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Association between fertilin β, protamines 1 and 2 and spermatid-specific linker histone H1-like protein mRNA levels, fertilization ability of human spermatozoa, and quality of preimplantation embryos

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Abstract: Fertilization involves a series of cellular interactions culminating in the fusion of gamete membranes, creating a zygote and then an embryo. During the process of human fertilization *in vivo* or in conventional *in vitro* fertilization (IVF), sperm must be capable of undergoing the acrosome reaction, binding to the zona pellucida (ZP), and penetrating the ZP to fuse with the oolema. The key role in this process is played by fertilin β [1-5]. Protamines and histones are the proteins that bind to sperm chromatin and contribute in chromatin remodeling during early spermiogenesis. It has been suggested that these proteins may also participate in successful fertilization and embryo development [6]. Using reverse transcription and real-time quantitative PCR reaction (QR-PCR) methods and zygote and embryo scoring, we compared fertilin β, protamine 1 (PRM1), protamine 2 (PRM2), spermatid-specific linker histone 1 (HILS1) mRNAs levels, *in vitro* fertilization ability of mature spermatozoa, and quality of embryos obtained from *in vitro* fertilization (IVF) [7,8]. We found significantly lower contents of fertilin β transcript in spermatozoa from patients in which IVF fertilization failed ($p<0.001$). We also noticed a correlation between high levels of fertilin β and increased quality of embryos ($p<0.05$). We observed an increase in PRM1 and PRM2 mRNA levels in spermatozoa obtained from patients with successful *in vitro* fertilization versus compared to the number of these transcripts isolated from spermatozoa of patients in which *in vitro* fertilization failed ($P<0.001$), ($P<0.001$), respectively. We found direct correlation between PRM1 and PRM2 mRNA levels to the quality of embryos ($r=0.31$, $P=0.012$), ($r=0.31$, $P=0.011$), respectively. The differences in HILS1 mRNA contents between these two groups were not statistically significant ($P>0,05$). We did not observe an association between HILS1 transcript contents and quality of embryos ($r=0.22$, $P=0.076$). We suggest that fertilin β and protamines contribute not only to successful fertilization, but may have an important impact in development of preimplantation embryos.

Key words: Fertilin β - PRM1 - PRM2 - HILS1 - Fertilization - Sperm - Z-SCORING

Introduction

Since Wykes *et al.* first described the existence of several mRNAs in human mature spermatozoa, paternal contribution in fertilization and embryo development has been studied and certain aspects elucidated [9]. It has been proved that the transcripts found in human mature spermatozoa are not transcribed de novo; rather, they represent remnants of cytoplasmic droplets that were not excluded during early steps of human spermatogenesis [10-13]. Out of over 3000 different transcripts identified in ejaculated spermatozoa, little is known as to which of these transcripts may be used in the prognosis of male reproduction ability and which can have therapeutic and practical applications.

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Table 1. Oligonucleotide sequences used for RQ-PCR analysis.

Transcript	Sequence (5'-3' direction)	Position	ENST number www. ensembl.org/	Exons	Product size (bp)
Fertilin beta	AACCACCAACTGCCATTATTC TTATTGACCGTATTTTCTCCG	86-107 220-241	00000104755	1, 2	155 bp
GAPDH	CTGCACCACCAACTGCTT TTCTGGGTGGCAGTGATG	555-574 642-659	00000105679	7, 8	105 bp
PRM1	CGGTGAGCTGCTGCCCAACT GCCTAAGTTCGACTGACCTG	183-203 316-336	00000312511	1, 2	153 bp
PRM2	GGATCCACAGCCGCCAGCATCGCT GCATGTTCTCTTCCTGGTTCTGCA	416-440 496-520	00000241808	3, 4	104 bp
HILS1	ACGTGCACACGCGTGTCGAG GCTGTGCCAACAAGAGTGTA	216-236 322-342	00000188662	2, 3	126 bp
GAPDH	CTGCACCACCAACTGCTT TTCTGGGTGGCAGTGATG	555-574 642-659	00000105679	7, 8	105 bp

Fertilin α and Fertilin β (also known as ADAM-1 and ADAM-2, respectively) form a heterodimer and belong to the ADAM family of integral membrane proteins [14-16]. Fertilin is located in the equatorial region of the sperm and the soluble extracellular domains of fertilin α and fertilin β can bind to the microvillar region of murine eggs [17,18]. Moreover, fertilin β peptides have been shown to inhibit the binding of sperm to oocytes and fusion of the gametes [19]. However, the role and impact of fertilin β on zygote formation and embryo development is still unknown.

