Clinical Experience with Synthetic Serum Substitute as a Protein Supplement in IVF Culture Media: A Retrospective Study*

NINA N. DESAI,[1,2] LEON A. SHEEAN,[1] DONALEE MARTIN,[1] VICTORIA GINDLESPERGER,[1] CYNTHIA M. AUSTIN,[1] HANNA LISBONNA,[1] BARRY PESKIN,[1] and JAMES M. GOLDFARB[1]

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Objective: *Our objective was to evaluate the efficacy of Synthetic Serum Substitute (Irvine Scientific—Materials Section, Santa Ana, CA), a globulin-enriched protein preparation containing human serum albumin for supplementation of IVF culture media.*

Design: *We retrospectively analyzed IVF cycles performed at MacDonald Womens Hospital between January 1992 and November 1994. IVF cycles were reviewed and classified according to the nature of protein supplementation used in the embryo culture medium. Three protein supplements utilized during this time period were compared: Synthetic Serum Substitute (SSS), Plasmanate (PL), and maternal serum (MS).*

Results: *Although clinical pregnancy rates among the three treatment groups were not statistically different, there was a definite trend toward a higher pregnancy rate with SSS supplementation (SSS, 38.2%; MS, 28.0%; and PL, 24.9%). Embryos grown in SSS-supplemented culture media had a significantly higher implantation rate (17.8 vs 10.4 and 10.3%, respectively, for MS and PL). Preliminary data also suggest that human embryo development and blastulation in vitro were enhanced by this protein supplement.*

Conclusions: *The higher implantation rate with SSS suggests that it may be superior to both maternal serum and Plasmanate in supporting human embryo development in vitro. Whether blastocysts derived from PL- and SSS-supplemented media are able to implant and give rise to clinical pregnancies remains to be seen.*

KEY WORDS: culture medium; human embryo; in vitro fertilization; protein supplement; synthetic serum.

INTRODUCTION

Culture media used in human in vitro programs are generally supplemented with a protein source. Classically, IVF programs have used either maternal serum or fetal cord serum as the protein source. However, there has been a trend in recent years away from maternal and fetal cord sera toward commercially available products. Several considerations have motivated these changes. Maternal serum can be inconvenient in that the patient must be available for collection and serum has to be prepared and heat inactivated for each individual patient. Pooled fetal cord serum is of concern to some because of potential for contamination. Each lot has to be tested for HIV, CMV, and hepatitis and pretested in the mouse bioassay for ability to support embryo development. Furthermore, the use of either fetal or maternal serum can introduce variables such as differences in antibodies, inflammatory mediators, etc., that cause inconsistencies in media between patients.

One alternative to serum has been the use of defined proteins such as human serum albumin (HSA) and bovine serum albumin (BSA) (1,2). However, there have been concerns regarding the lack of ability of HSA and BSA to support motility of sperm (3) and the toxic effects of BSA in mouse embryo studies (4). Synthetic serum preparations (5,6) and plasma protein fractions (7–9) have also been used

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[1] Department of Obstetrics and Gynecology, University MacDonald Women's Hospital, Case Western Reserve University, Cleveland, Ohio 44106.

[2] To whom correspondence should be addressed at Department of OB/GYN, University MacDonald Women's Hospital, 11100 Euclid Avenue, Cleveland, Ohio 44106.

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in IVF culture media with varying degrees of success. Two such products, Plasmanate and Plasmatein, both containing human serum albumin and mixed globulins, have been advocated as alternatives to serum in that they overcome some of the problems encountered with the use of HSA and BSA alone (8,9). Pool and Martin (9) reported an improvement in IVF outcome using Plasmatein for protein culture medium supplementation instead of HSA. They attributed the increase in pregnancy to the significant amounts of α- and β-globulin present in this protein preparation. In our program we first started using Plasmanate as an alternative to maternal serum in patients with endometriosis and/or unexplained infertility because of concerns regarding possible negative factors in maternal serum in these patients (10–12). Following favorable results in these patient groups, we decided to use Plasmanate in all cycles (Desai *et al.*, abstract).

