

RELATIONSHIP OF SUBNORMAL SEMEN PARAMETERS AND SUBSEQUENT ZONA PELLUCIDA THINNING FOLLOWING IN VITRO FERTILIZATION

J. H. CHECK
D. LURIE
J. LOCUNIAK
A. BOLLENDORF
D. SUMMERS-CHASE

The University of Medicine and Dentistry of New Jersey,
Robert Wood Johnson Medical School at Camden, Cooper
Hospital/University Medical Center, Department of Obstetrics
and Gynecology, Division of Reproductive Endocrinology
& Infertility, Camden, New Jersey, USA

Infertility due to male factor has been associated with reduced implantation rates despite normal fertilization. The mechanism responsible for lower pregnancy rates is not known. One cause of failure to achieve pregnancy despite transfer of embryos is impairment of zona pellucida (ZP) thinning, which inhibits the embryo from hatching despite initial cell cleavage. This study was designed to evaluate whether there is an association between the ability of the ZP to thin, as measured by ZP thickness on day of transfer, and subnormal semen parameters. Significant differences in ZP thickness ($p < 0.05$, Kruskal-Wallis) were noted according to median concentration of sperm, total motile sperm, and motile density in that these values were the highest in the group of embryos with the thinnest zona pellucida and lowest in the group with the thickest zona pellucida. Thus, some factor may be missing from sperm from subnormal specimens that normally assists in zona thinning.

Keywords in vitro fertilization, semen analysis, zona thinning

Data have been published demonstrating that an occult male factor may be present even when fertilization occurs. This could cause a detrimental effect on embryos with normal morphology and adequate cleavage, resulting in a low pregnancy rate (PR) following transfer. It may also be related to implantation defects. This discrepancy has been found in sperm that failed the hypoosmotic swelling (HOS) test [4] and the stress test [2]. The mechanism responsible for the poor PRs is not known. The zona pellucida glycoproteins may be affected by abnormal sperm and disrupt the fertilization/implantation process by interfering with zona pellucida thinning [9].

Address correspondence to Jerome H. Check, MD, PhD, 7447 Old York Road, Melrose Park, PA 19027, USA.

It was hypothesized that specimens with abnormal semen parameters might have a detrimental effect on the resulting embryo that impairs the ability of the zona pellucida to thin, thus yielding embryos with thicker zona pelluciditas on the day of transfer. This study was designed to investigate the relationship between zona thickness and semen parameters. The in vitro fertilization (IVF) parameters of stimulation protocol, indication for IVF cycle, and age of the female were also compared.

MATERIALS AND METHODS

Patient Data

This study is a retrospective analysis of 1479 embryos from 429 standard IVF cycles. Embryos resulting from intracytoplasmic sperm injection were excluded since there were no sperm surrounding the oocyte which might have an effect on the zona pellucida.

Semen Processing

The semen was processed by a discontinuous Percoll gradient of three 1-mL layers of 90, 60, and 45% (Sigma, St. Louis, MO). Percoll was made isotonic using 10× human tubal fluid (HTF) containing 5 mg/mL human serum albumin (Irvine Scientific, Santa Ana, CA). The pellet was washed twice with HTF, then resuspended in culture medium. An assessment was made of the volume, count, and motility. Count and motility were analyzed manually using the MicroCell counting chamber (Conception Technologies). Motile density (MD) was calculated by multiplying count/mL × % motility, and total motile sperm by volume × count/mL × % motility. Morphology was performed according to the Tygerberg strict criteria. Semen samples containing <4% normal forms were considered abnormal [7]. The HOS test was performed as previously described by Jeyendran et al. [6]. A result was considered subnormal when <50% of the sperm demonstrated swelling.

In Vitro Fertilization

Following collection, the oocytes were incubated at 37°C, humidified in organ culture dishes (Falcon) containing human tubal fluid (HTF) (Irvine Scientific), and covered with warmed mineral oil (Squibb, Princeton, NJ). Insemination of the oocytes was performed at least 4 h after collection. The insemination concentration was dependent on the strict morphology result from the male partner's initial analysis [7]. If the strict morphology was ≥10%, then each oocyte was inseminated with 50,000 motile sperm. However, if the strict morphology was <10% the insemination concentration was adjusted so that 10,000 motile sperm with normal morphology was added to each oocyte.

