BUILDING NEW CULTURE MEDIA FOR ASSISTED REPRODUCTIVE TECHNOLOGY

Mary N. Egbuniwe[a], H. Lee Higdon III.[b], and William R. Boone[b]

[a]Department of Biology, Wofford College, Spartanburg, SC
[b]Department of Obstetrics and Gynecology, Greenville Hospital System, Greenville, SC
bboone@ghs.org

ABSTRACT

In the last 20 years, researchers in the field of Assisted Reproductive Technology (ART) have made many advances in formulating media for use in in-vitro fertilization procedures. In an effort to improve our in-vitro production (fertilization and development), we compared a commercially prepared culture medium (Human Tubal Fluid [HTF]; Irvine Scientific, Irvine, CA) to our in-house prepared medium (Reproductive Endocrinology and Infertility [REI]). Following positive results in media comparison trials using mouse embryos, we performed a similar randomized controlled study with the use of oocytes from 77 women (mean age [yrs.] = 33 ± 4.3 [SD]). Overall fertilization rates for HTF and REI were 67.3% (376/559) versus 78.9% (471/597) respectively (Chi-square; P <0.0001). Using the oocyte as the test unit in a logistic model for predicting fertilization, and adjusting for subject (mother) and method of insemination (in-vitro fertilization versus intracytoplasmic sperm injection), the odds ratio (95% confidence interval) for REI medium versus HTF medium was 1.8 (1.4, 2.4; P < 0.0001). Thus, oocytes cultured in REI medium were more likely to fertilize when compared to oocytes cultured in HTF medium. Similar analyses for cell-stage development and embryo quality as defined by grade, demonstrated no differences between the two media. The increase in the number of fertilized oocytes when using REI media led to a larger cohort of embryos from which to select superior embryos at the time of transfer. Furthermore, this increase in number of fertilized oocytes provided more supernumerary embryos for cryopreservation.

INTRODUCTION

The term infertility is generally defined as the inability to conceive after one year of normal, regular heterosexual intercourse without the use of any contraception (Seibel, 1990). Based on a series of nationwide surveys conducted by the National Center for Health Statistics, it is estimated that approximately 3.3 million of the 40 million cohabitating couples of childbearing age in the United States are infertile (Sher et al., 1995). Individuals seeking treatment for their infertility often resort to Assisted Reproductive Technology (ART).

While human ART procedures are only slightly more than 25 years old, the concept of ART is well over 100 years of age (Steptoe and Edwards, 1978). In the 1890's, Walter Heape (1891, cited by Wood and Trounson, 1999) successfully transferred embryos between rabbits. He demonstrated the physiological possibility of recovering a pre-implantation stage embryo from a female animal without hindering development. Since

this discovery, many others have investigated and advanced the technology essential to the development of ART.

Besides the ova and sperm quality, two other important components involved in an ART procedure are incubator environment and the culture medium in which the gametes reside. The main elements involved in incubation are temperature, humidity, and gas composition. The standard temperature for culturing human embryos is 37°C. It is believed that incubator temperatures above 37°C induce poor development and provide an environment for irreversible morphological changes (Brinster, 1969). Conversely, temperatures below 37°C promote a slowing of development and potentially, developmental arrest (Brinster, 1969).

Humidity is considered important when the culture medium in which the embryos reside is not covered with mineral oil to prevent evaporation. When the medium is not overlaid with oil, the water in the culture medium will evaporate, resulting in an increase of osmotic pressure in the culture medium (Elder and Dale, 2000).

Many of the modern incubators control for nitrogen (N_2), oxygen (O_2) and carbon dioxide (CO_2). Considered to be inert, the concentration of N_2 varies depending on the amount of CO_2 and O_2. Whitten (1956, cited by Brinster, 1969) proved that an eight-cell mouse embryo can develop in an atmosphere of 5% CO_2 and 95% air, however the embryo cannot develop in 5% CO_2 and 95% N_2. In contrast, Auerbach and Brinster (1968, cited by Brinster, 1969) discovered that a two-cell mouse embryo had a significant and quantifiable need for atmospheric O_2 during the culture period. When the culture media was placed in a CO_2, O_2 and N_2 environment, development of the two-cell mouse embryo to the blastocyst stage occurred.

Also important to the optimization of the incubator environment is the type of buffer system employed by the medium. Such a buffer is bicarbonate (HCO_3), which affects the pH of the culture medium. In this case, attention must be paid to the concentration of HCO_3 in the medium and the gas composition inside the incubator. Brinster (1965, cited by Brinster, 1969) reported the optimal pH of an embryo culture medium is approximately 7.4. In order to maintain this pH, most culture media used for human embryos require 5% CO_2 in the incubator. By holding the HCO_3 concentration constant and increasing or decreasing the percentage of CO_2 in an incubator, media pH will respond accordingly.

