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ARTICLE

The relationship between sperm head retardance using polarized light microscopy and clinical outcomes

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Abstract In human sperm head, birefringence can be seen under polarized light resulting from highly ordered structures within the acrosome and nucleus. Selecting sperm with partial head birefringence improves success of clinical pregnancies in patients with severe male factor infertility. The aim of this study was to establish a range of retardance in sperm heads using polarized light microscopy to select an optimum sperm for intracytoplasmic sperm injection (ICSI). Sperm heads of 63 couples undergoing ICSI in women aged 38 years or younger were imaged at the time of ICSI and later analysed for retardance blinded to embryo and cycle outcomes. Sperm head retardance was similar irrespective of whether fertilization occurred. Quality of embryos on day 3 and day 5 were higher when sperm were selected with head retardance ranging from 0.56 nm or greater to 0.91 nm or less. Selection of sperm with head retardance ranging from 0.56 nm or greater to 0.91 nm or less was associated with higher clinical pregnancy rates (OR 3.74 95% CI 1.43 to 9.77). Optimum sperm for selection at the time of ICSI was with head retardance within the range 0.56 nm or greater to 0.91 nm or less.

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KEYWORDS: ICSI, polarized light microscopy, pregnancy outcome, sperm head birefringence, sperm head retardance

Introduction

Polarized light enables structures with molecular order to be observed (birefringence) when a single ray of polarized light is refracted into two polarized rays travelling at different speeds. The difference between these phases is otherwise referred to as retardance (Gianaroli et al., 2010; Oldenbourg, 1996; Oldenbourg and Mei, 1995). Although polarized light microscopy (PLM) has been used to visualize biological structures, sperm has not been widely imaged. Improvements to

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resolution, control of annotation and its computer software, PLM now allows visualization of the condensed chromatin in sperm (Oldenbourg, 1996, 2010; Oldenbourg and Mei, 1995).

It has been shown with transmission electron microscopy that sperm heads exhibit birefringence based on highly ordered structures within the acrosome and nucleus (Baccetti, 2004). The head of sperm are birefringent owing to anisotropic structures within the nuclear and acrosomal regions. The birefringence within the sperm nucleus is created by molecular order within nucleoprotein filaments oriented longitudinally. Similarly, the acrosome has birefringence caused by protein filaments oriented longitudinally (Baccetti, 2004; Gianaroli et al., 2008). The use of PLM has enabled DNA damage to be positively correlated with increased sperm head retardance (Damasceno-Vieira et al., 2008).

A study to distinguish clinical outcomes dependent on using PLM on sperm before intracytoplasmic sperm injection (ICSI) found no significant differences between fertilization and cleavage rates when using PLM (Gianaroli et al., 2008). Good-quality embryos, however, were significantly higher on day 3 in the group that used PLM to assess the sperm for injection, as well as implantation and ongoing pregnancy rates. Selection of sperm with birefringence may indicate a normal sperm structure as well as DNA integrity (Crippa et al., 2009; Gianaroli et al., 2008; Magli et al., 2012; Peterson et al., 2011).

A further study has suggested that birefringence in a sperm head can be evaluated as total or partial. The difference between the two depends on the completion of the acrosome reaction (Gianaroli et al., 2008). Partial birefringence is characteristic of the completion of the acrosome reaction. The selection of sperm with partial birefringence increases the potential of selecting a sperm with DNA integrity, and increasing clinical pregnancies of patients with severe male factor infertility (Gianaroli et al., 2008; Magli et al., 2012). With the use of the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling assay, it has been shown that sperm with total head birefringence present with significantly higher proportions of DNA fragmentation compared with sperm with partial head birefringence (Peterson et al., 2011). These studies suggest that a possible optimum range of retardance exists that reflects partial birefringence and reduce DNA fragmentation of single sperm. No studies have specifically defined the relationship between birefringence of sperm head and protoplasmic structures.

To our knowledge, no published data have quantified sperm head retardance in humans. Therefore, the aim of this study was to determine a range of retardance in human sperm heads using polarized light microscopy (PLM) that might enable the selection of the optimum sperm for intracytoplasmic sperm injection (ICSI).

