media

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This review travels the road of protein supplementation in embryo culture development—from whole crude plasma in the mid Twentieth century moving through to the completely genetically engineered human albumin with successful births at the beginning of the Twenty-first.

KEY WORDS: Albumin; culture media; human IVF; recombinant albumin; serum.

INTRODUCTION

Since the world's first in vitro fertilization (IVF) baby was born in 1978 (1), there has been an explosive expansion in the use of human assisted reproductive technologies, with more than 765 cycles of IVF being performed annually for every million inhabitants, in Europe alone (2). During that time, the chemical formulation of culture media that supports the growth of embryos, has undergone continuous development. In particular, the origin and uniformity of the protein fraction in these media has been a source of concern in the pursuit of consistently high success rates for clinics. Within the past 5–10 years, commercially produced culture media has largely superseded the practice of using "in house" made media in the majority of IVF clinics worldwide. One of the most challenging aspects for media-producing companies in attaining quality assurance in their products has been their choice of a reliable, consistent, and safe protein source. Reports of notorious LOT-to-LOT variability in protein sources have long been blamed for fluctuating pregnancy rates in clinics (3). Only now with the recent availability of recombinant albumin is it possible to create a culture medium where the molecular structure of the protein is completely defined.

This report, reviews the path that has led the IVF industry to use the purest available protein product in the culture of human embryos. The literature review begins in the 1940s when the protein component of media was more or less undefined—moving through to the 1970s when it became partially defined—and ending with the dawning of a new era in the 1990s of genetically defined albumin media additives. Experimental data from animal studies and human clinical trials that have resulted in the world's first IVF babies born from the use of recombinant albumin will also be presented.

INITIAL USE OF PROTEIN

Some of the earliest efforts to culture mammalian cells were published in the 1940s (4). At this time, culture media consisted solely of whole serum or plasma. These fluids provided the nutritional, physical, and chemical requirements of the embryo to some extent. Although a degree of success was experienced with this system, it was recognised as suboptimal in light of the knowledge that embryos in vivo do not come into direct contact with serum in the reproductive tract. It was not until the pioneering work of Eagles in the early 1950s, then later Krebs and Ringer (5), that a deeper understanding of the fundamental requirements of growing mammalian cells was developed, thus reducing the dependence on

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serum as the sole culture substrate (6). Their findings that cells require amino acids, vitamins, and balanced salts for normal metabolism, remain the basis for our media compositions today. Using these formulations, Whitten, Biggers, and Whittingham performed landmark mouse embryology experiments during the 1960s. They did however continue the inclusion of serum in the culture media, as both a protein source and agent to prevent the adherence of embryos to each other as well as to the plastic culture vessels (7,8,9).

ALBUMIN

The fact that albumin is universally added to most of human IVF culture media implies that it is widely considered to be of benefit. Indeed, the list of putative roles of albumin in culture media is extensive, including

- pH buffer
- Colloid osmotic regulation
- Membrane stabilization
- Carrier of growth promoting substances (amino acids, vitamins, fatty acids, hormones, growth factors, etc.)
- Surfactant (antiadhesion)
- Scavenger (toxins and heavy metals)
- Nutrient (breakdown to amino acids)

With this list of wide ranging roles of albumin, it is not surprising that it is used universally as a supplement to embryonic culture.

Albumin is the major soluble protein constituent of human blood (50–60%) with various physiological roles as stated above. It is also the most abundant macromolecule that the gametes come into contact with in the human oviduct (10). As a relatively large (mol.wt. 68 kD) multidomain protein, albumin exists as a single, nonglycosylated polypeptide chain. Because of its large surface area and the abundant binding sites, it functions as an effective carrier of various molecules such as water, salts, free fatty acids, vitamins, and hormones. Once bound, albumin transports these molecules between tissues and cells, both in vivo and in vitro. The binding capacity of albumin makes it an effective scavenger to remove toxic substances including pyrogens from the medium. However, it has been shown that the chelating and pH buffering role of albumin can be replaced by the addition of amino acids (11). The primary role of albumin as a regulator of osmotic pressure in blood possibly implicates it as also performing this function in embryo culture (3).

The reason for adding protein to culture media in both domestic animal and human IVF is historical and stems from the early mouse work previously referred to. Having stated this, it is still unclear whether protein is in fact essential for IVF and embryonic development. It is for example possible to culture viable mouse embryos to the hatching stage in vitro in a protein-free media and there have also been reports of human pregnancies achieved in such systems (12,13). Nevertheless, doubts remain about the ability of protein-free media to support development of embryos to the blastocyst stage, particularly, in light of recent evidence that endocytosed albumin enhances fetal growth to that which is comparable to in vivo developed blastocysts (14). It is possible that protein-free media, may result in survival of only those embryos that have the ability to adapt to utilization of alternative metabolites. Yet, it must also be remembered that the presence or absence of suitable alternative protein substitutes will have a bearing on the reporting of success or failure of a medium.