One major concern, however, in using either Plasmanate or Plasmatein has been the presence of preservatives and the possibility of lot-to-lot variation in globulin content and/or composition. These products are normally used as plasma volume expanders and have been formulated for their vascular effects. Recently, Synthetic Serum Substitute (SSS; Irvine Scientific), a globulin-enriched product manufactured specifically for IVF, has been suggested for routine use in place of serum (Weathersbee *et al.*, unpublished). This paper describes our experience using Irvine SSS as the exclusive source of protein in our IVF program over a 5-month period. These data are compared to earlier results using either maternal serum or Plasmanate as the protein source. This report, therefore, summarizes 68 consecutive cycles over a 5-month interval when protein supplementation of HTF represented the only laboratory variable in the management of IVF patients.

MATERIALS AND METHODS

During the 5 months from May 25 through November 5, 1994, all patients undergoing IVF at University Hospitals of Cleveland had Synthetic Serum Substitute (Irvine) used as the protein supplementation. A total of 97 patients underwent IVF procedures during this time. Of these, 68 were used as the study group. Patients eliminated were those involved in our surrogate/IVF program, oocyte donation program, micromanipulation program, or coculture program. The cycles of these 68 patients were compared with 263 similarly selected patient cycles performed between January 1, 1992, and December 31, 1993.

Controlled ovarian stimulation for all patients consisted of down-regulation with leuprolide acetate (Lupron; TAP Pharmaceuticals, Chicago, IL), followed by stimulation with human menopausal gonadotropins (Pergonal; Serono Laboratories, Randolph, MI) until two or more follicles reached 18 mm in mean diameter with an estradiol concentration appropriate to the ovarian response. Ten thousand units of human chorionic gonadotropin (hCG) (Profasi; Serono Laboratories) was then given. Transvaginal ultrasound directed oocyte retrieval was done 36 hr later. Luteal-phase support consisted of 2500 IU hCG on days 3, 6, 9, and 12 post-retrieval.

Maternal serum (MS) was prepared the day after the first hCG injection. Thirty milliliters of maternal blood was drawn, allowed to clot, and centrifuged at high speed to sediment erythrocytes. Serum supernate was removed and heat inactivated at 56°C for more than 30 min prior to supplementing the culture media. Serum was not used if it was hemolyzed or exhibited an extraordinary lipid content.

Human tubal fluid (HTF) Medium (Irvine Scientific) supplemented with either maternal serum, Plasmanate (PL) (Cutter Biological, Miles, Inc., Elkhart, IN), or Synthetic Serum Substitute (SSS) (Irvine Scientific) was used for oocyte/embryo culture and sperm preparation. Although "synthetic serum substitute" has been used to describe other protein preparations, in this study the abbreviation SSS refers exclusively to the synthetic preparation marketed by Irvine Scientific. Plasmanate and SSS are supplied as stock solutions containing 50 and 60 mg/ml of protein, respectively, and are quite stable. For insemination and sperm preparation, HTF was supplemented with either 10% maternal serum, 10% SSS, or 6% Plasmanate. All oocyte/embryo culture was performed in microdroplets (75–100 μl) under oil (Squibb) in NUNC dishes (60 cm^2). For embryo culture, growth medium containing HTF supplemented with either 15% maternal serum, 15% SSS, or 10% Plasmanate was prepared.

Aspirated oocyte–cumulus complexes were trimmed by sharp dissection, washed, and placed in microdroplets containing preequilibrated insemination medium. Droplets were inseminated with approximately 50,000 motile sperm, prepared by Percoll density-gradient centrifugation. Insemination dishes were incubated at 37°C in a humidified

atmosphere with 5% O_2, 5% CO_2, and 90% N_2 serving as the gas phase. Postfertilization, zygotes were transferred to new dishes containing preequilibrated growth medium. Embryo transfer was performed on Day 2 (40–48 hr postinsemination). Embryo selection was based upon cleavage stage and degree of fragmentation. Two to five embryos were transferred to the patient, depending on the number and quality of embryos available, the age of the patient, medical history, and finally, the couples' attitude toward multiple pregnancy. Embryo transfers were performed using a Tomcat catheter and 10–30 μl of the growth media.