Materials and methods

Patients and study design

63 fresh cycles were included that took place between August 2010 and January 2013. During this time, methodologies for ICSI remained unchanged within the laboratory. Inclusion criteria were women 38 years or younger undergoing ICSI, who had between five and 15 mature oocytes collected, blastocyst culture and elective single embryo transfer on day 5. Patients were excluded if they had had over three previous fresh cycles with no success, used donor gametes, used cryopreserved gametes or blastomere biopsy.

Sperm that were assessed as having normal morphology according to the World Health Organization (WHO, 2010) were selected for ICSI. Before injection, each sperm head was imaged using PLM. Images were saved. Birefringence of individual sperm heads were later analysed blinded to embryo and cycle outcomes. Retardance of PLM was compared with fertilization rates 18 h after ICSI, cleavage rates and quality at 66 h after ICSI on day 3, blastulation and utilization rates (i.e. number of embryos either transferred fresh or frozen for later use) at 113–121 h after ICSI on day 5 and clinical pregnancy rates. Clinical pregnancy was defined as the presence of a gestational sac (with or without a fetal heart) as per ANZARD, The Australia and New Zealand Assisted Reproduction Database (Macaldowie et al., 2013). International Review Board approval for the study was obtained from the IVF Australia Human Research Ethics Committee (approved 23 August, 2010).

Assisted reproductive technology procedure

Ovarian stimulation was carried out using Puregon FSH (Merck Sharp and Dohme, South Granville, Australia) or Gonal F (Merck Serono, Frenchs Forest, Australia) at a dosage between 150 and 300 IU per day. Monitoring of stimulation was achieved with regular blood tests and ultrasounds. Ultrasound-guided oocyte collection under sedation was scheduled 36 h after trigger injection of choriogonadotropin alfa (Ovidrel 250 μg, Merck Serono, Frenchs Forest, Australia).

All day 0 culture dishes containing COOK cleavage medium and COOK culture oil (COOK Medical, 2013, Brisbane, Australia) were prepared the day before ultrasound-guided oocyte collection and equilibrated overnight in 6% carbon dioxide humidified gas atmosphere at 37.0°C.

Once oocytes were collected, they were taken to the laboratory where they were denuded of their cumulus and coronal cells. Oocytes were assessed for maturity. Metaphase I and Metaphase II oocytes were kept separate and transferred into the culture dish.

Timing of ICSI occurred 39–41 h after trigger injection to allow for optimum oocyte maturity and fertilization (Kilani et al., 2009) within a glass-bottomed tissue culture dish (Flurodish, World Precision Instruments, Florida, USA). Each mature oocyte (maximum 8) was placed into a 5 μL droplet of warmed COOK gamete buffer along with two droplets of 7% polyvinylpyrrolidone (PVP) (Sage-Cooper Surgical, Connecticut, USA), one droplet containing an aliquot of the prepared sperm. The droplets were then covered with warmed COOK culture oil. The ICSI dish was prepared and placed on 37°C warm plate for 20 min before gametes were transferred into and ICSI occured to ensure all media were pre-warmed.

Sperm preparation

Sperm was prepared by placing 2.0 ml of each patient's semen sample over a discontinuous gradient separated using 40% and 80% Puresperm (Nidacon, Molndal, Sweden). The

discontinuous gradient was centrifuged for 10 min at 300 g. The sperm pellet was then removed and placed into a clean test tube and washed with 2.0–3.0 ml of pre-warmed COOK gamete buffer. The sperm solution was then centrifuged for 5 min at 300 g. Once complete, the suspension was removed leaving 0.2–0.5 ml of the final sperm preparation in the tube. The temperature of the final preparation was maintained at 37°C until ICSI was complete.

Imaging and measuring sperm head retardance

Imaging was achieved using Oosight™ software (Cambridge Research Instruments, USA), using a 546 nm filter attached to an inverted Olympus IX-70 with Normaski DIC optics, visualized through a 55.9 cm monitor. Fresh sperm were selected for ICSI based on normal morphology and rapid progressive motility. The sperm were immobilized using a micropipette within the PVP droplet. The sperm were then moved into the PVP droplet not containing the aliquot of the prepared sperm sample. The sperm were positioned so that the bilaterally flattened side of the headpiece was lying flat against the Flurodish in a vertical or horizontal position. An image of head birefringence was taken at x40 magnification followed by ICSI. This process was repeated by selecting the next sperm to be immobilized, imaged then followed by ICSI. Once proficient, an embryologist undertook the immobilizing and imaging of a single sperm in less than 30 s. For eight oocytes, therefore, the whole process took 8–10 min.