It is widely accepted that the presence of albumin/protein in sperm preparation media provides protective properties that help to maintain motility and viability. There is evidence that albumin acts as a potent inhibitor of lipid peroxidation by binding hydroperoxy fatty acids that can be damaging to sperm membranes in solution (15). Indeed, the well-documented toxin-scavenging action of albumin suggests that it be eliminated from sperm survival test media to ensure the assay sensitivity (16). For fertilization, it is possible that albumin may play an important role in sperm capacitation and/or the acrosome reaction. While the amount of protein present in the cumulus oophorus may render the addition of albumin in the fertilization media unnecessary, there is on the other hand no evidence to suggest that it is detrimental.

The role of albumin as a direct source of nutrients for the embryo has been debated, especially at the early cleavage stages prior to compaction (17). However, at the blastocyst stage of development, albumin has been shown to be endocytosed into the embryo from the medium (18). It is possible that albumin could be catabolized thus providing free fatty acids as a substrate for the citric acid cycle that is active at this stage of embryonic development (18). Regardless of whether albumin is a direct source of nutrient or not, it may still contribute to the overall mitogenic

potential of the medium by binding growth promoting substances and releasing them to the cell (19). One example of this can be found in the reports that albumin acts as a carrier for embryo derived platelet activating factor (PAF), which is a well characterised embryonic growth factor. Ammit and O'Neill's work has shown that the optimal effects are exhibited when albumin and PAF are equimolar (20). In the absence of all protein, PAF is not released at normal levels in mouse embryos and therefore does not have the opportunity to exert autocrine effects. It is hypothesized that albumin may therefore perform an important role as a chelating agent to partner embryo-derived PAF.

From a purely practical level, albumin has a role in facilitating the handling of embryos in vitro. Most of the culture vessels used in the field of embryology are made of polystyrene, which is prone to developing an electrostatic charge (3). In a solution with no protein the charge on the dish will attract and hold any other protein (i.e. embryos), making the handling and manipulation of embryos difficult for the embryologist. To avoid this, the dish must be saturated with protein or other macromolecules such as polyvinyl pyrolidone (PVP), so that the charge on the dish is eliminated. This will also prevent the embryos from adhering to each other or the handling pipette in a protein-free medium.

THE RISKS OF USING SERUM AS A SOURCE OF ALBUMIN

Until the early 1990s, the most commonly used protein sources in human IVF and embryo culture was human serum. Traditionally this was obtained either from pooled adult human donor serum or fetal cord serum (21). Donor serum was usually obtained from either a local blood bank or from a group of female donors known to the clinic. Fetal cord serum was obtained by drawing venous blood from the umbilical cord into sterile tubes immediately after delivery of the baby. In both cases the blood sample was centrifuged, the serum aspirated, followed by heat inactivation at +56°C for 30 min and sterile filtration. Inactivation of blood complement is traditionally considered necessary to ensure that cell lysis does not occur through the action of the membrane attack complex (MAC) that is induced by an antigen–antibody reaction (22). However, Imoedemhe *et al.* (23) published data suggesting that heat inactivation may be an unnecessary step. While there is a report in the literature of a human pregnancy resulting from the culture of embryos in whole serum (24), conventionally serum is added to media at a concentration of 10%.

One of the major drawbacks of using pooled serum is the necessity for screening the donors and the risk of contamination with an infectious disease such as human immunodeficiency virus (HIV) or hepatitis. One incident that highlights this risk has been reported in the literature (25). In 1988, a group of 128 women were infected with hepatitis B virus via contaminated donor serum that had been added to the IVF culture media. A hepatitis-B infection was detected in 79 of the women but no serious forms of hepatitis occurred. Three of the women became infected carriers and none of the partners or children became infected. To date there are no known reports of HIV transmission resulting from IVF treatment via culture media.

In an attempt to minimize the risk of disease transmission, there was a worldwide trend in the 1980s of using maternal serum for the culture media (26). Blood was drawn from each patient, and the prepared serum was used exclusively for that patient's culture media. While some claimed that this was the only method to guarantee safety against disease transmission, others argued that there was an increased potential for mix-ups using this system. Other unpopular aspects of this method are that it is time consuming in preparation and involves more discomfort for patients. But perhaps the greatest pitfall with using maternal serum is that certain patients were found to have embryo toxic factors in their serum preparation. This aspect was clearly demonstrated in a report by Levelle *et al.*, where some maternal sera supported mouse embryonic development, while others did not (27). Dokras *et al.* also found that serum from women with unexplained infertility can inhibit both mouse and human embryo growth in vitro (28).

