Analysis of the Human Zona Pellucida During Culture: Correlation with Diagnosis and the Preovulatory Hormonal Environment

J. RICARDO LORET DE MOLA,[1,2] WILLIAM T. GARSIDE,[1] JENNIFER BUCCI,[1] RICHARD W. TURECK,[1] and SUSAN HEYNER[1]

Submitted: December 17, 1996
Accepted: March 5, 1997

Purpose: *The objective of this study was to analyze sequentially the human zona pellucida changes in an in vitro fertilization program as it relates to several variables.*
Methods: *The zona pellucida thickness was measured daily in zygotes and cleavage-stage embryos on a Nikon inverted microscope equipped with Hoffman modulation contrast optics, using an ocular micrometer. A total of 512 embryos from 96 patients was evaluated.*
Results: *There was a highly significant direct correlation between zona thickness and preovulatory estradiol and basal day 3 FSH levels ($P < 0.02$ and $P < 0.0006$, respectively). This relationship showed a rapid reversal following 48 hr of culture; embryos from patients with the highest FSH levels had thinner zonae prior to transfer ($P < 0.0007$). The zonae from patients with unexplained infertility were thicker (19.4 ± 2.7 μm) than those from patients with endometriosis (17.7 ± 2.2 μm), tubal (17.5 ± 2.4 μm), or male-factor infertility (16.4 ± 2.7 μm) ($P < 0.0001$) on the first day of culture.*
Conclusions: *We hypothesize that the thickness of the human zona pellucida is influenced by the preovulatory hormonal environment and diagnosis. These factors should be considered as part of the embryo quality evaluation prior to transfer or when assessing the possibility of using assisted hatching. More studies are needed to understand the factors regulating the thickness of the human zona pellucida.*

KEY WORDS: human; zona pellucida; assisted hatching; follicle stimulating hormone; estradiol; age; infertility.

[1] Department of Obstetrics and Gynecology, Center for Research on Women's Health and Reproduction, University of Pennsylvania, Philadelphia, Pennsylvania 19104
[2] To whom correspondence should be addressed at Case Western Reserve University, Department of Reproductive Biology, 11100 Euclid Avenue, 7th Floor, Cleveland, Ohio 44106.

INTRODUCTION

The zona pellucida is an acellular structure critical for maintaining the three-dimensional integrity of the embryo. It protects against microorganisms, viruses, and immune cells that can access the embryo through a gap in the zona. The zona is vital to the embryo, allowing free passage through the oviduct, prevent early aggregation and implantation on the tubal wall (1). In precompacted mouse embryos, a gap in the zona alters hatching (2,3) and impairs the developmental potential after transfer (4). There is considerable experimental evidence that certain zona changes provide a barrier to hatching and implantation. It is not clear whether zona hardening or excessive thickness is due to the harsh culture conditions encountered during in vitro fertilization (IVF) or to factors not previously identified (5). There are few reports providing a detained analysis of zonae changes in culture. Further, there is virtually no information regarding other external and endocrine factors that could affect zona thickness. Our objective was to correlate patient's hormonal status and infertility diagnosis with sequential changes in the thickness of the zona pellucida following oocyte retrieval and in vitro culture.

MATERIALS AND METHODS

Patients

The zona thickness was measured in 512 embryos from 96 patients undergoing IVF. The mean age in the study group was 33.8 ± 4.2 years. Of this group of patients, 35 had tubal infertility, 10 had male infertility, 36 had endometriosis, and 15 had unexplained infertility. A total of 20 pregnancies was achieved among

1058-0468/97/0700-0332$12.50/0

these patients (20.8%). All patients had basal day 3 follicle-stimulating hormone (FSH) levels evaluated during their work-up, consisting of a serum level on the third day after menses in the natural cycle preceding the assisted reproduction cycle. Elevated levels were defined as >20 pg/ml. Two stimulation protocols were utilized in these patients. In the "flare-up" protocol leuprolide acetate stimulation was started on day three of the menstrual cycle, followed by human menotropins (CD3)(n = 13). The second protocol was luteal-phase down regulation with leuprolide acetate followed by human menotropins (LDL)(n = 83). Human chorionic gonadotropin (hCG) was administered after at least two follicles reached an average diameter of 16 mm, concomitant with a serum estradiol (E_2) level of at least 150 pg/ml per leading follicle. Informed consent was obtained from all patients participating in our program.