Images were stored for later analysis. The embryologist was unaware of the result at the time of ICSI. Measurement of sperm head retardance was carried out at 200% magnification of the original image. The oval region tool on the selection panel (Oosight™) software was used for measuring the total head area. The region retardance, appearing on the measurement statistics tab, was used in this study.

Fertilization and embryo scoring

Fertilization was assessed 18 h after ICSI. Normal fertilization was indicated by two pronuclei (PN) and polar bodies. Abnormal fertilization included 1PN, 3PN and no evidence of fertilization. With the use of single-embryo culture, fertilized oocytes were graded on day 3 for cleavage. This study recognized normal embryo cleavage as having between six and 10 cells at 66 h after ICSI. These embryos were graded as: 'A' (≤20% fragmentation), or 'B' (>20% fragmentation). The percentage accounts for the total zona volume. Abnormal day 3 cleavage in this study referred to embryos less than six cells, A or B grade. COOK blastocyst media was used from the morning of day 3 after cleavage assessment was complete.

Blastocyst and utilization assessment occurred on day 5 using the Gardner's scale grading system (Sathananthan, 2010). The grading of an A, B, or C depicts the quality of the inner cell mass and the trophoblast, respectively.

Utilization referred to blastocysts of high quality whose stage was 3–6 (i.e. blastocyst to hatched blastocyst) with inner cell mass and trophectoderm grade with any combination of A or B. These embryos were either transferred into the patient or vitrified individually, for identification, within COOK vitrification media according to the manufacturer's protocol (http://www.cookmedical.com).

Statistical analysis

An independent samples *t*-test (equal or unequal) was used to measure quantitative variables. A receiver's operation curve (ROC) was used to evaluate the quality or performance of testing methods, as well as to identify the optimal threshold value. The ROC curve was achieved by plotting the true positive rate (sensitivity) against the false positive rate (specificity) for different cut off points. A testing method is considered poor if the ROC curve follows the line of equality, and more reliable the closer it becomes to the coordinates (0,1). The point on the curve closest to (0,1) is considered to be the cut-off value for the test. A chi-squared analysis or Fisher's exact test was conducted to test categorical variables.

Results were considered significant at $P < 0.05$. The Statistics Package for Social Sciences (SPSS version 19.0 for Mac, IBM Corp., USA) was used for all data analysis.

Results

A total of 63 patients (median 33.7 years; range 21.6–37.8) derived from 101 fresh and subsequent cryopreserved cycles ($n = 38$) were included in this study. A total of 486 sperm from partners (median 34.7 years; range 22.1–56.3) were used for ICSI and measured for head retardance.

The indications for ICSI were teratozoospermia (32%), oligozoospermia (29%), asthenozoospermia (18%), reduced fertilization rate from previous IVF (10%), presence of sperm antibodies (2%) and unexplained infertility (9%). Sixty-four per cent of patients were on their first fresh cycle, 23% on their second and 13% on their third.

Sperm head retardance

The mean ± SD head retardance for the 486 sperm measured was 0.92 ± 0.25 nm and the range was 0.40–2.38 nm (**Figure 1**). A high frequency of head retardance was observed between 0.75 nm and 0.91 nm. A total of 2.3% of sperm measured had a head retardance less than 0.56 nm.

Embryological development and sperm head retardance

Although no differences were found in sperm head retardance between normal or abnormal fertilization, good-quality embryos on day 3 were more likely to come from oocytes fertilized from sperm with lower head retardance ($P = 0.001$). When sperm head retardance was greater than 0.91 nm, embryo quality decreased ($P = 0.002$). A total of 169 out of 204 embryos (83%) were of high quality on day 3 when retardance was within the range 0.56 nm or greater to 0.91 nm or less compared with 107 out of 160 (67%) if the embryos incorporated retardance less than 0.56 nm or greater than