A quantitative serum β-hCG test was performed 14–16 days after embryo transfer. Positive results were confirmed with a second hCG level 1 week later and an ultrasound approximately 4 weeks from transfer. Clinical pregnancy was confirmed by the presence of a heartbeat on ultrasound 4–6 weeks after embryo transfer.

Clinical pregnancy rate was expressed as the number of pregnancies per the total number of retrievals. The implantation rate was calculated on the basis of the number of fetal heartbeats per total embryos replaced. The percentage blastocyst formation was expressed as the number of blastocysts formed per total "spare embryos" cultured. The percentage blastocysts with an obvious inner cell mass was calculated in a similar manner. Statistical significance was determined using the *t* test and chi-square analysis. *P* values <0.05 are reported as significant.

RESULTS

IVF cycles were reviewed and classified according to the nature of protein supplementation used in the embryo culture medium. Of the 263 IVF cycles performed between January 1992 and December 1993, 213 utilized Plasmanate and 50 used maternal serum. SSS was used in 68 consecutive cycles in 1994. There were no changes in patient selection, ovarian stimulation, or laboratory techniques.

Although the clinical pregnancy rates among the three treatment groups were not in themselves statistically different (Table I), there was a definite trend toward a higher pregnancy rate with SSS supplementation [38.2% SSS vs 24.9% PL ($P < 0.05$) and 28.0% MS]. It should also be noted that the average number of embryos per transfer was lower in the SSS treatment group [3.38 ($P < 0.05$) vs 3.92 and 3.95, respectively, with MS and PL]. Embryos grown in SSS-supplemented culture medium had a higher implantation rate higher (17.8%; $P < 0.05$) compared to those cultured in maternal serum (10.4%)- or Plasmanate (10.3%) supplemented medium. The average patient age and number of oocytes recovered per cycle were very similar among all three treatment groups (Table I). Interestingly, overall oocyte fertilization was lower when SSS was used for insemination medium supplementation compared to Plasmanate (59.1%, vs 68.1% with PL).

The major variable between the treatment groups was patient diagnosis (Fig. 1A). Initially, Plasmanate supplementation was restricted to patients with unexplained infertility, endometriosis, or immunologic factors. Patient indication in the maternal serum group was therefore primarily tubal infertility. With increasing confidence in Plasmanate's ability to support early embryo cleavage and positive pregnancy outcomes, maternal serum usage was completely dropped. Patients diagnosed with tubal factor or unexplained infertility comprised 36.6 and 40.8%, respectively, of the Plasmanate study group, but clinical pregnancy rates did not vary much with diagnosis (20.4 and 25%, respec-

Table I. Comparison of Patient Parameters and IVF Cycle Outcome

	Maternal serum	Plasmanate	SSS	*P*
Age	34.0 ± 3.4	34.7 ± 3.8	34.7 ± 3.5	NS
Retrievals	50	213	68	
Embryo transfers	49	209	63	
Total oocytes	530	2440	779	
No. oocytes/retrieval	10.6 ± 7.2	11.5 ± 6.5	11.5 ± 6.8	NS
No. embryos/transfer	3.92 ± 1.3	3.95 ± 1.3	3.38 ± 1.1	<0.05
Fertilization rate	64.2	68.1	59.1	<0.05
Clinical pregnancy rate per retrieval	28.0	24.9	38.2	NS
Implantation rate	10.4	10.3	17.8	<0.05