Design

We undertook a prospective study of the zona's thickness from day 1 through day 3 of *in vitro* culture. Day 1 was defined as 24 hr after transvaginal oocyte retrieval, and embryos were routinely transferred 72 hr after retrieval (Day 3). The thickness of the zona pellucida surrounding oocytes was not evaluated at the time of retrieval due to the difficulty in obtaining an accurate zona measurement through the cumulus cell mass. Stripping the cumulus could be detrimental to pregnancy rates and was not performed at that time. Zygotes and cleavage-stage embryos were evaluated as previously described (6). Daily measurements were performed at the same time of the day. These measurements were made with a Nikon inverted microscope equipped with Hoffman modulation contrast optics, using an ocular micrometer. The micrometer was calibrated in order to provide a direct value for the zona's thickness. Each zygote/embryo had four measurements made at two perpendicular equators, photographed, and "rolled over" to repeat the measurements and photograph. Three individuals made all the measurements form both the microscope and the photographs. The mean zona thickness for each stage was initially calculated from both the direct measurements and the prints (n = 100). Since there was an excellent correlation between methods, only the photographs were used for the remainder of the study (n = 412). Only the photographic measurements were used in data analysis.

Data Analysis and Statistics

The data were analyzed using a Macintosh Power PC 7100/66 personal computer, using StatView 4.01 (Abacus Software Concepts 1992, Berkeley, CA). The κ statistic would determine if the correlation between examiners was better than chance. The results are presented with the standard error of the mean. For evaluating the differences between groups with categorical data, multiple linear regression and one-way analysis of variance (ANOVA) were applied. When a significant F ratio was defined by ANOVA, the groups were compared using Fisher's PLSD post hoc test. For nominal data the Kruskal–Wallis test was applied; at $P < 0.05$ the differences were considered to be statistically significant.

RESULTS

There was an excellent intra- and interobserver correlation among the three examiners taking measurements (κ = 1.0, $P < 0.001$). We found that the average zona thickness was 18.2 ± 0.2 μm on day 1, 16.0 ± 0.2 μm on day 2, and 13.9 ± 0.16 μm on day 3; linear regression analysis revealed a highly significant correlation between the average zona thickness and the day of culture ($P < 0.0001$).

There was also a direct correlation between the zona thickness on day 1 and E_2 levels on the day of hCG administration ($P < 0.02$) (R^2 = 0.013) as shown in Fig. 1. A similar linear correlation was also noted on the first day of culture with cycle day 3 FSH levels, suggesting that the average zona pellucida is thicker

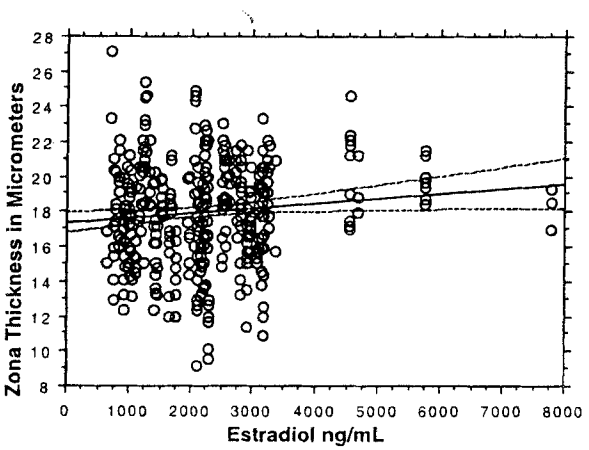

Fig. 1. The zona pellucida was thicker among embryos from patients who had the highest E_2 levels on the first day of culture. The lines indicate the standard error of the mean.