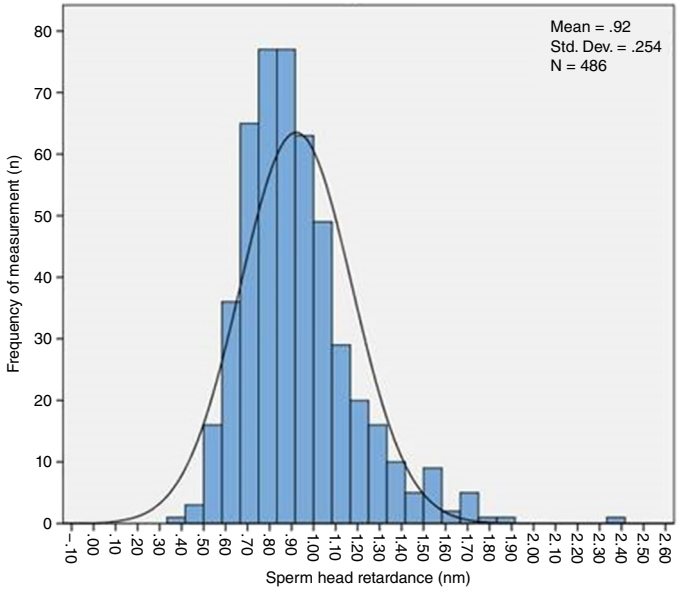

Figure 1 Sperm head retardance frequencies of the combined sperm sample ($n = 486$) from 63 patients. Mean ± SD sperm head retardance = 0.92 ± 0.25 nm and range = 0.40 nm to 2.38 nm.

0.91 nm. No differences were found in sperm head retardance comparing blastulation. A significant difference was found in utilization rates where embryos used (transferred, cryopreserved, or both) came from a sperm head retardance that was significantly lower than the discarded embryos (0.88 ± 0.2 nm versus 0.93 ± 0.27 nm; $P = 0.04$) (**Table 1**).

Clinical pregnancies and sperm head retardance

Median (range) female and male age of the observed 34 clinical pregnancies was 32.3 (23–37.5) years and 34.4 (28–56.3) years, respectively. Clinical pregnancies were formed from significantly lower sperm head retardance (0.81 ± 0.16 versus 0.91 ± 0.24; $P = 0.011$) (**Table 1**). Most of the pregnancies ($n = 27$; 79.4%), however, were from a sperm head of 0.91 nm or less retardance compared with ($n = 7$; 20.6%) head retardance greater than 0.91 nm (≤0.91 nm versus >0.91 nm; $P = 0.005$) (**Figure 2**).

Optimal sperm head retadance and clinical pregnancy rates

Of the 101 embryos transferred, 61 (60.4%) embryos had sperm head retardance within the range 0.56 nm or greater to

Table 1 Comparisons of sperm head retardance (mean ± SD) with embryological and clinical outcomes.

Outcome	*Sperm analysed (n = 486)*	*Sperm head retardance (nm)*		
		Mean ± SD	P	P ≤ *0.91 nm*[a]
Fertilization	364	0.91 ± 0.25	NS	
No Fertilization	122	0.95 ± 0.27		
Cleavage six to 10 cells A/B grade	276	0.89 ± 0.23	0.001	0.002
Cleavage less than six cells A/B grade	88	0.98 ± 0.28		
Blastulation	235	0.90 ± 0.24	NS	
No blastulation	129	0.93 ± 0.26		
Utilized	151	0.88 ± 0.20	0.040	
Discarded	213	0.93 ± 0.27		
Clinical pregnancy (fresh and cryopreserved embryo transfers[a]	34	0.81 ± 0.16	0.011	0.008
Fresh embryo transfers	20	0.83 ± 0.17	NS	
Cryopreserved embryo transfers	14	0.78 ± 0.16	0.009	
No clinical pregnancy	67	0.91 ± 0.24		

[a]Cut-off value resulted from receiver operator curve analysis.
NS = not statistically significant.