Protamines belong to the small sperm nuclear specific proteins, which replace somatic histones and transition proteins during maturation phase of spermatogenesis. After binding of PRM1 and PRM2 to the sperm nucleus, chromatin is highly condensed and transcriptionally silent. Several lines of experiments described an increased correlation between the protamines' transcript and protein concentrations, sperm motility, and fertilization ability [20-23].

The spermatid-specific linker histone (HILS1) functions as a protein, is expressed in late spermatids, and is involved in sperm chromatin condensation [24-26]. Yan *et al.* postulated that HILS1 may also regulate specific gene transcription and DNA repair during spermatid differentiation [24,27-29].

The aim of this study was to determine the association between fertilin β, PRM1, PRM2, and HILS1 transcript contents, fertilization ability of human sperm, and the development of embryos after *in vitro* fertilization (IVF).

Materials and methods

A total of 83 infertile couples' selected to IVF were investigated in the Division of Infertility and Endocrinology of Reproduction in Poznan, between September 2005 and December 2006. All patients enrolled in this study were treated by conventional IVF. The couples were excluded from our studies when the female partners were over 39 years of age, folliclestimulating hormone (FSH) concentration at the start of menstrual cycle was more than 12 mIU/mL or when the women suffered from extensive endometriosis (II and IV degree) or polycystic ovarian syndrome. Male partners had at least 5 million of spermatozoa per 1 mL of ejaculate with minimum 10% of sperm motility (A + B category), in total.

For ovarian stimulation, 150-300 IU human menopausal gonadotrophin (Menopur, Ferring) injections were administered daily, beginning on day 3 of the menstrual cycle, after verification of pituitary suppression by the gonadotrophin - releasing hormone agonist triptorelin (Decapeptyl 0.1 mg/day, Ferring) which was given daily on day 21 of the menstrual cycle preceding the treatment cycle. Final oocyte maturation was triggered with 10 000 IU of human chorionic gonadotrophin (HCG, Pregnyl) when the leading three follicles were >17 mm in diameter. Transvaginal ultrasound guided oocyte retrieval was performed under conscious sedation at 35-36h post - HCG. Oocytes were collected directly into gassed (6.0% CO_2) and warmed (37±C) Vitrolofe medium (Sweden) and placed in CO_2 incubator (Heracell, Heraeus).

For fertilization, conventional IVF procedure were used.

About 16-18h after insemination, oocytes were examined for the presence of pronuclei and polar bodies. Fertilization was considered normal when two clearly distinct pronuclei were present. The zygotes were scored according to the Z-scoring system [8]. The system takes into account nuclear size and alignment, number of nucleoli (nucleolar precursor bodies, NPB), and their distribution. Embryonic morphology was noted 68h (day 3) after insemination. The number of blastomeres, blastomere uniformity, and cytoplasmic appearance were scored. Additionally, embryos were scored as grade A-D according to the degree of cytoplasm fragmentation on days 2 and 3. Grade A: 0-10% cytoplasm fragmentation, grade B: 11-20% cytoplasm fragmentation, grade C: 21-50% and grade D: over 50% cytoplasm fragmentation.

