Increasing Synthetic Serum Substitute (SSS) Concentrations in P1 Glucose/Phosphate-Free Medium Improves Implantation Rate: A Comparative Study

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Purpose: To assess the comparative efficacy of IVF medium (MediCult, with 5.2 mM glucose) and a glucose/phosphate-free medium, P1 (Irvine Scientific), and to investigate the influence of increasing the serum supplementation (synthetic serum substitute; SSS; Irvine Scientific) to P1 on embryo development and implantation.

Methods: Patients were randomly assigned to IVF medium (Group 1, cycles n = 172) or P1 supplemented with 10% SSS (Group 2, cycles n = 229) according to the medium scheduled for use on the day of oocyte retrieval. Another 555 IVF consequent cycles (Group 3) were performed using increased SSS concentrations (20%) in P1 medium.

Results and Conclusion: In this large series of IVF cycles, we herein demonstrate that significantly higher pregnancy and implantation rates were found when embryos were cultured in glucose/phosphate-free medium P1 supplemented with 20% SSS compared to supplementation with the lower SSS concentration and with IVF medium.

KEY WORDS: glucose; implantation rate; IVF culture medium; pregnancy; serum.

INTRODUCTION

The culture environment of preimplantation embryos affects postimplantation events. It is, therefore,

important to define the precise substrate requirements of every developmental stage to optimize the efficacy of the embryo culture media. We had routinely used IVF medium (Medi Cult, Copenhagen, Denmark, with 5.2 mM glucose) in our unit for fertilization and embryo development unit Day 3. However, several scientific reports demonstrated that the culture requirements for early stage embryos of most mammalian species are vastly different from those of the majority of somatic cells, since they are unable to utilize glucose for development (1–6). While pyruvate support human embryo development to the blastocyst stage, embryos cannot use glucose to compensate for the lack of pyruvate in the medium (7). According to these investigations, the embryos seemed to grow better in glucose/phosphate-free media than they do in media containing the typical levels of glucose found in the ones used for somatic cell culture (2.5–5.5 mM). Based on these data, a commercial glucose/phosphate-free medium preimplantation Stage 1 (P1) medium for human IVF (Irvine Scientific, Irvine CA) was developed. In the first part of the current study, we compared the efficacy of IVF medium and P1.

Several studies have reported improved embryo development with serum-supplemented medium (2,8). While serum may provide beneficial factors to the cumulus milieu, it can also introduce detrimental constituents (9,10). In the second part of this study, we assessed the influence of increasing the serum supplementation, synthetic serum substitute (SSS; Irvine Scientific) prepared from human serum albumin (hSA) and human globulins, on embryo development and implantation.

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MATERIALS AND METHODS

The investigation was conducted in two steps. In the first step, (Sept.–Nov. 1997), a prospective study, patients were randomly assigned to IVF medium (Group 1; $n = 172$ cycles) or P1 supplemented with 10% SSS (Group 2; $n = 229$ cycles) according to the medium that was being used for oocyte retrieval on that day. Specifically, the protocol dictated that one of the two mediums would be selected for use on a given day according to a randomized list prepared beforehand, and only that medium was used for all the patients scheduled for retrieval on that day.

In the second step (Jan.–May 1998), a total of 555 IVF/ICSI consecutive cycles were performed using 20% SSS in P1 medium for the first 3 days (Group 3). It is important to emphasize at this point that this large group of patients, although recruited consecutively, resembles Groups 1 and 2 in age, number of previous IVF cycles, cause of infertility, ovulation induction protocol, and in vitro culture conditions, differing only in the parameter of incubation culture medium. Therefore, although Group 3 was not sampled prospectively, it is statistically comparable with Groups 1 and 2.

Ovulation induction was performed using a routine protocol of gonadotrophin-releasing hormone analogue (GnRH-a; 900 $\mu\text{g/day}$ of busereilin acetate nasal spray ($\text{D-Ser}[\text{TBU}]^6$ -ethylamide-luteinizing hormone-releasing hormone (LH-RH), Suprefact; Hoechst AG, Frankfurt, Germany) and three ampules of human menopausal gonadotrophin (hMG; Pergonal; Teva Pharmaceutical Industries Limited, Petah Tikva, Israel). Oocyte retrieval was scheduled 35–36 h after administration of 10,000 IU human chorionic gonadotrophin (hCG; Chorigon; Teva Pharmaceutical Industries Ltd.).

The cumulus oocyte-complexes were isolated into IVF medium (Group 1), P1 medium supplemented with 10% SSS (Group 2), or P1 medium supplemented with 20% SSS (Group 3). SSS (Irvine Scientific) contains 6% protein components consisting of 84% pharmaceutical grade hSA (50 mg/ml). Therefore, when 10% SSS is added to the P1 medium, the actual hSA concentration would be 0.5%. It then follows that 20% SSS supplementation is equivalent to 1% hSA. IVF medium (Medicult) also contains 1% hSA. The remaining 16% protein component of SSS is human globulins.