Early in in-vitro production (IVP) procedures, embryonic culture medium resembled the ionic composition of serum. Whitten (1956, cited by Brinster, 1969) found that when Ca^{2+}, Mg^{2+}, and K^{+} were omitted from the medium, there was no growth of the eight-cell mouse embryo. He also reported that development was delayed when phosphate (HPO_4^{-}) was absent from the medium.

While Brinster found that two-cell mouse embryos could develop to the blastocyst stage in medium with an osmolarity between 200 and 354 mOsm, he observed that the maximum number of two-cell mouse embryos reached the blastocyst stage when the osmolarity of the culture medium was 276 mOsm. The culture medium used by Brinster contained those specific ions (Ca^{2+}, Mg^{2+}, and K^{+}) that Whitten found to be essential to embryonic development (Brinster, 1969).

Drawing on the above knowledge of media building, the purpose of this study is to compare our in-house REI culture medium to a standard culture medium to determine whether differences in fertilization rate and stage and grade of the resulting embryos can be observed.

MATERIALS AND METHODS

Patient Population. Data for this study were taken from 77 women undergoing a fresh, non-donor IVP procedure in our tertiary care hospital-based ART program. All patients, regardless of infertility diagnoses, were included in this analysis unless they failed to undergo embryo transfer.

Our institutional review committee approved this prospective, cohort study, and patients signed consent forms indicating awareness that their data might be used to monitor and possibly improve methodologies used in ART.

General Endocrine Information. Ovarian stimulation drugs were administered to produce several mature oocytes instead of the single oocyte (egg) that normally developed each month. By developing more than the single oocyte, the patient increased her chance of becoming pregnant.

Drugs such as leuprolide acetate (Lupron; TAP Pharmaceuticals, North Chicago, IL) were used to suppress the normal ovarian function in order to better control the stimulation process. Following the initiation of Lupron, ovarian stimulation drugs, called gonadotropins, such as follicle stimulating hormone (FSH; Fertinex, Gonal-F, Serono Laboratories, Randolph, MA) and possibly, human menopausal gonadotropin (hMG; Repronex, Ferring Pharmaceuticals Inc., White Plains, NJ) were used to stimulate the ovaries to produce multiple oocytes.

Hormone values and follicular size were monitored over the course of the stimulated cycle as a means to determine the optimal oocyte harvest time. In general, patients that were stimulated for nine to eleven days, had three follicles 16 mm or greater in diameter, and had a blood estradiol level between 1,500 and 3,000 pg/mL were injected with human chorionic gonadotropin (hCG; Profassi, Serono Laboratories, Randolph, MA). Thirty-six hours following hCG injection, oocytes were retrieved transvaginally with a specially fitted ultrasound probe.

Stimulation Protocols. Our patient stimulation protocols have been described previously (Nichols et al. 2001). Before their IVP cycle, couples underwent screening procedures that included a physical examination, uterine evaluation (saline infusion sonohysterogram, hysterosalpingogram or hysteroscopy), basal hormone levels (follicle stimulating hormone [FSH], dehydroepiandrosterone sulfate, prolactin, and thyroid stimulating hormone), semen analysis and a review of previous stimulations or infertility treatment cycles. All women with basal FSH levels < 10 mIU/mL (measured on day 3 of the menstrual cycle) and no previous history of poor gonadotropin stimulation or cycle cancellation, underwent a standard protocol starting with luteal phase Lupron for pituitary suppression.

The standard protocol included the administration of Lupron (0.5 to 1 mg daily) beginning on approximately cycle day 21 of the menstrual cycle preceding gonadotropin stimulation. Lupron was decreased to 0.25 or 0.5 mg, respectively, at the start of gonadotropins and continued daily until the day of hCG administration. After at least 14 days of Lupron, FSH, with or without hMG, was initiated on cycle day 2, 3, or 4 beginning at a dose of 150-450 IU daily. Adjustments were made to gonadotropin

dosages after at least 4 days and thereafter depending upon follicular sizes and estrogen levels. We gave 10,000 IU of hCG intramuscularly, when three follicles, measured by transvaginal ultrasound, were ≥ 16mm in diameter.

Women not undergoing the standard protocol received a modified microdose flare protocol. After at least 21 days of oral contraceptives, the women were administered 40 μg of Lupron subcutaneously, twice-daily beginning on the second day of withdrawal bleeding. At least 450 IU of gonadotropins were given daily to each woman beginning two days after the start of Lupron. As in the standard protocol, the hCG injection was administered when there were three follicles ≥ 16 mm in size.