Sperm purification method. All semen samples were analyzed according to standard seminological procedures (World Health Organization, 1999). After liquefaction, semen samples were analyzed for sperm density, motility, and morphology. Ejaculated spermatozoa were purified by centrifugation through discontinuous SpermGrad and prepared using swim-up method. The purity of the spermatozoa was examined by using an optical microscope

Table 2. Correlation of number of PRM1, PRM2 and HILS1 transcripts copies and quality of zygotes and embryos. The number of PRM1, PRM2, and HILS1 transcript copies is presented per 106 sperm cells or per fmol of GAPDH. Grade A: 0-10% of cytoplasm fragmentation, grade B: 11-20% cytoplasm fragmentation, grade C: 21-50% and grade D: over 50% of cytoplasm fragmentation. ^{a}P>0,05 and ^{b}P>0,05 represent statistically significant correlations of PRM1, PRM2, and HILS1 transcript contents in spermatozoa obtained from patient groups and quality of zygotes and embryos (correlation of high transcript contents with grade A embryos (**bold**) and low transcript contents with grade D embryos) after IVF method.

Number of transcript copies	Zygotes Z1-Z4	Embryos			
		A	B	C	D
Fertilin β/10^6	P >0,05	^{a}**P<0,05**	P >0,05	P >0,05	^{a}**P<0,05**
Fertilin β/ GAPDH	P >0,05	^{b}**P<0,05**	P >0,05	P >0,05	^{b}**P<0,05**
PRM 1/ 10^6	P >0,05	^{a}**P<0,05**	^{b}P <0,05	^{b}P <0,05	^{a}P < 0,05
PRM1/GAPDH	P >0,05	^{b}**P<0,05**	^{b}P <0,05	^{b}P <0,05	^{b}P<0,05
PRM2/ 10^6	P >0,05	^{a}**P <0,05**	^{b}P <0,05	^{b}P <0,05	^{a}P<0,05
PRM2/GAPDH	P >0,05	^{b}**P <0,05**	^{b}P <0,05	^{b}P <0,05	^{b}P<0,05
HILS1/ 10^6	*P* >0,05	^{a}P >0,05	^{a}P >0,05	^{a}P >0,05	^{a}P >0,05
HILS1/GAPDH	*P* >0,05	^{b}P >0,05	^{b}P >0,05	^{b}P >0,05	^{b}P >0,05

equipped with 100 x oil objective, and spermatozoa contaminated by somatic or round cells were discarded. The purified samples of spermatozoa were subsequently used to isolate RNAs, which were reverse transcribed into cDNA [30].

RQ-PCR analysis of PRM1, PRM2, and HILS1 transcript contents in spermatozoa. Each RNA sample was isolated from one million spermatozoa according to Chomczyński and Sacchi (1987) [7]. The purity of the RNA samples was verified spectrophotometrically at 260 and 280 nm. RNA was treated with DNase I (Promega Co. Madison, USA) and reverse-transcribed into cDNA using random hexamer priming and reverse transcriptase (RT), (Sigma Co. St. Louis, USA). Quantitative analysis of PRM1, PRM2, and HILS1 cDNA was performed by RQ-PCR SYBR Green I analysis (Light Cycler, Roche Diagnostics GmbH, Mannheim, Germany). Fertilin β, PRM1, PRM2, and HILS1 cDNAs were amplified using pairs of primers (Table 1). For amplification, 2 µl (corresponding to 10^6 spermatozoal RNA) of cDNA was added to 18 µl of PCR mix containing HotStart*Taq* DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, 2.5 mM MgCl2, and primers (Qiagen Inc. Valencia, Ca, USA). Quantification of copy number was derived from a standard curve of a known amount of synthetic DNA template. Since GAPDH transcript exhibited similar levels in spermatozoa of both groups of donors, we adjusted the quantity of fertilin β, PRM1, PRM2, and HILS1 transcripts in each sample to use the same amount of GAPDH. The levels of fertilin β, PRM1, PRM2, and HILS1 mRNAs are expressed as the number of these transcripts per 10^6 spermatozoa or adjusted to fmol of GAPDH mRNA.

Statistical analysis. Each of these experiments was performed at least in triplicate. Results were estimated using Student's t-test and one-way analysis of variance (ANOVA) with Newman-Keule's post-hoc test. P value was determined by Student's t-test, with P<0.05 as the level of significance.