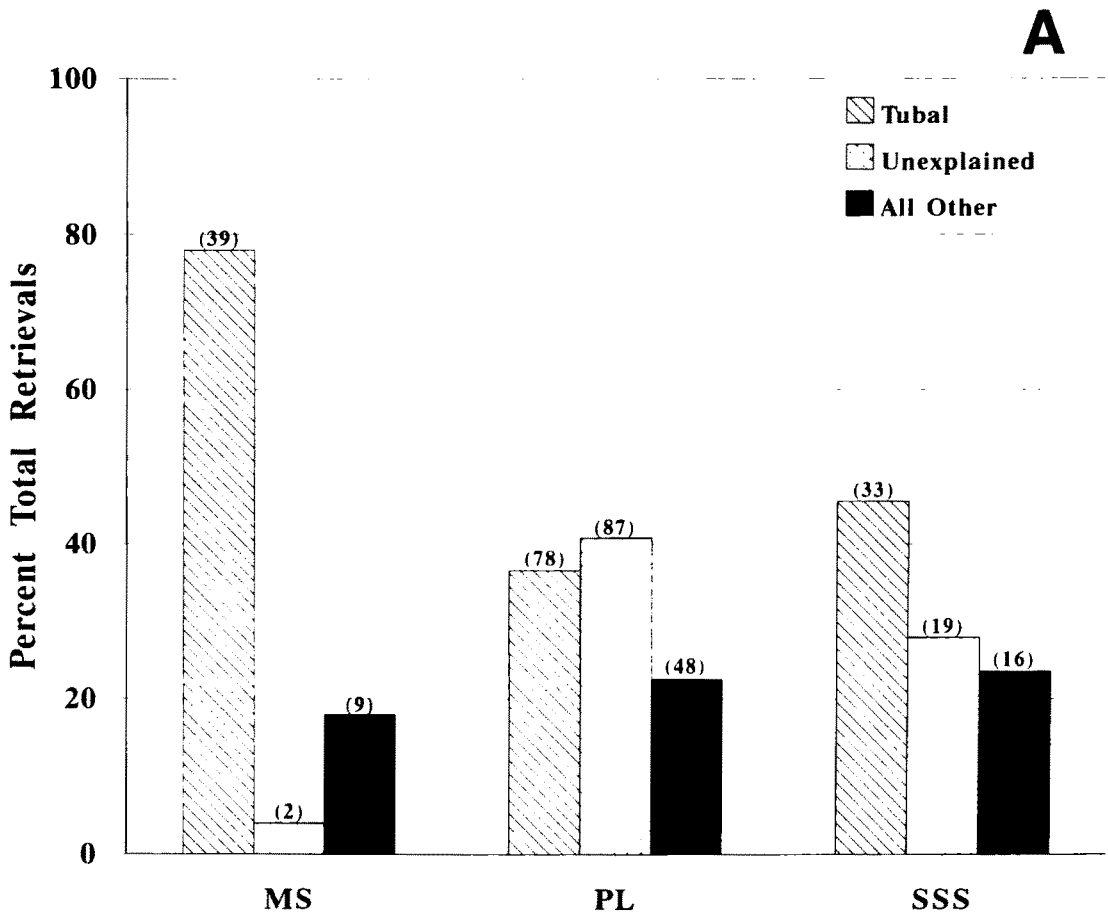

Fig. 1. Indication for IVF treatment. (A) Patient diagnosis is compared in the three treatment groups (MS, PL, and SSS). (B) Patient diagnosis during study period I, when IVF medium was supplemented with either MS or PL, is contrasted to the second study period, when only SSS supplementation was used.

tively). In the SSS group, indication for IVF was tubal factor in 48.5% and unexplained infertility in 27.9% of the total cycles analyzed. From Fig. 1B it can be seen that patient indication for infertility treatment during this study period was similar to that seen between January 1992 and December 1993, when either maternal serum or Plasmanate was being used for IVF medium supplementation. The clinical pregnancy rate was significantly higher for tubal infertility with SSS supplementation (48.5%; $P < 0.05$) (Fig. 2).

Figure 3 depicts the percentage of "spare embryos" (poor morphology, unsuitable for early freezing, and not transferred) that were capable of advancing to the blastocyst stage after continued culture, in each treatment group. The percentage of fully expanded blastocysts with an easily discernable inner cell mass is also shown. These blastocysts were cryopreserved for future transfer.