Oocyte (Egg) Retrieval and Incubation. Thirty-four to 36 hours following hCG injection, patients were taken to the operating room where, under mild sedation, transvaginal ultrasound harvest of oocytes was performed through the vaginal wall. The ultrasound probe emitted high-frequency sound waves that were translated into images of the reproductive tract shown on a monitor screen. Once the follicles were visualized, a needle was inserted into the follicle and the contents of the follicle (fluid and oocyte) were aspirated.

After retrieval, oocytes were placed into 50 μl drops of Human Tubal Fluid (HTF; Cat. No. 9962, Irvine Scientific, Santa Ana, CA) or culture media made in our laboratory (Reproductive Endocrinology and Infertility; REI). The REI media was a modification of the media described by Parrish et al. (1988). The drops were prepared in Falcon #3002 Petri dishes (Becton-Dickinson Labware, Lincoln Park, NJ) one to three hours prior to oocyte retrieval with media that were equilibrated overnight in a 36.7°C incubator containing a 5.0% CO_2 in air and high (95-100%) relative humidity atmosphere. The drops were covered with washed, equilibrated mineral oil (Cat. No. M-8410, Sigma Chemical Co., St. Louis, MO). Both culture media were supplemented with a serum source. The HTF medium was supplemented with synthetic serum substitute (SSS; Cat. No. 99193, Irvine Scientific, Santa Ana, CA) at a concentration of 10% (vol/vol). The REI medium was supplemented with human serum albumin (Cat. No. A-1887, Sigma Chemical Co., St. Louis, MO) at a concentration of 6 mg/mL.

Oocyte Insemination and Culture. Fertilization of the oocytes was achieved using either co-incubation of the oocytes with 20,000 motile sperm (Johnson and Boone, 2000) or by intracytoplasmic sperm injection (ICSI) in which a sperm is injected into the cytoplasm of the oocyte (ICSI; Johnson et al. 2004). Those oocytes that fertilized remained in their specified culture media until their transfer 72 hours later. Embryo quality was assessed on the morning of transfer, with Grade 1 being the best quality and Grade 5 being non viable. This system of evaluation was similar to Veeck (1991).

Determination of Pregnancy. Twelve to 14 days following the embryo transfer, patients had blood drawn and assayed for the presence of beta-hCG. A clinical pregnancy was defined as the presence of appropriately rising beta-hCG levels and the presence of a gestational sac demonstrated by ultrasound 6 to 7 weeks after oocyte retrieval. A biochemical pregnancy, defined as an initial positive beta-hCG result that failed to rise appropriately and no gestational sac on ultrasound, was reported as a failure to conceive.

RESULTS

Overall, fertilization rates for HTF and REI media were 67.3% (376/559) versus 78.9% (471/597), respectively (Chi-square $P < 0.0001$). With the use of the oocyte as the test unit in a logistic model to predict fertilization, and adjusting for subject (mother) and method of insemination (IVF versus ICSI), the odds ratio (95% confidence interval) for REI culture medium versus HTF medium was 1.8 (1.4, 2.4; $P < 0.0001$). Thus, oocytes cultured in REI medium had an increased chance of fertilization when compared to oocytes cultured in HTF medium.

Similar analyses for cell-stage development and embryo quality as defined by grade showed no significant differences. Mean cell stages (standard deviation) in the two groups were 6.1 (2.1) and 6.2 (2.4) for the REI and HTF groups, respectively ($P = 0.6$). Mean cell grades (standard deviation) in the two groups were 2.3 (1.0) and 2.4 (1.1) for the REI and HTF groups, respectively ($P > 0.4$; 1 = Excellent and 5 = Dead).

Representative samples of a human oocyte, zygote, four-cell, and eight-cell embryo are shown in Figure 1.