Results

Using real-time quantitative PCR (RQ-PCR) reaction we evaluated the number of fertilin β, PRM1 and PRM2 mRNA in human spermatozoa from infertile couples undergoing *in vitro* fertilization (IVF).

We found a significantly lower content of fertilin β transcript in spermatozoa of IVF couples with failed fertilization as compared to those where zygotes appeared (P<0.001). The median of fertilin β mRNA contents in patients with failed and successful IVF method was 326.3 (range 12.98 - 345.8) and 77790 (range 4578 - 856700) copies per million of spermatozoa, respectively (P<0.001) (Figure 1A). We also adjusted fertilin β transcript number to fmol GAPDH mRNA, which confirmed significant differences between both groups (P<0.001) (Figure 1B).

We studied correlations in the number of fertilin β transcripts and quality of zygotes and embryos. No correlation was observed between the number of fertilin β transcripts to the quality of zygotes (p>0.05) (Table 2). However, an increase in the number of fertilin β correlated to an increase in the quality of embryos (p<0.05) (Table 1). Conversely, the number of good quality embryos (7-9 blastomers with 0-10% of cytoplasm fragmentation) decreased as the number of copies of fertilin β transcripts decreased (p<0.05) (Table 2).

In IVF-treated couples, we observed a significantly decreased content of PRM1 and PRM2 transcripts in spermatozoa that did not yield fertilization compared to those where zygotes appeared (P<0.001, P<0.001, respectively) (Figure 2, 3). An association between PRM1 and PRM2 mRNA contents of spermatozoa and preimplantation embryo quality was shown using zygote scoring (Z-scoring) and grading of embryo quality. Significantly lower levels of PRM1 and PRM2 mRNA were observed in the group of patients with failed *in vitro* fertilization as compared to patients with successful *in vitro* fertilization (P<0.05, P<0.05, respectively) (Table 2).

We did not observe differences in HILS1 transcript contents between spermatozoa obtained from patients

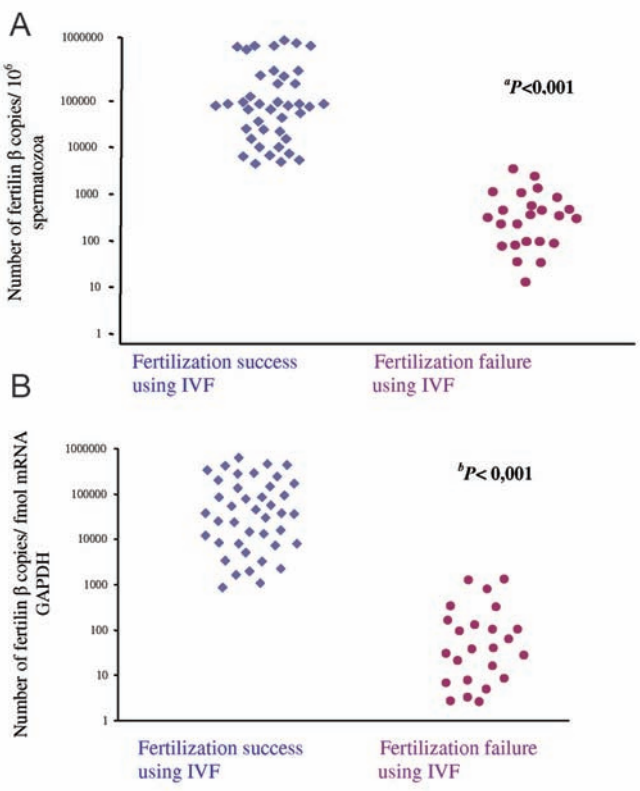

Figure 1. Number of fertilin β transcript copies in spermatozoa isolated from IVF patients. The number of fertilin β transcript copies is presented per 10^6 sperm cells (**A**) or per fmol of GAPDH (**B**). ^aP and ^bP represents statistical differences between fertilin β transcript contents in spermatozoa from IVF patients.