DISCUSSION

Although there have been reports of successful IVF procedures in which medium was prepared without protein supplementation (13), it is generally accepted that protein is desirable in preparation of media for human in vitro fertilization. The addition of proteins to culture media seems to accelerate the cleavage rate of human embryos (14) and to decrease DNA damage (15).

This paper reports our recent experience with SSS and compares it to our previous experience with maternal serum and Plasmanate. Although the SSS was not used concurrently with the other two protein sources, the IVF protocols, other than type of protein supplementation, were the same. Similarly patient ages (Table I) and indication for IVF (Fig. 1B) were comparable between the two time periods.

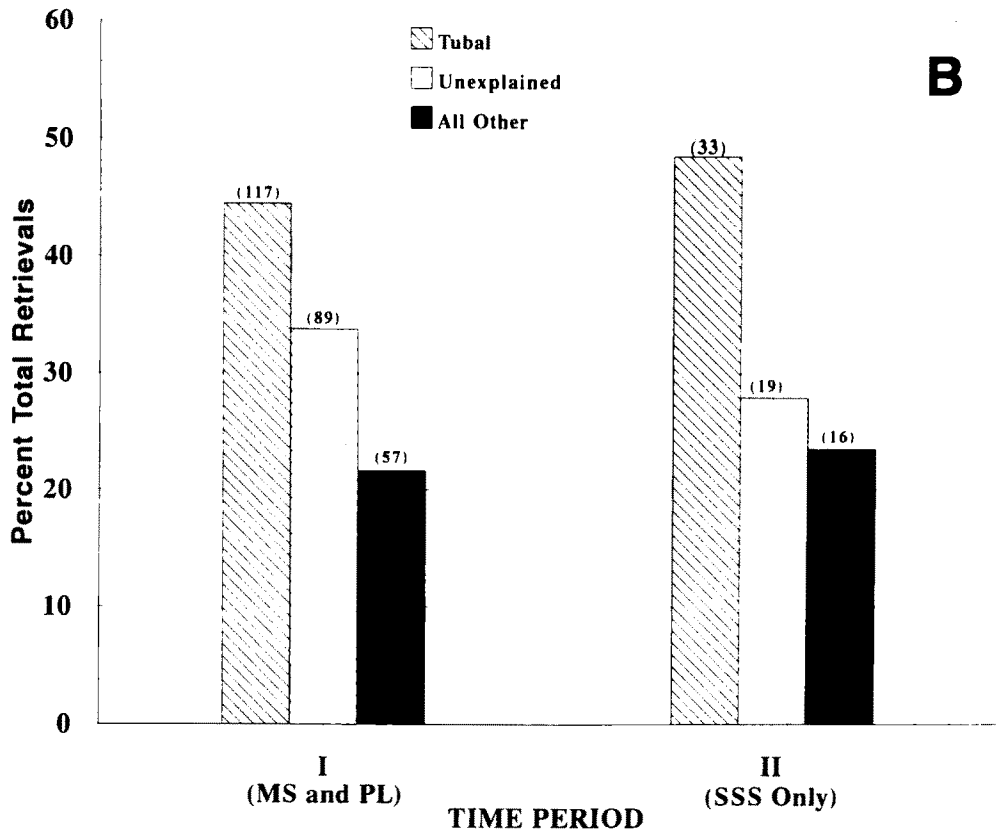

Fig. 1. continued.

The trend toward an increased clinical pregnancy rate and the improved implantation rate in the SSS group (Table I) is very reassuring as to its potential for routine use in IVF. While the decrease in oocyte fertilization rate with the SSS supplement (59.1 vs 68.1% PL) is of statistical significance, the degree of difference may have little clinical significance, particularly when offset by the higher implantation rate. Nonetheless, fertilization outcome of patients with SSS supplementation should be further investigated in a controlled randomized study, in patients with both normal and impaired semen parameters.