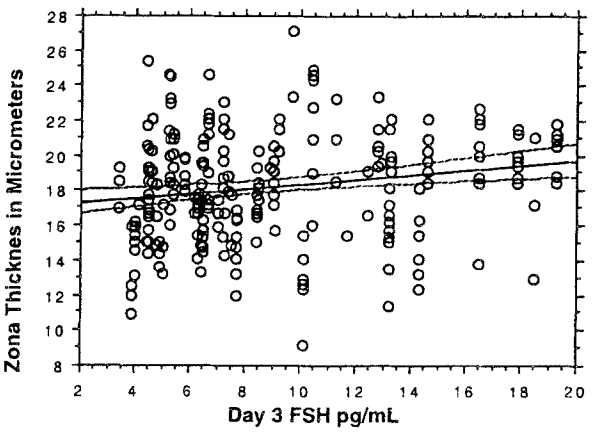

Fig. 2. Zona thickness on the first day of culture from patients who had the highest preovulatory day 3 FSH levels. The lines indicate the standard error of the mean.

among patients with the highest basal FSH values (R^2 = 0.039; $P < 0.0006$) as shown in Fig. 2. However, this relationship changed rapidly in culture; by day 2 the zygote zona thickness was similar across all FSH levels, and by day 3 this relationship was reversed ($P < 0.0007$; $R^2 = 0.058$) as shown in Fig. 3. There was also a reversal in the thickness of the zona with higher estradiol levels, but it did not reach statistical significance ($P = 0.14$). Comparing the effects of the stimulation protocols on the zona thickness as measured on day 1 of culture, the zona was similar for both groups. However, as the time in culture lengthened, it became apparent that embryos from the LPL protocol (mean, 15.2 ± 0.1 μm) had thinner zonae compared to the CD3 protocol (mean, 16.2 ± 0.5 μm) ($P < 0.05$).

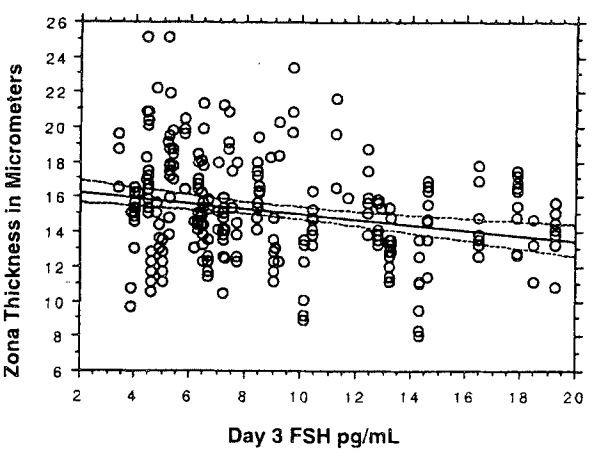

Fig. 3. The zona pellucida changes are reversed after 48 hr in culture for patients who had the highest preovulatory day 3 FSH levels. The lines indicate the standard error of the mean.

When the diagnosis for infertility was analyzed, the average zona thickness was significantly greater during the first day in culture among patients with unexplained infertility (19.4 ± 2.7 μm) as compared with patients diagnosed with endometriosis (17.7 ± 2.2 μm), tubal (17.5 ± 2.4 μm), or male-factor infertility (16.4 ± 2.7 μm) ($P < 0.0001$). That relationship persisted for the remainder of the culture period among all groups.

DISCUSSION

The zona pellucida is formed during oogenesis (7,8) and is largely responsible for determining the species specificity of gamete interactions during fertilization. Multiple functions have been ascribed to this acellular investment, including the maintenance of the embryo's cellular cohesiveness, as well as serving as a barrier to environmental and immune factors. However, our understanding of the mechanisms underlying normal zona changes is rather limited. Several theories have been postulated including that the zona is simply mechanically "stretched" as the embryo grows. The blastocyst and zona pellucida undergo expansion and thinning prior to hatching, including cycles of contraction and expansion (9). However, more recent data argue against mechanical factors being important in affecting zona changes (10).