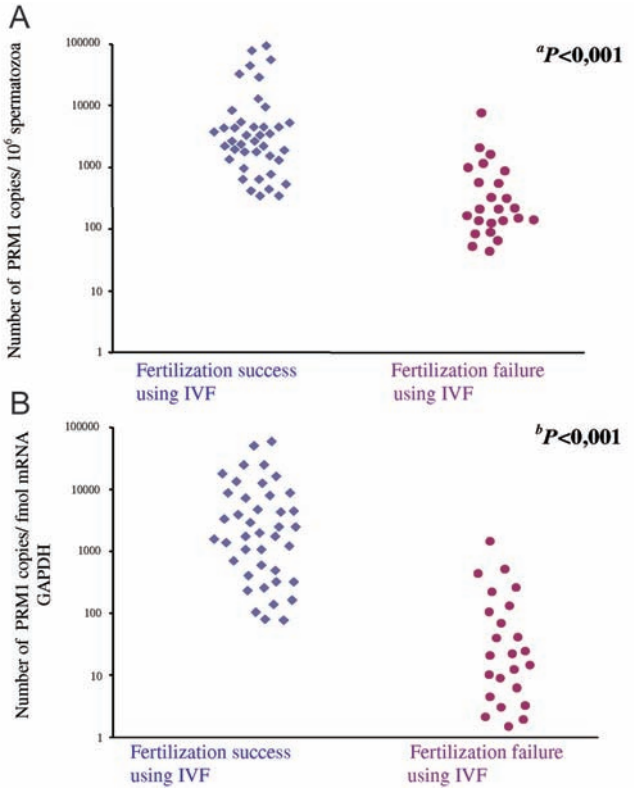

Figure 2. Number of PRM1 transcript copies isolated from spermatozoa obtained from patients with successful *in vitro* fertilization and patients with failed *in vitro* fertilization. The number of PRM1 transcript copies is presented per 10^6 sperm cells (**A**) or per fmol of GAPDH (**B**). ^aP and ^bP represent statistical differences between PRM1 transcript contents in spermatozoa obtained from patients with successful *in vitro* fertilization and patients with *in vitro* fertilization failure.

with successful *in vitro* fertilization and patient with *in vitro* fertilization failure (P>0.05) (Figure 4). Moreover, we did not find an association between HILS1 transcripts and quality of embryos (P>0.05) (Table 2).

Discussion

Fertilization can be defined as the physical union of the sperm and the ovum to yield a zygote. The molecular contribution of the ovum at fertilization is well understood, whereas our understanding of the role of sperm is still evolving [31]. Sperm have been considered the delivery vehicle of the paternal genetic complement to the ovum, but several data have shown that sperm contribute more than just their DNA [32]. In fact, they deliver practically their entire contents during fertilization, including a host of novel RNAs [33]. The most comprehensive description of the sperm RNA transcripts present in healthy fertile men was published in 2002 [34, 35]. The fertilin β transcript can be found among the examined sperm transcripts [36].

Our data shows that fertilin β may play a crucial role in the process of sperm/egg fusion. Bronson, *et al.* has suggested that fertilin β binds to an oolemmal integrin, and it is proposed that the tripeptide FEE (Phe-Glu-Glu) is the integrin recognition sequence in human fertilin β. He proposes that a fertilin-like molecule is functionally active on human spermatozoa and that its interaction with an oolemmal integrin receptor plays a role in fertilization in humans [37]. A similar conclusion is inferred by Zhang J, *et al.*, whose research has demonstrated that fertilin β plays an important role in fertilization via its disintegrin domain as a sperm-specific antigen. His paper suggests that fertilin β is an important target antigen and has a very promising value in the development of a human immunocontraceptive vaccine [38].