Assessment of fertilization by documentation of pronuclei took place 16–21 h following insemination. Two pronuclear (2PN) embryos were transferred to fresh culture dishes and examined at both 48- and 72-h intervals postretrieval. The embryos were observed for cell stages and grades using a Nikon Diaphot inverted microscope. Transfers were then performed 72 h postretrieval.

Measurements of the Zona Pellucida

The microscope, equipped with a camera, was connected to a monitor with an IMAGEN Omnex Imaging system. Measurements were taken along 4 points of the zona pellucida for

each embryo. The measurements of these 4 points were averaged and the mean thickness/embryo was determined.

Statistical Analysis

Embryos were classified into 3 groups based on their mean zona measurement: thin (<1 SD below mean), average (within 1 SD of the mean), or thick (>1 SD above mean). The relationships of semen parameters, age, indication for IVF, and stimulation protocol were compared on the day of embryo transfer using Kruskal-Wallis analysis of variance and chi-square analysis. A *p* value of 0.05 was used. Since assisted hatching was performed when the zona pellucida were thick, PRs were not evaluated.

RESULTS

The zona pellucida thickness of the embryos on the day of transfer ranged from 6.1 to 29.7 μm with a mean \pm SD of $17.3 \pm 3.1 \mu\text{m}$ and a median of 17.2 μm . The zona pellucida thickness was normally distributed. A total of 206 embryos were classified as thin, with zona pellucida thickness less than 14.3 μm ; 1054 embryos were classified as average, with thickness between 14.3 and 20.4 μm ; and 219 embryos classified as thick, with zona pellucida of greater than 20.4 μm .

A comparison of zona pellucida, by patient's age, showed that the distribution of zona pellucida thickness was the same for patients ≤ 35 years of age as for those older than 35 years (Figure 1). Therefore, all embryos were combined for further analysis. The stimulation proto-

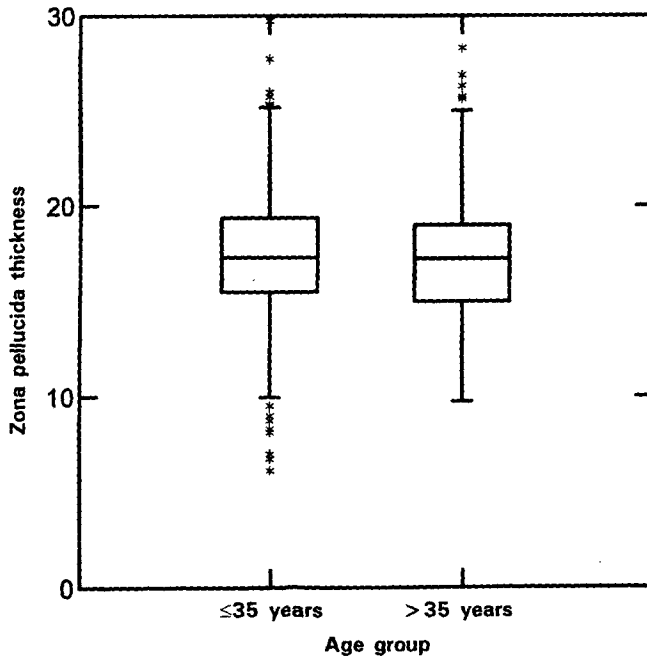


Figure 1. Comparison of zona pellucida thickness by patient's age.

Table 1. Comparison of semen parameters by zona pellucida thickness

	Zona pellucida thickness		
	(<14.3 μm) (<i>n</i> = 206 embryos)	(14.3–20.4 μm) (<i>n</i> = 1054 embryos)	(>20.4 μm) (<i>n</i> = 219 embryos)
Concentration (million/mL)*	48 (5–278)	44 (2–484)	38 (2–172)
Motility (%)	61 (18–88)	60 (10–90)	60 (11–90)
Motile density (million/mL)*	38 (1–153)	28 (1–250)	27 (1–223)
Total motile (million)*	61 (1–384)	53 (1–384)	44 (1–187)
HOS (%)	73 (35–91)	73 (21–96)	72 (34–90)
Strict morphology (%)	10 (2–35)	10 (2–35)	11 (1–24)

Note. Data are presented as median ranges. * $p < 0.05$, Kruskal–Wallis ANOVA.

col used, the number of oocytes retrieved, and the fertilization rate were not correlated with the thickness of the zona pellucida (Table 1). However, when the indication for IVF was analyzed, there were three times as many cases of male factor in the thick zona-pellucida group than in the thin zona pellucida group (8.2 vs. 2.9%).