The importance of the combined effects of hormone-dependent uterine, embryonic, and culture factors has recently been recognized in zona thinning and hatching (11). The presence of specific free amino acids in the culture medium are necessary for proper hatching, attachment, and outgrowth of mouse embryos (12). Studies have reported that 70 to 75% of embryos fail to hatch in culture (13,14), and suboptimal culture conditions can be responsible for the spontaneous "hardening" of the zona (15,16). The absence of serum, follicular fluid, or fetuin in the medium will induce zona hardening (15,17,18), and this is more significant with aging (19). Oophorectomy causes a 24-hr delay in zona hatching, implying that hormone-dependent uterine lytic factors facilitate this process (20,21). It has also been postulated that a lytic agent is actively secreted by mouse embryos, since zonae from unfertilized oocytes persist longer in the uterus than from normal zygotes. A trypsin-like proteinase, strypsin, is secreted by mouse embryos during culture and may participate directly in the zona thinning and hatching process (22). This proteinase has been specifically identified in the mural trophectoderm, suggesting that

in vitro hatching is initiated by limited proteolysis of the zona overlying the mural trophectoderm.

The present study addresses the relationship between the sequential changes in the zona and variables such as the preovulatory hormonal environment and diagnosis. Since cleavage-stage embryos with a good prognosis for pregnancy have a thinner zona (presumably in preparation for subsequent hatching) (23), we became interested in any factors that could affect or predict these changes. Since there are experimental data suggesting that zona thinning and subsequent hatching are affected by hormone-dependent factors (11,20,21), we considered the possibility that such changes were initiated or induced during follicular development. Exogenous gonadotropins are known to induce changes in the appearance and constitution of the zona pellucida. It has been recognized that the zona pellucida can undergo changes after stimulation with pregnant mare's serum, as evidenced by a reduction in PAS-positive material present within the zona pellucida (24). After the addition of hCG, the PAS-positive material within the zona is depleted further, suggesting that changes in the protein–carbohydrate composition of the zona are gonadotropin and/or hCG dependent. Our findings suggest that the preovulatory hormonal environment has a profound effect on the thickness of the human zona pellucida, in agreement with those observations. The data analysis provides evidence that an elevated day 3 FSH is an independent variable affecting the zona thickness, even after controlling for age. Our previous report also emphasized that patients over age 35 had thinner zonae compared to younger age groups (23). A recent study by Bertrand *et al.* (11) suggests that estradiol levels can affect the thickness of the zona pellucida. In contrast to our study, they found that the zona is thinner among patients with the highest estradiol values. However, their study did not address the zonae changes throughout the entire culture period, but only after fertilization was assessed. Our study did find that the zona is affected by estradiol levels, but the thinning occurs later during culture (days 2 and 3) than in the Bertrand *et al.* paper; this is due to the rapid reversal in the zona thickness after culture. Despite these minor discrepancies, both papers seem to be in agreement that estradiol and/or gonadotropins affect the human zona.

Our study supports the concept that changes in the thickness of the human zona can be correlated with the preovulatory hormonal environment and the diagnosis for infertility. The effects of the hyperstimulation protocol were not surprising, since patients using the LDL protocol were younger and had higher E_2 levels at the time of retrieval. The finding that patients with unexplained infertility had a thicker zonae than other groups emphasizes a potentially unrecognized etiologic factor for infertility among these patients. These observations may provide some insight into the expected zona thickness during culture and how it is affected after various preovulatory hormonal states and infertility conditions. Criteria for selective assisted hatching should take these variables into account, particularly when evaluating the desirability of assisted hatching on embryos with a thick zona from patients with elevated day 3 FSH levels (25). Patients with previous cycles where there was a lack of fertilization may have oocytes with an unusually thick zona pellucida, and ICSI will overcome some of those difficulties (11,26). Studies designed to evaluate the role of assisted hatching in patients with unexplained infertility will also be of interest. Future studies will be aimed at explaining some of the mechanisms involved in the thinning of the human zona pellucida, in order to assess the current zona thickness criteria used for assisted hatching.

ACKNOWLEDGMENTS

We would like to extend our thanks to Janine Claycomb and Barbara Kuczinski-Brown for their technical assistance and help during this project.

