Human chorionic gonadotropin is a glycoprotein hormone comprising 2 subunits, α and β, joined noncovalently (1). It is the most extensively studied hormone of pregnancy and is primarily produced by the embryo and later by the syncytiotrophoblast. HCG appears in maternal blood approximately 6 to 9 days after conception (2). The hormone has been detected at variable levels in culture media of blastocysts (3–6). Although its expression has been documented beginning 2 days after fertilization (5, 7–9), the presence of the hCG molecule has not been reported to date. The objective of our study was to develop an in-house enzyme-linked immunosorbent assay (ELISA) procedure to detect and quantify hCG from day 2 spent embryo culture media and to determine the association of hCG with the developmental potential of the embryo.

MATERIALS AND METHODS

A total of 102 day 2 spent embryo culture media from 11 women undergoing intracytoplasmic sperm injection IVF procedures were analyzed for hCG. Institutional Review Board approval was not required for the present study because it does not directly involve any patients or their surgical waste, and the study data were coded and managed in a way that excluded the identification of patients.

Spent Embryo Culture Media Collection

Following intracytoplasmic sperm injection, each oocyte was placed into individual droplets of 50 μL of culture medium and incubated. They were examined for fertilization 16 hours following intracytoplasmic sperm injection. Those having 2 pronuclei were maintained in the same culture media for another 30 to 31 hours. The embryos were then transferred to a different culture medium for further embryo development. The spent embryo culture media, in which the embryos were incubated for approximately 48 hours (i.e., until the embryos were transferred), were kept frozen individually in labeled microcentrifuge vials at −20°C until analysis. A sample of pure culture media incubated under the same conditions but without an embryo was also kept frozen and used as a blank control.

SECRETION OF hCG BY DAY 2 SPENT EMBRYO CULTURE MEDIA

To detect hCG in spent embryo culture media at day 2 after intracytoplasmic sperm injection and to assess the relationship of hCG to embryo development.

Intervention(s): The culture media samples were tested for hCG by ELISA.

Main Outcome Measure(s): Quantity of hCG produced by embryos and correlation with the embryos’ developmental status.

Result(s): hCG was found in 93 of 102 culture media tested by enzyme-linked immunosorbent assay. The correlation analysis revealed that the concentration of hCG was independent of embryo developmental status.

Conclusion(s): The ability to detect hCG from day 2 spent culture media may be used as a marker for embryo competence. (Fertil Steril 2011;96:615–7. ©2011 by American Society for Reproductive Medicine.)

Key Words: hCG, embryo development, ELISA, spent culture media

hCG Determination by ELISA

A quantitative sandwich ELISA was developed in-house and was performed following the optimization on spent embryo culture media. Goat polyclonal antibody to hCG (Abcam) was diluted in coating buffer (0.1 M bicarbonate buffer [pH 9.2]) (Sigma), and polystyrene microtiter plates (Greiner Biotech) were sensitized overnight at 4°C followed by blocking with Tris-buffered saline blocking buffer (Thermo Fisher Scientific) for 1 hour at room temperature. The plates were washed 3 times with phosphate-buffered saline containing 0.05% Tween 20 (Sigma) for 1 hour at room temperature. Synthetic hCG protein standards (Abcam) prepared in appropriate prediluted culture media to avoid matrix difference and samples diluted in phosphate-buffered saline containing 0.05% Tween 20 were pipetted onto the wells and incubated for 1 hour at room temperature. A pure embryo culture medium blank was included in all assays performed. After any unbound compounds were washed away, a mouse monoclonal antibody to hCG-β (Abcam) was added to the wells and incubated for another 1 hour at room temperature. Following a wash, goat anti-mouse IgG conjugated to horseradish peroxidase (Leinco Technologies Inc.) was added to the wells and incubated for 30 minutes at 37°C. SureBlue reserve TMB microwell substrate solution (KPL, Inc.) was added to the wells after the plate was washed with phosphate-buffered saline containing 0.05% Tween 20 and the reaction stopped with 1 M phosphoric acid stop buffer. The optical density of the color developed was measured using ultra microplate reader EL808 (Molecular Devices, Inc.) at 450 nm. The optical density values from the ELISA reader were directly transferred to Microsoft Excel, and the concentrations of the samples were determined.
Reliability of hCG ELISA Procedure

Pure embryo culture media spiked with known concentrations of hCG were used to determine the recovery rate of hCG and the reliability of the ELISA procedure. Recovery of hCG was calculated as the concentration determined divided by the expected concentration and expressed as a percentage. To determine interassay and intra-assay variations, hCG spiked culture media were assayed in triplicate on 3 different plates and repeated on at least 3 different days.

Embryo Development Monitoring

The embryo culture media were collected on day 2, and embryo quality was monitored on days 3 and 5 based on cleavage rate and morphological features, as previously described (10–13). One or more blastocyst-based morphological characteristics were transferred from the cohort of embryos to all 11 women.

Statistical Analysis

Pearson correlation and the significance between hCG concentration and embryo development were calculated. *P* < .05 was considered statistically significant. To calculate the 99% cut-off value for hCG concentrations from the day 2 spent embryo culture media, the results were log transformed for analysis. HCG results are expressed as means ± SD (range).