A comparison of sperm parameters by zona pellucida thickness is seen in Tables 2 and 3. The median sperm concentration of the initial semen sample decreased inversely with zona pellucida thickness. The sperm concentration was lowest for embryos with thick zona pellucida and highest for embryos with thin zona pellucida ($38.2 \times 10^6/\text{mL}$ versus $47.5 \times 10^6/\text{mL}$) ($p < 0.05$, Kruskal–Wallis ANOVA).

Of the embryos with thick zona pellucida, 22.2% resulted from cycles where the concentration was $<20 \times 10^6/\text{mL}$ as compared to 15% of thin embryos ($p = 0.09$, chi-square).

Similarly, the median motile density decreased inversely with zona pellucida thickness. In the thin group, 14.6% had low motile density ($<10 \times 10^6/\text{mL}$) as compared to 21.7% of embryos in the thick group ($p = 0.11$, chi-square). The median total motile sperm was also inversely related to zona pellucida thickness. No association was found between zona pellucida thickness on day of transfer and HOS test scores or strict morphology.

Table 2. Comparison of proportion of embryos with low semen parameters by zona pellucida thickness

	Zona pellucida thickness		
	(<14.3 μm) (<i>n</i> = 206 embryos)	(14.3–20.4 μm) (<i>n</i> = 1054 embryos)	(>20.4 μm) (<i>n</i> = 219 embryos)
Concentration (<20 million)	31 (15%, 12%)	173 (17%, 69%)	48 (22%, 19%)
Motile density (<10 million/mL)	30 (15%, 12%)	173 (17%, 69%)	47 (22%, 19%)
HOS (<50%)	6 (4%, 8%)	58 (6%, 77%)	11 (5%, 15%)
Strict normal morphology (<4%)	5 (3%, 6%)	72 (7%, 79%)	14 (7%, 15%)

Note. Data are presented as frequency, column percentage (% of embryos in each thickness group) with low semen parameters, and row percentage (% embryos with low semen parameter in each thickness group). $p =$ not significant.

Table 3. Comparison of female and IVF parameters by zona pellucida thickness

	Zona pellucida thickness		
	(<14.3 μ m) (n = 206 embryos)	(14.3–20.4 μ m) (n = 1054 embryos)	(>20.4 μ m) (n = 219 embryos)
Age (years)	36 (26–51)	35 (24–51)	35 (26–51)
No. mature oocytes	10 (1–32)	9 (0–37)	10 (2–30)
Fertilization rate (%)	75 (20–100)	75 (0–100)	75 (21–100)
Indication for IVF			
Tubal	68 (33%)	317 (30%)	76 (35%)
Endometriosis	18 (9%)	142 (14%)	22 (10%)
Male	6 (3%)	56 (5%)	18 (8%)
Unexplained	21 (10%)	107 (10%)	23 (11%)
Ovulatory	7 (3%)	51 (5%)	5 (2%)
Recipient	23 (11%)	89 (8%)	16 (7%)
Other	6 (3%)	17 (2%)	2 (1%)
Multiple factors	57 (28%)	275 (26%)	57 (26%)
Stimulation			
CC/HMG	10 (5%)	59 (6%)	17 (8%)
Flare	76 (37%)	374 (36%)	78 (36%)
Long lupron	87 (42%)	471 (45%)	89 (41%)
Recipient	31 (15%)	142 (14%)	34 (16%)
Other	2 (1%)	8 (1%)	1 (0%)

Note. *p* = not significant.

No association was found between zona pellucida thickness and insemination protocol used. Approximately 54% of embryos in each thickness group were fertilized through the insemination of 50,000 motile sperm per oocyte and 46% of embryos were fertilized using the insemination concentration adjusted for strict morphology.