REFERENCES

1. Edwards RG: Cleavage of one and two-cell rabbit eggs in vitro after removal of the zona pellucida. J Reprod Fertil 1964;7:413–415
2. Talansky BE, Gordon JW: Cleavage characteristics of mouse embryos inseminated and cultures after zona pellucida drilling. Gamete Res 1989;4:180–187
3. Malter HE, Cohen J: Partial zona dissection of the human oocyte: A nontraumatic method using micromanipulation to assist zona pellucida penetration. Fertil Steril 1989;51:139–148
4. Nichols J, Gardner RL: Effect of damage to the zona pellucida on development of preimplantation embryos in the mouse. Hum Reprod 1989;4:180–187
5. Tucker MJ, Luecke NM, Wiker SR, Wright G: Chemical removal of the outside of the zona pellucida of day 3 embryos has no impact on implantation rates. J Assist Reprod Genet 1993;10:187–91
6. Veeck LL: Atlas of the Human Oocyte and Early Conceptus, Vol 2. Baltimore, MD, Williams & Wilkins, 1991
7. Edwards RG: Conception in the Human Female. London, Academic Press, 1980
8. Wasserman PM: The zona pellucida: A coat of many colors. Bioassays 1987;6:161–166

9. Cole RJ: Cinemicrographic observation on the trophoblast and zona pellucida of the mouse blastocyst. J Embryol Exp Morphol 1967;17:481–490
10. Gordon JW, Dapunt U: A new mouse model for embryos with a hatching deficiency and its use to elucidate the mechanism of blastocyst hatching. Fertil Steril 1993;59:1296–1301
11. Bertrand E, Van den Beergh M, Englert Y: Clinical parameters influencing human zona pellucida thickness. Fertil Steril 1996;66:408–411
12. Spindle Al, Pedersen RA: Hatching attachment and outgrowth of mouse blastocysts in vitro: Fixed nitrogen requirements. J Exp Zool 1973;186:305–318
13. Fehilly CB, Cohen J, Simons RF, Fishel SB, Edwards RG: Cryopreservation of cleaving embryos and expanded blastocysts in the human: A comparative study, Fertil Steril 1985;44:638–644
14. Dokras Am Sargent IL, Ross C, Gardner RL, Barlow DH: The human blastocyst: Its morphology and hCG secretion in vitro. Hum Reprod 1991;6:1143–1151
15. DeFelice M, Salustri A, Siracusa G: "Spontaneous" hardening of the zona pellucida of mouse oocyte during in vitro culture. II. The effect of follicular fluid and glycosaminoglycans. Gamete Res 1982;12:227–235
16. DeFelice M, Siracusa G: "Spontaneous" hardening of the zona pellucida of mouse oocytes during in vitro culture. Gamete Res 1982;6:107–113
17. Downs SM, Schroeder AC, Eppig JJ: Serum maintains the fertilizability of mouse oocytes matured in vitro by preventing hardening of the zona pellucida. Gamete Res 1986;15:115–122
18. Schroeder AC, Schultz RM, Kopf GS, Taylor FR, Becker RB, Eppig JJ: Fetuin inhibits zona pellucida hardening and conversion of ZP to $ZP2_f$ during spontaneous mouse oocyte maturation in vitro in the absence of serum. Biol Reprod 1990;43:891–897
19. Longo FJ: Changes in the zonae pellucidae and plasmalemmae of aging mouse eggs. Biol Reprod 1981;25:399–411
20. McLaren A: The fate of the zona pellucida in mice. J Embryol Exp Morphol 1970;23:1–19
21. Mintz B: Control of embryo implantation and survival. Adv Biosci 1971;6:317–334
22. Perona RM, Wassarman PM: Mouse blastocysts hatch in vitro by using a trypsin-like proteinase associated with cells of mural trophectoderm. Dev Biol 1986;114:42–52
23. Garside WT, Loret de Mola JR, Bucci J, Tureck RW, Heyner S: Sequential analysis of zona thickness during in vitro culture of human zygotes: Correlation with embryo quality, age and implantation. Mol Reprod Dev 1997;47:99–104
24. Fowler RE, Grainge C: A histochemical study of the changes occurring in the protein-carbohydrate composition of the cumulus-oocyte complex and zona pellucida in immature mice in response to gonadotrophin stimulation. Histochem J 1985;17:1235–1249
25. Cohen J: Assisted hatching of human embryos. J Assist Reprod Genet 1993;8:179–190
26. Bertrand E, Van den Beergh M, Englert Y: Does zona pellucida thickness influence the fertilization rate? Hum Reprod 1995;10:1189–1193