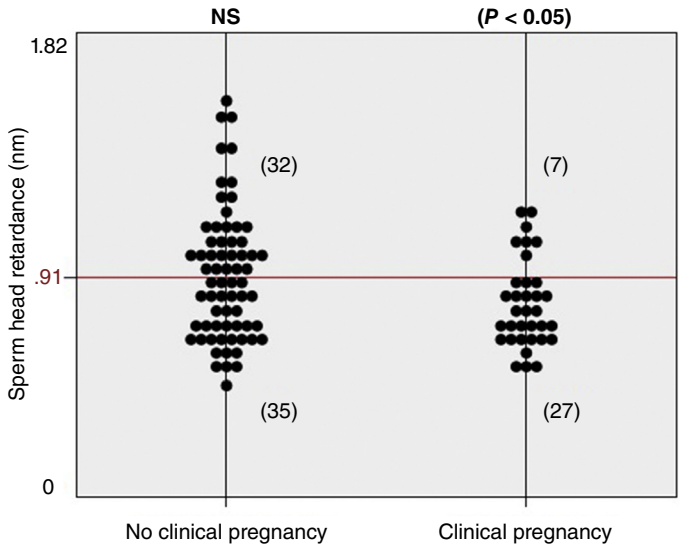

Figure 2 Clinical pregnancies plotted against sperm head retardance where 0.91 nm or less cut-off value resulting from receiver operator curve analysis (Table 1) was considered optimal. In the clinical pregnancy group, significantly more pregnancies resulted from sperm with head retardance 0.91 nm or less ($P < 0.05$). NS = not statistically significant.

0.91 nm or less, with a clinical pregnancy rate of 44.3%. The remaining 40 (39.6%) embryos had sperm head retardance within the range less than 0.56 nm to greater than 0.91 nm, with a clinical pregnancy rate of 17.5% ($P = 0.005$) (Table 2). When comparing the 63 fresh embryo transfers only, 41 (65.0%) of the embryos had sperm head retardance within the range 0.56 nm or greater to 0.91 nm or less, with a clinical pregnancy rate of 39.0%. The remaining 22 (35.0%) embryos had sperm head retardance within the range less than 0.56 nm to greater than 0.91 nm, with a clinical pregnancy rate of 18.2% ($P = 0.155$) (Table 2). When comparing 38 cryopreserved embryo transfers only, 20 (52.6%) embryos had sperm head retardance within the range 0.56 nm or greater to 0.91 nm or less, with a clinical pregnancy rate of 55.0%. The remaining 18 (47.4%) embryos had sperm head retardance within the range less than 0.56 nm to greater than 0.91 nm, with a clinical pregnancy rate of 16.7% ($P = 0.021$) (Table 2).

The odds ratio for a clinical pregnancy occurring when sperm head retardance was within the range 0.56 nm or greater to 0.91 nm or less was 3.74, with a 95% confidence interval of (1.43, 9.77; $P = 0.007$). The minimum sperm head retardance within an embryo that resulted in a clinical pregnancy was 0.56 nm. The results show that most clinical pregnancies resulted from sperm head retardance within the range 0.56 nm or greater to 0.91 nm or less (Table 2). The median (range) age of all the women with oocytes injected with sperm of optimal head retardance was 33.5 (21.6–37.9) years. This age was identical to the group of women with oocytes injected with non-optimal sperm head retardance 33.7 (21.6–37.9) years.

Discussion

As far as is known, this is the first time that the degree of sperm head retardance has been related to embryological and clinical outcomes. Previous studies have focused on the presence (total or partial) or absence of birefringence and

Table 2 Sperm selected for intracytoplasmic sperm injection within the optimal head retardance range 0.56 nm or greater to 0.91 nm or less as well as suboptimal ranges less than 0.56 nm and greater than 0.91 nm and embryological and clinical outcomes.

Outcome	*<0.56 nm*[a] n *(%)*	*≥0.56 nm to ≤0.91 nm*[b] n *(%)*	*>0.91 nm*[c] n *(%)*	*P*
Sperm selected at intracytoplasmic sperm injection	11/486 (2.3)	264/486 (54.3)	211/486 (43.4)	–
Normal fertilization (two pronuclei)	7/11 (63.6)	204/264 (77.3)	153/211 (72.5)	NS
Good-quality embryos on day 3	5/7 (71.4)	169/204 (82.8)	102/153 (66.7)	0.0005
Blastocyst formation	4/7 (57.1)	136/204 (66.7)	95/153 (62.1)	NS
Embryos utilized	1/7 (14.3)	90/204 (44.1)	60/153 (39.2)	NS
Clinical pregnancies fresh embryo transfers	0/1 (0.0)	16/41 (39.0)	4/21 (19.0)	NS
Clinical pregnancies cryopreserved embryo transfers	0/0 (0.0)	11/20 (55.0)	3/18 (16.7)	0.021
Cumulative clinical pregnancies	0/1 (0.0)	27/61 (44.3)	7/39 (17.9)	0.005

P-values: sperm head retardance range [b] comparing [a] and [c].
Data represent all patients ($n = 63$) and sperm ($n = 486$) analysed within the study.
NS = not statistically significant.

clinical outcomes. This study indicates that sperm with head retardance between 0.56 nm or greater to 0.91 nm or less produce better outcomes with ICSI.