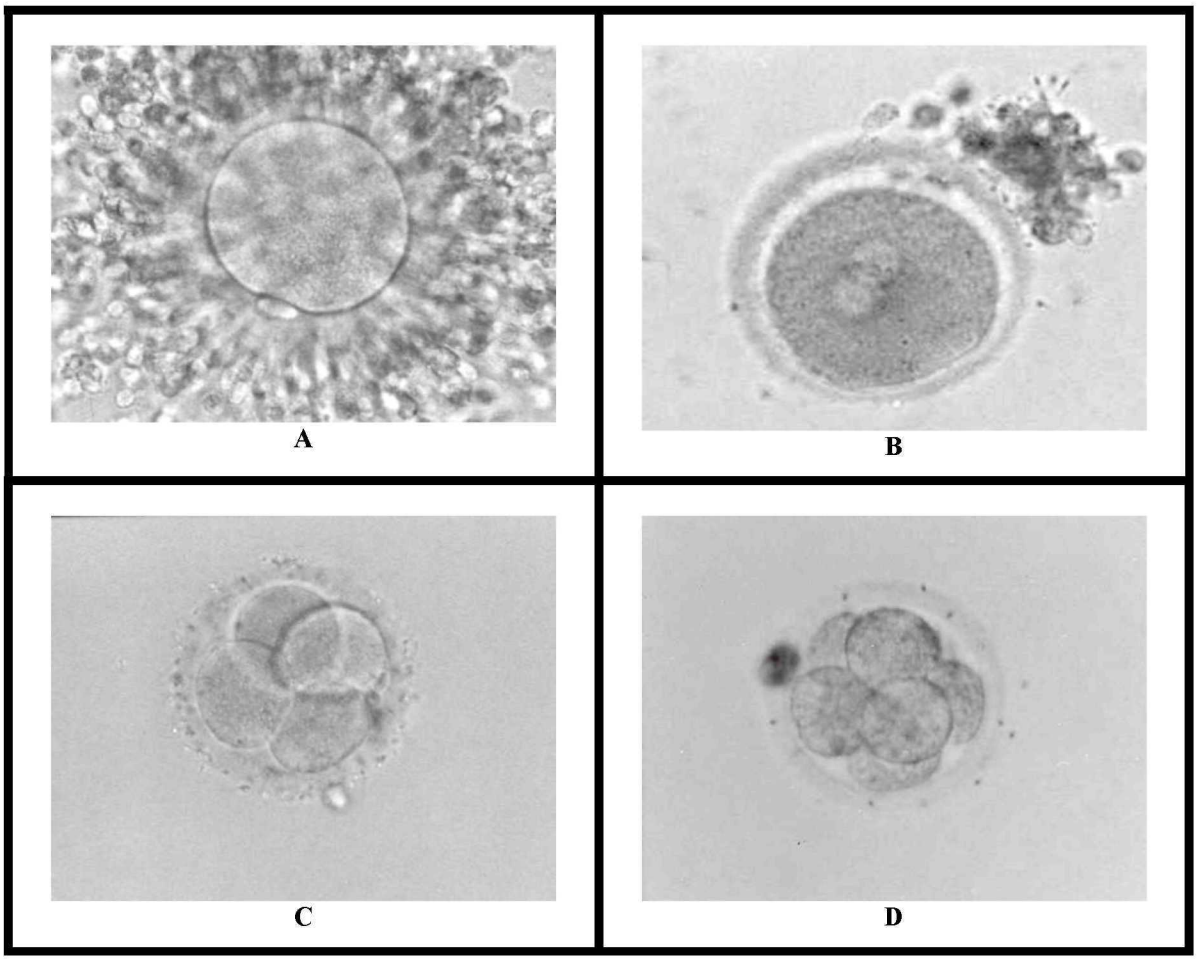

Figure 1. Representative samples of a human oocyte (A), zygote (B), four-cell embryo (C), and eight-cell embryo (D).

DISCUSSION

In an effort to improve existing IVP success, we developed a new culture medium (REI) and compared it to the current, widely-used, HTF media. The REI media outperformed the HTF media for fertilization, which resulted in more embryos being available for fresh transfer or for a future transfer using frozen/thawed embryos.

Over the past 10 years, many advances have been made in mammalian embryo culture media. Today, ART professionals can make decisions about media based on quality control procedures applied during manufacturing, cost effectiveness, and length of the shelf life. While exact needs of embryos during early development have not been completely defined, stage-specific and chemically distinct medium are now being developed for use in IVP programs. These media attempt to recreate the natural in vivo environment by taking into consideration modifications in embryo metabolism and physiology that transpire during the pre-implantation stage.

Unfortunately, incubator technology has not changed dramatically over the same period of time. The basic dual (CO_2 and air) and triple gas (CO_2, O_2 and N_2) incubators used in the early days of ART are still used today. Research regarding optimal incubator environment for specific medium is virtually nonexistent. It seems that because there has been success in fertilization and embryo culture in dual and triple gas incubators, both are considered adequate for IVP. Although ART fertilization rates are relatively high, insufficient incubation technology may inhibit progress. This area needs further research.

In conclusion, REI medium provides more embryos for the patient because of the increased percentage of fertilized oocytes. This increase in embryo number leads to a larger cohort of embryos from which to select superior embryos at the time of the transfer. Furthermore, supernumerary embryos can be cryopreserved. Cryopreservation provides couples with future embryo transfer attempts without the added risk of another stimulated cycle; furthermore, frozen embryo cycles offer a sizable reduction in cost.

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