DISCUSSION

The zona pellucida is a glycoprotein structure that encases the developing preimplantation embryo and must be shed prior to implantation in the endometrial lining [8]. The zona pellucida of the human gradually thins over time after fertilization [3]. It has been theorized that lysins found in the female reproductive tract may thin the zona pellucida as the embryo travels down the fallopian tube, enabling it to hatch out and implant upon reaching the uterus.

With IVF the embryo does not have access to the lysins produced by the female reproductive tract and yet thinning has been observed [5]. Lysins may be produced by the embryo itself [10] or thinning may be a function of increasing pressure caused by the increasing number of blastomeres [3]. In some cases, zona thinning does not occur and may result in a hatching impairment [5]. Lack of zona thinning may be due to a lack of zona lysins on the part of the embryo or caused by the in vitro conditions [1].

The in vitro embryos may not be able to benefit from the lysins produced by the fallopian tubes to aid in the thinning of the zona. However, they are exposed to one other cell type after the oocyte retrieval: the sperm. It may be that sperm of good count and motility are superior

and aid in producing the agents necessary for the thinning of the zona pellucida once 2PN embryos are moved to the fresh dishes. The samples with the subnormal semen parameters may be devoid of whatever zona thinning factor is needed. Consequently, the male factor patients may have embryos with thicker zones.

The results demonstrate that sperm parameters may possibly have an effect on the zona pellucida of developing embryos. In cases where there are subnormal semen parameters, the zona pellucida may have a zona thinning impairment, which may lead to lower implantation and PRs. These data did not show any difference in the zona pellucida thickness by female's age, indicating that the impairment to the mechanism that allows the zona pellucida to thin may be a function of male as well as female factors. The hatching impairment caused by the thick zona can be overcome by assisted hatching, resulting in PRs that are similar to the groups with the average and thin zones [5].

The data presented here demonstrate that male factor patients may not have the adequate zona thinning factor that might be present in non-male factor samples. The result is that the embryos that are transferred have a thicker zona, which does not allow the embryo to hatch out easily, causing poor implantation.

REFERENCES

1. Alikani M, Cohen J (1992): Advances in clinical micromanipulation of gametes and embryos: assisted fertilization and hatching. *Arch Pathol Lab Med* 116:373-378.
2. Alvarez JG, Minaretzis D, Barrett CB, Mortola JF, Thompson IE (1996): The sperm stress test: a novel test that predicts pregnancy in assisted reproductive technologies. *Fertil Steril* 65:400-405.
3. Chan PJ (1987): Developmental potential of human oocytes according to zona pellucida thickness. *In Vitro Fertil Embryo Transfer* 4:237.
4. Check JH, Stumpo L, Lurie D, Benfer K, Callan C (1995): A comparative prospective study using matched samples to determine the influence of subnormal hypoosmotic test scores of spermatozoa on subsequent fertilization and pregnancy rates following in-vitro fertilization. *Hum Reprod* 10: 1197-1200.
5. Cohen J, Alikani M, Trowbridge J, Rosenwaks Z (1992): Implantation enhancement by selective assisted hatching using zona drilling of human embryos with poor prognosis. *Hum Reprod* 7:685-691.
6. Jeyendran RS, Van der Ven HH, Perez-Palaez M, Crabo BG, Zaneveld LJD (1984): Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. *J Reprod Fertil* 70:219-228.
7. Kruger TF, Acosta AA, Simmons KF, Swanson RJ, Matta JF, Oehninger S (1988): Predictive value of abnormal sperm morphology in in vitro fertilization. *Fertil Steril* 49:112-117.
8. Tucker M, Cohen J, Massey JB, Mayer MP, Wiker S, Wright G (1991): Partial zona dissection of frozen-thawed human embryos may enhance blastocyst hatching, implantation, and pregnancy rates. *Am J Obstet Gynecol* 165:341-345.
9. Wasserman P (1990): Regulation of mammalian fertilization by zona pellucida glycoproteins. *J Reprod Fertil* 42:79-87.
10. Wright G, Wiker S, Elsner C, Kort H, Massey J, Mitchell D, Toledo A, Cohen J (1990): Observations on the morphology of pronuclei and nucleoli in human zygotes and implications for cryopreservation. *Hum Reprod* 5:109-115.