The retrospective nature of this study necessitates our having to draw comparisons among three treatment groups where the final protein content in fertilization and culture media were not uniform. Inseminations were performed in 6% PL-, 10% SSS-, or 10% MS-supplemented media. The calculated protein content of PL-, SSS-, and MS-supplemented media for insemination (based on stock solutions) was 3.0, 6.0, and approximately 7.5 mg protein/ml, respectively. Embryo culture media contained 5.0, 9.0, and 11.25 mg protein/ml in Pl-, SSS-, and MS-supplemented media, respectively. However, as shown in Table II a wide range of protein concentrations has been used in IVF fertilization and culture media. The effectiveness of the various protein preparations has been related largely to their composition. Psalti *et al.*, using UltraSerG, a synthetic serum preparation containing albumin and a mixture of purified growth factors and adhesion factors, reported an adverse effect on both oocyte fertilization and embryo development (6). In contrast, Medicult's chemically defined Synthetic Serum Replacement Medium (SSR-2) used with highly purified HSA (10 mg/ml) is an extremely effective protein supplement for IVF work (5). Staessen *et al.* demonstrated that while Albumninar-20, a therapeutic albumin preparation with a low globulin content, enhanced fertilization, early cleavage, and clinical pregnancy rates, subsequent development of supernumary embryos was distinctly impaired (2). The synthetic protein preparation (SSS) utilized in this investigation is a defined mixture of HSA and α- and β-globulins, formulated to mimic the protein content of Plasmatein. The globulin content of SSS

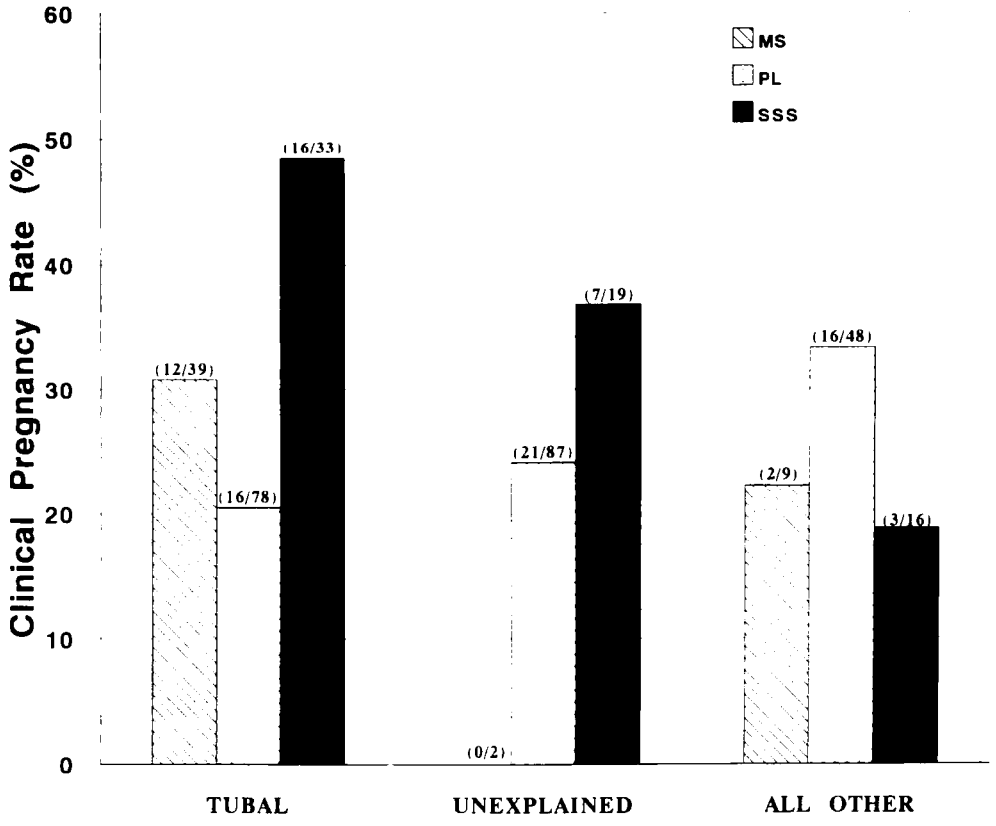

Fig. 2. The IVF cycle outcome for patients within each infertility diagnosis is compared among the three treatment groups. Clinical pregnancies confirmed by the presence of a fetal heartbeat on ultrasound are shown. *Statistically significant difference ($P < 0.05$).