Our results suggest that fertilin β may have influence on early embryo development. It has been proved that spermatozoal transcripts (mRNAs), such as fertilin β, are present in cytoplasmic droplets in mature ejaculated human sperm. In recent studies using the hamster sperm penetration assay, sperm-specific human RNAs were shown to be delivered to the oocyte on fertilization [39-41]. The half life of each RNA varied from 5 hours to at least the time of activation of the embryonic genome, which is the 8-cell stage in human

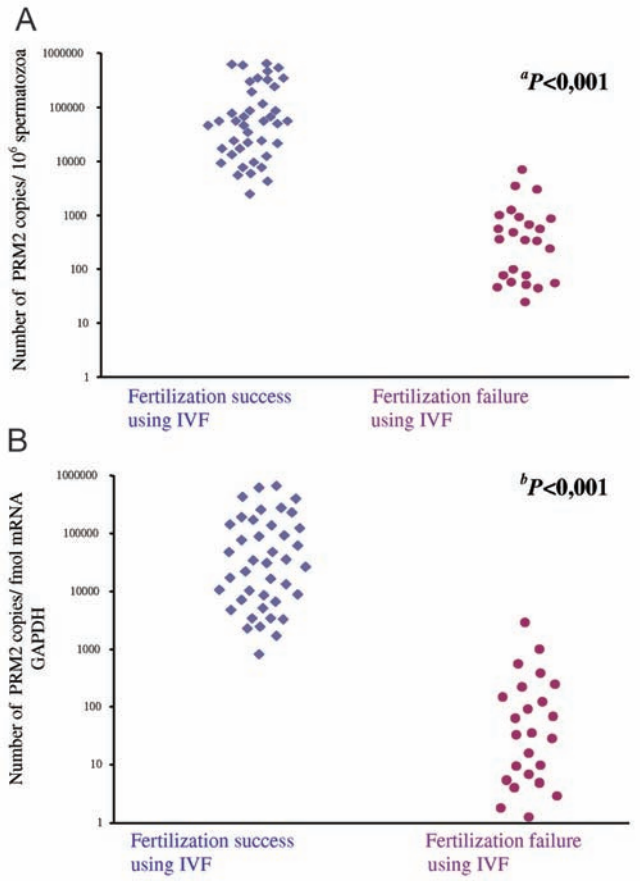

Figure 3. Number of PRM2 transcript copies isolated from spermatozoa obtained from patients with successful *in vitro* fertilization and patients with failed *in vitro* fertilization. The number of PRM2 transcript copies is presented per 10^6 sperm cells (**A**) or per fmol of GAPDH (**B**). ^aP and ^bP represent statistical differences between PRM1 transcript contents in spermatozoa obtained from patients with successful *in vitro* fertilization and patients with *in vitro* fertilization failure.