A body of literature describing reports of abnormal fetal development in domestic species embryo culture now exists. Although the exact cause and mechanism is still under intense investigation, this phenomenon has been linked to the use of serum and in particular human serum (29) in culture media. The types of abnormalities include abnormal ultrastructure of the mitochondria (29), abnormal energy metabolism (30), premature blastocoel development and the birth of abnormally large fetuses (29). Although there is to date no evidence to suggest that these abnormalities exist in human assisted reproductive technologies,

these data do serve as a word of caution about the addition of nonphysiological compounds to embryo culture (31).

THE SHIFT FROM SERUM TO PURIFIED ALBUMIN PRODUCTS

For all the disadvantages of serum outlined above, scientists have continued to search for more highly defined sources of albumin. Reports on the use of blood bank preparations of human serum albumin (HSA) date back to the infancy of IVF; however, it has really only gained widespread use since the 1990s. Other reported albumin sources used in human IVF include some therapeutic albumin preparations such as Albuminar-5® (32), Plasmatein® (33), and Plasmanate® (34). The latter two products are plasma expanders and have limitations in that they are designed to optimize vascular physiology and not embryo culture. Reports that the higher globulin content of Plasmatein® maybe beneficial for embryo culture led to the development of a so-called synthetic serum substitute (SSS), and these have been present in some commercially prepared media since 1995 (35,33). It may be noted that the word "synthetic" is misleading since the product components are not synthetic.

It has been generally believed that the positive effect of serum addition to culture media was due to constituents such as cyclic adenosine monophosphate, catecholamines, vitamins, growth factors, lipids, and albumin (36). However, major limitations in identifying these agents, their concentrations, and embryotrophic effects, have been partly due to the LOT-to-LOT variation that exists in serum (37). It was Menezo *et al.* who first showed that HSA performed equivalently to serum in IVF culture (38). Since that report, there have been other studies comparing serum verses albumin preparations that support Menezo's findings (39). Two papers that conflict with this conclusion are by Hargreaves *et al.* and Staessen *et al.* (40,41).

A criticism frequently raised in human IVF is the inclusion of blood products, such as serum albumin in the culture system. Although HSA is significantly more purified, tested, and regulated than serum, the use of any blood products raises the issue of potential contamination. Currently, most manufacturers are adhering to disclaimers and place liability for the use of blood derived products in the hands of IVF clinics. From a sales and marketing perspective this has major implications for countries, such as Australia, that prohibit the importing of products containing constituents derived from human/animal products unless they have been approved by the Therapeutic Goods Association (TGA).

Commercial HSA products are heat treated at +60°C for 10 h and/or cold ethanol extracted during the course of their manufacture as a means of minimizing viral transmission (42,43). While the risk of transmitting a viral disease through HSA products is almost negligible, there remains the risk of contamination with other pathogenic agents such as those responsible for the lethal neurodegenerative disorder called Creutzfeldt-Jakob Disease (CJD) (44). The disease appears to be due to a new putative class of infectious agents/proteins called prions. These proteins have a molecular weight in the range of 30 kD, replicate like viruses, but seem to be devoid of nucleic acid genomes. In 1995, Baxter International (Deerfield, IL) recalled batches of HSA that were used for (among other things) IVF culture, when they suspected one of their blood donors had died from CJD (45). It is still unknown as to the level of risk of transmission of a prion protein via HSA, however with the advent of techniques such as intracytoplasmic sperm injection and nuclear transfer, the manufacturing industry remains particularly vigilant about monitoring progress in this area.

It is now widely accepted that HSA is the most well defined protein additive to culture media that is available on the market today. Even so, LOT-to-LOT variation is still a problem because of the inherent variability in donor blood and processing techniques used by different manufacturers. The degree of albumin purification also remains one of the most disappointing aspects of this product in scientists' pursuit of complete control over media composition. Most HSA is produced either by purification through an ion exchange/precipitation method or a Cohn fractionation process. This accounts for the variation of contaminants that have been detected between HSA brands (46). An interesting aspect of these differences is the possibility that they could be associated with differences in embryonic development rates. Elucidating which electrophoresis patterns are responsible for improved embryotrophic activities is an active area of research. One such study has identified citrate in a BSA product, as being a growth promoting contaminate (47). It may for example be possible in the future to treat albumin with known embryotrophic compounds.