Insemination of the retrieved oocytes was performed by standard IVF insemination (40–50% of the cycles) or ICSI (50–60% of the cycles) according

to sperm parameters. A routine semen analysis was done according to World Health Organization (WHO). Standard IVF insemination was performed with approximately 200,000 spermatozoa/mL at 39–41 h post-HCG. For the ICSI procedure cumulus and corona radiata cells were removed 2–3 h after oocyte retrieval, by aspiration of the oocytes through glass pipettes (150–200 μm inner diameter) in culture medium containing 80 IU hyaluronidase/mL (Type VIII Sigma, Aldrich, U.K.). The specific culture medium was Sperm Medium (Medicult) in Group 1 and modified human tubal fluid (HTF) medium (Irvine Scientific) supplemented with 5% SSS in Group 2. Only those oocytes found to be at metaphase II were injected. Injections were performed at 37–41 h post-HCG in the same medium used for oocyte denudation, on a heated microscope stage (37°C). Sperms were immobilized in 10% PVP droplet (Medi Cult, Copenhagen, Denmark) by breaking the tail with the shaft of the injection pipette. Oocytes were held by gentle suction on holding pipette with the polar body in the 6 o'clock or 12 o'clock position. The immobilized spermatozoon was advanced to the tip of the injection pipette, which was then pushed through the zona at the 3 o'clock position. Oolemma puncture was confirmed by a sudden shift of ooplasm into the injection pipette, before depositing the spermatozoon. After the ICSI procedure, the oocytes were returned to the culture dish for further incubation in their original medium at 37°C and 5% CO_2 in air. All the oocytes were inspected for fertilization 16–20 h later. Embryo cleavage and quality were evaluated on Day 3 prior to transfer. Embryo morphology was scored from 1 to 4 according to the shape of the blastomeres and the amount of detached anuclear fragments: Grade 1 – excellent embryos, Grade 2 – good, Grade 3 – fair, and Grade 4 – poor, as detailed previously (11). Pregnancy was confirmed by the detection of two consecutive and increasing β -hCG levels (>10 IU/L). Clinical pregnancies were defined by a sonographic demonstration of a gestational sac at 6.5–7 weeks of pregnancy. Implantation rate (IR) was defined as the number of gestational sacs divided by the total number of embryos transferred.

Statistical Analysis

The nonparametric Kruskal–Wallis one-way ANOVA test was used for comparing female age, number of retrieved oocytes, fertilization, cleavage, embryo quality, and embryo transfer. Chi-square analysis was used when comparing pregnancy and

Table I. Patient Characteristics and Fertilization and Embryo Quality in the Different Culture Media

	IVF medium (Group 1)	P1 + 10% SSS (Group 2)	P1 + 20% SSS (Group 3)
No. of cycles	172	229	555
Mean female age ^a (year)	33.5 ± 0.4	33.6 ± 0.4	34.1 ± 0.3
Mean no. of oocytes retrieved per cycle	9.6 ± 0.5	8.7 ± 0.4	10.6 ± 0.3
<i>n</i>	1654	1992	5877
Two pronuclear fertilization ^a (2PN/OPU)	55.9 ± 1.9	53.2 ± 1.7	56.0 ± 1.0
Embryo quality (% of total) ^a			
Grade 1	42.2 ± 3.1	51.8 ± 2.9	48.1 ± 1.7
Grade 2	34.3 ± 2.5	30.0 ± 2.3	35.4 ± 1.5
Grade 3	19.6 ± 2.2	14.7 ± 1.9	12.1 ± 1.0
Grade 4	3.5 ± 1.1	3.1 ± 0.9	4.1 ± 0.7

Note. Values are means ± SEM; SSS – synthetic serum substitute.

^a Kruskal–Wallis one-way ANOVA test showing no significant difference ($P > 0.05$) between groups 1, 2, and 3 when comparing female age, fertilization rate, and embryo quality.

IRs. A P value of <0.05 was considered statistically different.

RESULTS

The results of 956 IVF cycles were analyzed according to the medium in use for fertilization and embryo development, i.e., 172 with IVF medium for culture (Group 1) and 784 cycles with P1 medium in different SSS concentrations (Groups 2 and 3, Table I). There were no statistically significant differences among the groups for female age, fertilization rate, embryo quality, and the mean number of embryos transferred per patient (Tables I and II, $p > 0.05$). Almost all patients had embryos for transfer, yielding a high embryo transfer (ET) rate in all three groups (96.5, 93.0, and 98.9%, respectively, $P > 0.05$, Table II). Positive β -hCG per ET was 21.1, 22.5, and 28.5% for Groups 1, 2, and 3, respectively, with that value of Group 3 reaching a comparatively higher significance ($P < 0.05$,

Table II). The embryos that developed in P1 medium supplemented with 20% SSS demonstrated a significantly higher IR (9.9%, Group 3) compared to 7.3 and 7.0% in Groups 1 and 2, respectively ($P < 0.05$, Table II).