Although fertilization rates were not related to sperm head retardance, the best quality embryos (i.e. six to 10 cells A and B) were more likely to have lower sperm retardance, with optimum retardance being 0.91 nm or less but 0.56 nm or greater ($P = 0.0005$). At blastulation, there was a trend but no significant difference. For the blastocysts that were either transferred or cryopreserved, however, sperm head retardance was lower than those that were discarded owing to poor quality ($P = 0.040$). Clinical pregnancies were more likely to occur if sperm retardance was low ($P = 0.011$), with optimum retardance being between 0.56 nm or greater to 0.91 nm or less ($P = 0.005$).

To explain these findings, lower sperm head retardance is likely to reflect completion of the acrosome reaction, and intact DNA macromolecular structure. It has been shown that DNA fragmentation is associated with high retardance (Damasceno-Vieira et al., 2008) and also little or no birefringence (Boudjema et al., 2009; Crippa et al., 2009), and, therefore, very low retardance. In our study, all sperm had some degree of birefringence, which may reflect methodological differences. It may also be that the sperm assessed were the best quality, as all were selected for injection based on normal morphology. A further potential bias is that the embryologist injecting the sperm also took the images for analysis. Although actual measurements of sperm retardance were not obtained until later, there is a chance the embryologist would have been aware of the intensity of birefringence. Until results were later analysed, however, the degree of birefringence was unknown at the time of the experiment. The lower and higher ranges of retardance that we obtained, namely 0.4–0.56 nm and greater than 1.19 nm were not associated with a clinical pregnancy. Small numbers (11/486 [2.3%]) of sperm were assessed as having head retardance less than 0.56 nm, and of these one sperm (9.1%) generated an embryo used for transfer that resulted in no clinical pregnancy. We therefore surmize that, despite normal morphology, these sperm may have had significant DNA fragmentation. The exclusion of sperm in these ranges, more so greater than 1.19 nm, may improve pregnancy rates in ICSI.

It was not surprising that fertilization rates were not related to sperm appearance or sperm head retardance, as it is well recognized that the influence of the sperm genome is not expressed until late cleavage stage (Tesarik, 2005; Tesarik et al., 2002, 2004). This lack of difference at fertilization is consistent with previous sperm selection studies using Motile Sperm Organelle Morphology Examination (MSOME) (Hazout et al., 2006). Use of MSOME allows high magnification visualization of structural abnormalities of the sperm that may appear normal under conventional ICSI magnification (×20–40) (Bartoov et al., 2002). The effect of the paternal genome commences on day 3 and so it would be expected that poor sperm quality would begin to affect embryo development. This would explain the statistically significant difference found in the rate of best quality embryos developing from sperm with lower head retardance in this study. This result was comparable with a previous study using MSOME (Hazout et al., 2006). That effect continues into blastulation, with the significantly higher rate of good quality blastocysts either transferred or cryopreserved being derived from sperm with lower head retardance ($P = 0.040$).

When comparing our clinical pregnancy data to similar studies testing sperm birefringence, our results were comparable (Gianaroli et al., 2008, 2010). These studies have indicated that clinical pregnancies increase if selected sperm have partial head birefringence owing to its association with a reacted acrosome as well as DNA integrity (Magli et al., 2012). The sperm with reacted acrosomes would have reduced retardance owing to their loss in molecular order within its acrosome. Sperm with lower head retardance resulted in higher clinical pregnancy rates ($P = 0.011$). Selection bias may potentially occur in clinical pregnancy rates because increasing female age has a significant negative effect on success rates. The age of women in the group containing sperm head retardance within the optimal range, however, was the same as women outside the range of optimal head retardance.

To the best of our knowledge, our results show for the first time that sperm head retardance between 0.56 nm and 0.91 nm is associated with optimal embryo quality, development and clinical pregnancy rates. If this observation is valid, only 54.3% of oocytes undergoing ICSI in this study used sperm within this range, compromising the potential of almost one-half of oocytes to develop into good-quality embryos or clinical pregnancies.

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