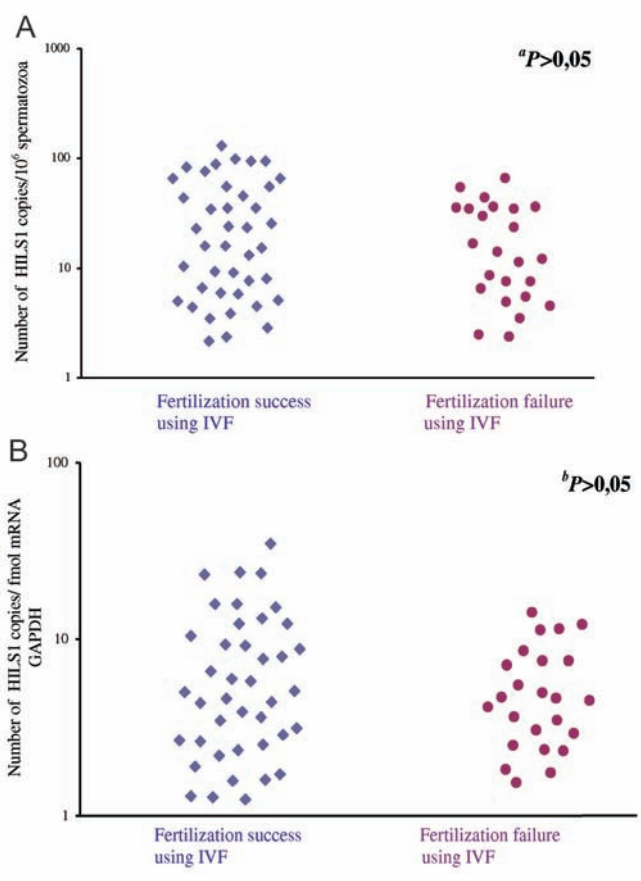

Figure 4. Number of HILS1 transcript copies isolated from spermatozoa obtained from patients with successful *in vitro* fertilization and patients with failed *in vitro* fertilization. The number of HILS1 transcript copies is presented per 106 sperm cells (**A**) or per fmol of GAPDH (**B**). ^aP and ^bP represent statistical differences between HILS1 transcript contents in spermatozoa obtained from patients with successful *in vitro* fertilization and patients with *in vitro* fertilization failure

embryos [42]. This is likely to occur as part of the destruction of maternal RNAs that takes place until the activation of the zygotic genome. This mechanism might target a host of mRNAs that would be detrimental to development. The ability of these RNAs to be translated when injected into human oocytes, and the short time that they reside in the zygote before activation of the zygotic genome, might reflect that they have important roles during early embryogenesis [43, 44, 45].

Since the role of protamines in sperm chromatin condensation and head morphology has been clearly proved, we analyzed the possible function of protamines in sperm fertilization ability and quality of embryos obtained from IVF procedure [46-48]. It is well known that the PRM1 and PRM2 genes are transcribed in the post-meiotic haploid spermatid [49, 50]. The subsequent translation of PRM1 and PRM2 genes and the binding of their protein products to DNA cause complete condensation of chromatin, leading to the eventual arrest of transcription. Recently, it has been suggested that an abnormal spermatozoal PRM1 and PRM2 transcript and protein ratio may be associated with abnormal chromatin condensation and lead to male infertility [22]. Ejaculated spermatozoa purified by Percol gradient centrifugation from normozoospermic and asthenozoospermic men exhibited different amounts of PRM1 and PRM2 transcripts [32]. Our data shows the association between PRM1 and PRM2 transcripts and fertilization ability of sperm IVF patients.

The role of chromatin remodeling and protamines in embryo development was also investigated by D'Occhio *et al.* [31]. Using sperm chromatin structure assay, they found significant correlation between sperm chromatin structure and embryo survival. Inappropriate sperm chromatin structure and PRMs deficiency seems to be not only a "male factor" of infertility, but also an important component in embryo devel-

opment, which may lead to an increased incidence of embryonic mortality [31]. Our results also demonstrate that protamines may contribute not only in successful fertilization but also in quality and developmental competence of preimplantation embryos.

HILS1 is highly expressed in elongated spermatids and contributes in the process of chromatin remodeling through a mechanism distinct from that of linker histones [24, 47]. We are the first to investigate the possible association between sperm HILS1 mRNA contents, *in vitro* fertilization outcome, and developmental competence of embryos. The lack of statistical differences in HILS1 mRNA contents between spermatozoa obtained from patient group with IVF fertilization success and patient group with IVF fertilization failure may suggest that HILS1 expression during early steps of spermatogenesis does not contribute to fertilization ability of human sperm. Although it was proved experimentally that somatic histones participated in chromatin remodeling during early embryogenesis [31, 48], our results demonstrated that HILS1, as the spermatid specific linker histone, do not participate in this process.

Fertilization is a complex process that involves both maternal and paternal gametes [32]. The discovery that spermatozoal mRNAs are delivered into the oocyte during fertilization give us a deeper insight into the molecular basics of embryo development [50]. It has given an impression of spermatozoal mRNA as a complex component of the ejaculated cell and hopefully dispelled any doubts as to the origins of this mRNA. The suggested involvement of RNA in chromatin repacking in spermatogenesis and in genomic imprinting requires further experimental validation. As a useful diagnostic resource, however, spermatozoal RNA has obvious advantages over more traditional techniques of infertility research and, clearly, the necessary groundwork has now been completed, leaving the way open for more extensive independent investigations [28].

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