It is worth bearing in mind that the mean number of previously failed IVF cycles of our patients is 6.2 ± 4.5 and 20% are ≥ 40 years of age.

DISCUSSION

The natural environment of the preimplantation human embryo is the oviduct where it is surrounded by oviductal cells and a very thin film of fluid. The composition of this fluid varies with the day of the cycle. During fertilization, which occurs at midcycle after ovulation, the pyruvate concentration is 0.32 mM, lactate reaches 10.5 mM, whereas glucose concentrations decrease to 0.5 mM (4). Albumin and immunoglobulin are the most abundant proteins in the

Table II. Pregnancy Rates in the Different Culture Media

	IVF medium (Group 1)	P1 + 10% SSS (Group 2)	P1 + 20% SSS (Group 3)
Embryo transfer rate (%)	96.5	93.0	98.9
Mean no. of embryos transferred per cycle ^a	3.1 ± 0.09	3.0 ± 0.09	3.1 ± 0.05
<i>n</i>	530	681	1727
β Hcg per embryo transfer	35/166	48/213	158/549
Percentage [†]	21.1	22.5	28.5 [‡]
Clinical pregnancies per embryo transfer (%) [‡]	28/166 (16.9)	39/213 (18.3)	126/549 (22.9)
Implantation rate (%) [‡]	39/530 (7.3)	48/681 (7.0)	171/1727 (9.9 [†])

Note. Values are means ± SEM; SSS – synthetic serum substitute.

^a Kruskal–Wallis one-way ANOVA test: no significant difference between Groups 1, 2, and 3 ($P > 0.05$).

[†] Chi-square test: Group 3 is significantly different from Groups 1 and 2 ($P < 0.05$).

[‡] Chi-square test: No significant difference between Groups 1, 2, and 3 ($P > 0.05$).

oviduct fluid (12). The media now available for IVF fertilization and development of human embryos attempt to mimic the physiological milieu. More precise identification of the substrate requirements of the embryos will enable the development of a medium composition approximating the natural *in vivo* one.

Although human embryos can be cultured in completely protein-free media, their development and implantation potential were seen to improve when a protein supplement was added to the culture media (2,8). Our results demonstrated that there was a significantly higher IR when the embryos were cultured in P1-medium supplemented with 20% SSS compared to supplementation with lower SSS concentrations. As mentioned earlier, when SSS is added at a concentration of either 10 or 20% in P1 medium, the actual hSA concentration is 0.5 and 1%, respectively. In addition, the protein component of SSS includes 16% human globulins. It is noteworthy that the presence of globulins with hSA has been shown to enhance the rate of embryo development and continuing pregnancy rates beyond the levels achieved for hSA alone (13,14).

The scientific reason for the benefit of adding serum is not known. However, it is well accepted that a major biological role of serum albumin is to compensate for whatever essential components are missing from the medium by serving as a reservoir for many of the beneficial constituents, such as different energy substrates, vitamins, steroids, fatty acids, amino acids, and growth factors. In addition, the serum serves as a protective compound by scavenging ions and small molecules secreted from the growing embryo (2,15).

The presence of glucose and phosphate in the culture medium has been demonstrated by several groups to inhibit early embryonic development of hamster and mouse (3,5). The P1 medium is essentially a modification of the HTF medium (2.8 mM glucose) in which all glucose and inorganic phosphate (Pi) is removed. One preliminary study demonstrated that a glucose/phosphate-free medium produced superior embryo development and IRs in human IVF as well (16). It was then confirmed in an expanded report of this study (17,18). In this large series of IVF cycles, we herein demonstrate that maintenance of human gametes and embryos in P1 medium for 72 h is conducive to good embryo development and pregnancies. It was recently demonstrated that human embryos developed more rapidly and were of higher quality morphologically with the use of glucose/phosphate-free medium (19). One explanation for the improvement in results achieved when glucose

and phosphate are omitted came from the study of Seshagiri and Bavister (1). They observed a two- to threefold increase in oxygen consumption in hamster embryos cultured without glucose and Pi. They suggested that early exposure of preimplantation embryos to these substrates appears to shunt embryonic metabolism into anaerobic glycolytic respiration that is energetically costly to the embryo. By eliminating glucose and phosphate, oxidative metabolism through the Krebs cycle is maintained, thereby yielding improved embryonic viability. Very recently Biggers (2001) reported evidence that glucose is not always an inhibitor of mouse preimplantation development *in vitro* and that the effect of glucose and KH_2PO_4 may depend on the background composition of the media in which they are studied (20).

In light of our large cohort, we could conclude that increased SSS in P1 medium yielded a better IR. However, to show a definite advantage of glucose-free media for embryo development and implantation, a similarly large study needs to be carried out using the same medium formulation (such as P1) with the same serum concentrations, with or without glucose.

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