(16%) is higher than that found in Plasmanate (12%). Pool and Martin have contended that a high concentration of globulins in culture media is desirable, creating a physiochemical microenvironment for the embryo which more resembles that found in the fallopian tube (9). For the growing embryo this might translate into shorter intermitotic intervals, accelerated cleavage, timely genomic activation, and higher implantation potential.

Sperm function in SSS-supplemented media needs to be critically assessed. The absence of normal plasma lipids (and other plasma components) distinguishes the Irvine-SSS product from both Plasmatein and Plasmanate. Whether these variables impact upon sperm function, capacitation, or fertilization requires further study.

To date little information has been available in the literature on the efficacy of SSS and Plasmanate in supporting in vitro human embryo development beyond the six- to eight-cell stage. This information can be essential since poorer-quality embryos (not suitable for freezing) are often carried in culture until Day 6. The necessity of examining the prolonged effect of any IVF protein supplement on embryo development was clearly demonstrated by the experience of Staessen *et al.* with Albuminar-20 (2). Timing of development in vitro, especially completion of the third cell cycle, appears to be critical in the evaluation of an embryo's potential for further development (16,17). Huisman *et al.* have reported an implantation rate of 41% for cavitating morula-stage embryos transferred on Day 4, compared to an implantation rate of 11% for embryos that were developmentally lagging on the day of transfer (18).

In the present study, development of 30.5% of the "spare," poorer-quality embryos to the blastocyst stage in SSS-supplemented medium was encouraging. Blastulation rates ranging from 23 to 42% have been reported (1,19,20,21) for good-quality normally fertilized zygotes grown in protein-supplemented HTF, T6, or EBSS media. Even lower blastocyst transformation rates were obtained