Over the years, there has been an ongoing, yet unsubstantiated concern that the stabilizing and preservative components of almost all of the commercial

HSA available may be embryo toxic. All HSA solutions approved by the FDA for therapeutic purposes in the USA are stabilized by the addition of sodium caprylate and sodium acetyltryptophanate. The purpose of these compounds is to preserve the conformation and solubility of the albumin protein during the 10-h incubation at +60°C (48). This incubation at high temperatures is intended to inactivate the hepatitis virus. In addition to these stabilizers, some products also contain a preservative in the form of malelic acid.

The concerns of batch to batch variability and incomplete purity of HSA have fuelled a search for alternative macromolecules. Synthetic polymers such as polyvinylalcohol (PVA) and PVP have both been investigated and used in IVF (3). However, neither can be considered a physiological alternative to protein and there has been some speculation about the teratological nature of these compounds. A physiological alternative to albumin is hyaluronate, a polysaccharide that is expressed in the uterus at increasing amounts in the human uterus around the time of implantation. Although preliminary trials with hyaluronate in mouse culture appear very promising (49), some leading scientists involved in developing media formulations believe that a completely pure albumin source would be the most desirable option.

RECOMBINANT ALBUMIN

With all of the concerns about the LOT-to-LOT variation and the relative impurity of the albumin products currently available, the appearance of recombinant albumin onto the market, could not have been more timely for human IVF. After approximately 10 years of development, the British based biotechnology company Delta Biotechnology Ltd., has released a recombinant albumin (rHA) under the trade name Recombumin™. In addition to the obvious clinical therapeutic applications such as blood expanders and drug formulation, it is the ideal candidate for embryo culture. Yeast cells (Saccharomyces cerevisiae) genetically engineered to express the human albumin protein are grown in a nutrient that encourages the production of albumin. The extracelluar albumin product is purified using several chromotography processes that ensure the product possesses minimal yeast-derived impurities, glycosylated forms, and antifoaming agents. Safety and tolerability of Recombumin was determined by carrying out pharmacokinetic studies in both rats and rabbits, then later clinical trials on healthy human volunteers who received repeated intramuscular and subcutaneous administration of the rHA. These toxicological evaluations performed by Centeon to date, have shown that Recombumin has a safety profile that is comparable to HSA.

While Recombumin has been proven to be structurally identical to plasma-derived HSA, it does possess several advantages including higher LOT-to-LOT consistency, greater homogeneity, and less endotoxin levels than HSA. Moreover, Recombumin is free from viral/prion contamination risk and plasma-derived impurities. For all of these listed characteristics, Recombumin is considered a highly desirable substitute for HSA in human embryo culture media.

Initial experiments investigating the efficacy of rHA in a mouse embryo culture system suggested that it can successfully replace HSA to support development of viable blastocysts. Gardner and Lane found that pronuclear embryos cultured in media supplemented with an optimal concentration of 1.25 mg/mL rHA produced equivalent rates of blastocyst development and equivalent cell number in the inner cell mass and trophectoderm compared to embryos cultured with 5 mg/mL HSA. Furthermore, equivalent implantation rates (63% rHA vs. 65% HSA) and fetal development rates (43% rHA vs. 46% HSA) were seen in embryos transferred in the random-bred strain of mice used (49).

Clinical trials of Recombumin supplementation for use in IVF media began early in the year of 2000. The first pregnancy Case Study was reported by Bungum *et al.* in a Danish clinic (50), where each of the three women (29–36-years-old) had their oocytes randomly allocated to culture media containing either HSA or rHA. In all the three cases, the embryos cultured in rHA media were considered of superior morphological quality and were chosen for transfer. A total of four implantations resulted from six embryos transferred in these women (one singleton miscarriage, one ongoing singleton, and one twin delivery). Clearly, it is necessary to now perform carefully designed randomized control trials to statistically confirm the positive indicators that are apparent from these initial trials of Recombumin. Naturally a follow-up of long-term health in children born from media supplemented with rHA will be warranted, as it is for all innovative changes to all IVF procedures.

CONCLUSION

For an oocyte that is removed from its natural environment, a culture medium that meets the essential requirements for its survival and growth in vitro is

required. Albumin is the most abundant macromolecule in the human oviduct (10), and for that reason alone, it is certain to remain a recommended supplementation of media. Albumin substitutes such as polymer macromolecules will not satisfy the requirement of providing an in vitro environment that is based on physiological compounds. In the quest to replicate the in vivo environment, it is likely that the media of the future will incorporate a careful blend of growth factors that may require albumin as a vehicle for their action. This area of intense research is currently hindered by the lack of available pure albumin.

With the emergence of recombinant albumin onto the commercial market, it is now possible for the first time in the history of embryo culture to produce a media product that is completely defined. The availability of this new tool will allow scientists to make further significant advances toward the ultimate goal, which is to provide the IVF clinics with a product that is consistent, safe, and results in optimum pregnancy rates.

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