RESULTS

The reliability of the in-house ELISA was evaluated following optimization, and the recovery of hCG was found to be more than 90%. The coefficient of variation for the intra-assay and interassay was 5.4% and 6.5%, respectively. Of the 102 spent embryo culture media, 9 had no detectable amounts of hCG. However, 7 of the 9 embryos developed into blastocysts. It therefore appears that the failure to detect hCG may be due to technical error rather than embryo quality because we could not rerun these samples and determine the concentrations or find out the cause because of the low sample volumes available for testing. Further, these samples might have had hCG values near or below the lowest detection limit of the assay. Overall, a possibility of more than 1 factor involved in the failed detection of hCG cannot be ruled out. Incidentally, none of these 9 embryos were transferred and hence were excluded from further analysis.

The means ± SD (range) of hCG results per 50 μL for the remaining 93 spent embryo culture media was 16.3 ± 3.6 pg (1.4–225 pg).

DISCUSSION

The grading systems based on stage of embryo development to identify embryos with high implantation potential have led to significant improvements in pregnancy rates but have not reduced the number of embryos transferred to either minimize or eliminate twins or higher-order multiple gestations. This has led many investigators to pursue other techniques to assess embryo competence.

It has been reported that embryos that result in pregnancy are different in their metabolomic profile compared with embryos that do not (14, 15). Several metabolic parameters using a number of noninvasive techniques on the developing embryos have been measured (14–16). Similarly, other investigators in the past have attempted to evaluate the unique embryo-secreted molecules, such as soluble human leukocyte antigen G from spent embryo culture media, with limited success (17–21).

Although each of these markers has demonstrated some usefulness in selecting embryos for transfer, controversy still exists regarding their efficacy. This is probably because not all embryos fail to implant for the same reason. When a single test gives normal results, this does not mean that other defects are not present that may compromise the quality of the embryo. The embryo is complex and becomes compromised when any one of a number of biochemical or morphological entities are disturbed. Normalcy of a single entity or even several entities does not guarantee that the other entities are normal. Unless a large battery of tests are applied and results of each test are graded for their significance in regard to the quality

| hCG concentration in day 2 spent embryo culture media from embryos that were transferred following ICSI IVF procedure and pregnancy outcome among 11 women studied. |
|---|---|---|---|---|---|---|---|---|---|
| hCG produced by embryos that were transferred (pg/50 μL) | P | P | P | P | P | P | P | NP | NP | NP | NP |
| >50.0 |  |  |  |  |  |  |  |  |  |  |  |
| 25.1–50.0 |  |  |  |  |  |  |  |  |  |  |  |
| 10.1–25.0 |  |  |  |  |  |  |  |  |  |  |  |
| 5.5–10.0 |  |  |  |  |  |  |  |  |  |  |  |
| 2.6–5.4 |  |  |  |  |  |  |  |  |  |  |  |
| 0–2.5 |  |  |  |  |  |  |  |  |  |  |  |

Note: P = pregnant (n = 7); NP = nonpregnant (n = 4). The bold line represents the 99th percentile (5.5 pg/50 μL; 110 pg/mL; 1 mIU/mL) of accepted levels of hCG.

Ramu. hCG in day 2 spent embryo culture media. Fertil Steril 2011.
of embryo, it will not be possible to state, with any degree of certainty, the reproductive potential of the embryo. Therefore, multiple markers are necessary to identify the one embryo most probable to implant and give rise to a healthy live birth.

Although the hCG molecule was the first marker produced by an embryo identified in the spent embryo culture media (3), no further attempts to establish its use as a potential marker of embryo competence has been reported to our knowledge. Its specific function at this stage has been reported to our knowledge. Its specific function at this stage of embryonic development is currently not known (3, 22, 23).

However, recent studies have suggested that the hCG molecule may be involved in the growth and development of the embryo at the early stages and implantation at the later stages of development (23). Therefore, we tested day 2 spent embryo culture media collected from 102 embryos and subjected them to ELISA for the quantification of hCG. Our study attempted to establish another marker to be incorporated in the whole gamete of potential markers to assess embryo competence.

A poor correlation between hCG levels and embryo development observed in the present study therefore makes the hCG levels more desirable because it indicated that a different and new entity of the embryo is being measured. A high accepted level of hCG (99th percentile) was selected to ensure that the most competent embryo would be identified. This stringent selection resulted in identifying 22 embryos as poor. Of these 22 embryos, 11 (50.0%) developed to the blastocyst stage. Sixteen of 71 (22.5%) embryos identified as having an acceptable amount of hCG also developed into blastocysts. This may suggest that the embryo’s morphological development is independent of the physiologic development, confirming previously reported findings (4, 6). Also, lack of significant correlation observed between the level of hCG and embryo development on day 2, 3, or 5 (r² = .001 each; P > .05) in the present study support the above conclusion.

Only 9 (8.8%) of the 102 embryos did not yield any hCG results in their spent culture media. However, 7 of the 9 did develop into blastocysts. This may suggest that the failure of hCG detection was due to a technical error. But the possibility that the embryos either did not produce any hCG or produced hCG below the assay detection level cannot be ruled out because hCG production is independent of the morphological development of the embryo.

Because more than 1 embryo from a cohort of embryos from the same women was transferred based on the morphological development of the embryo, one could not associate the concentration of hCG with that of the IVF outcome. However, only 1 embryo from a cohort of embryos transferred that resulted in pregnancy had hCG levels lower than 5.5 pg/50 μL (110 pg/mL; 1 μU/mL) compared with 44.4% of the embryos that did not yield pregnancy (Table 1). A more detailed prospective study needs to be designed in which many other molecular markers are identified and analyzed simultaneously in the spent embryo culture media to determine the one embryo most probable to implant and give rise to a healthy live birth.