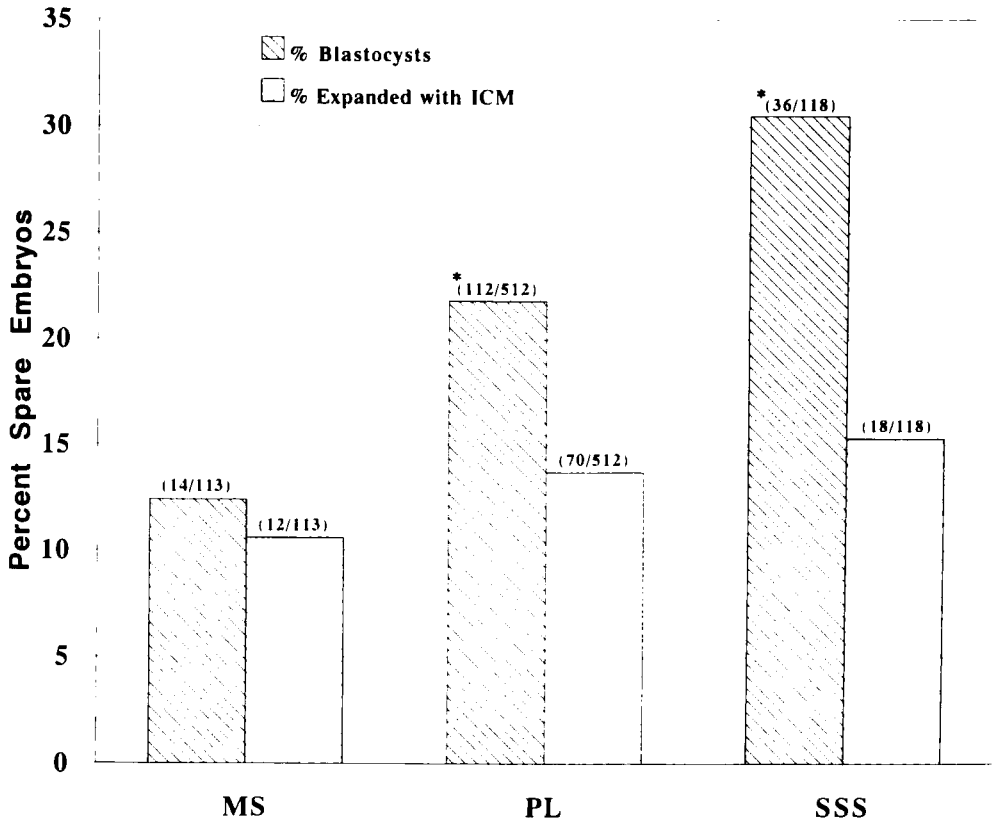

Fig. 3. Development of spare embryos in culture. Percentage reaching the blastocyst stage and percentage fully expanded with an ICM. *Significantly different ($P < 0.05$) for percentage blastocysts obtained with SSS or PL vs MS.

when only the poorer-grade embryos were cultured. Critical evaluation of cell number, proliferation, allocation of cells to the trophectoderm and inner cell mass (20), and ultimately implantation rates will be necessary to assess the efficacy of HTF/SSS-supplemented medium for prolonged in vitro culture of human embryos. It has been suggested that simple media such as HTF and EBSS, regardless of the

Table II. Comparison of Protein Preparations and Supplementation of IVF Culture Media

Brand name	Composition	Fertilization medium (mg protein/ml)	Culture medium (mg protein/ml)	Ref. no.
Albuminar-20	Therapeutic albumin, low globulin content	4.5	4.5	2
Medicult SSR2	Synthetic serum replacement, devoid of protein lipids and glycans. Contains chelators and a synthetic replacement for transferrin. Added to culture medium with purified HSA	10.0	10.0	5
Plasmatein	Therapeutic albumin with high α,β-globulins (<17%), γ-gamma globulins (<1%), and plasma lipids	5.0	5.0	9
UltraSerG	BSA with purified growth factors and adhesion proteins	1.5	1.5	6
Plasmanate	Therapeutic albumin with α,β-globulins (12%), γ-globulins (<1%), and plasma lipids	3.0	5.0	8[a]
Synthetic Serum Substitute (SSS) (Irvine)	HSA with α,β-globulins (16%), γ-globulins (<1%). No plasma lipids	6.0	9.0	—[a]
Maternal serum	All serum components (75 mg protein/ml)	7.5	11.25	—[a]

[a] Final protein concentrations in treatment groups in this study.

type of protein supplementation, are inadequate in supporting blastulation and that perhaps more complex culture media such as MEM-α and Ham's F12 should be used for culture to the blastocyst stage (21). Testing of SSS in combination with these more complex media is therefore warranted.

The human embryo is usually at the late morula or early blastocyst stage when it enters the uterus and implants shortly thereafter (22). The current practice of transferring IVF embryos to the patient's uterus on Day 2 at the two- to four-cell stage rather than at more advanced cleavage stages has been adopted primarily to sidestep difficulties encountered in prolonged culture of human embryos. But this also means that the growth potential of embryos selected for transfer cannot be adequately assessed. This is especially critical since the human embryonic genome is not activated until around the eight-cell stage (around Day 3), after which it is able to direct protein synthesis and, ultimately, its own development (23). With the development of better and more standardized IVF medium, it should be possible to delay embryo transfer until at least the third day postfertilization and, ultimately, to the blastocyst stage, allowing the "best" embryos with the greatest potential for implantation to be selected for transfer. Synthetic protein preparations may play a vital role in the development of specialized culture media for the extended in vitro development of human embryos.

CONCLUSIONS

The use of commercial protein preparations such as SSS and Plasmanate increases laboratory efficiency and allows greater standardization of media compared to the use of maternal serum, without any negative effect on pregnancy rates.

The higher implantation rate with SSS suggests that it may be superior to both maternal serum and Plasmanate in supporting human embryo development in vitro. While both Plasmanate and, to a greater extent, SSS allowed development of embryos to the blastocyst stage, it remains to be seen if blastocysts derived from either media can implant and give acceptable pregnancy rates.

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