

**RELATIONSHIP BETWEEN FERTILIN B MRNA
EXPRESSION AND THE FERTILIZING POTENTIALS OF
HUMAN SPERMATOZOA**

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ABSTRACT

Fertilin is a sperm surface protein that belongs to the ADAM (a disintegrin and a metalloproteinase) family of integral membrane proteins. It mediates adhesion and fusion between the sperm and egg plasma membranes that may play important roles in mammalian fertilization. This study aimed to explore the relation between fertilization ability of human sperm and fertilin β mRNA expression in non-fertile men. Expression of fertilin β gene mRNA was determined by RT-PCR from 141 semen samples (infertile n=81 and normal fertile n=60 men). Total RNA was extracted from sperm pellets. cDNA was generated then amplified. Computer assisted semen analysis and acrosine activity test were also performed. ELISA was done to measure FSH, LH, testosterone and prolactin. The study showed lowered sperm fertilin β expression in infertile group together with decreased sperm motility and other sperm functions. Also, there was significant positive correlation between fertilin β expression and testosterone levels. Furthermore, normal morphology may influence changes of fertilin β as shown by multiple regression analysis. From this study it can be concluded that infertility which is associated with decreased expression of sperm fertilin β could be due to failure of sperm migration into the oviduct. Moreover, defective protease can produce incomplete fertilin β processing with improper maturation of sperm proteins. Here in this study showed possible direct role of fertilin- β in fertilization and also in sperm morphology and hence sperm capacitation.

Keywords: Fertilin β expression, Infertility, Hormonal profile, Sperm function.

INTRODUCTION

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 (Howes et al., 2001, Geraci and Giudice, 2006, Vieira and Miller 2006).

The ADAM (a disintegrin and metalloprotease) family of sperm cell-surface proteins, including fertilin α/β (ADAM1/2) and cyritestin (ADAM3), provide the motif that serves as recognition site to bind integrin $\alpha 6\beta 1$ on oocytes to promote successful fusion adhesion and fusion between the sperm and egg plasma membranes (Primakoff and Myles, 2000). Moreover, ADAM7 is required for normal fertility and is important for the maintenance of epididymal integrity and for sperm morphology, motility, and membrane proteins (Choi et al., 2015).

Fertilin α and β form a heterodimer and belong to the ADAM family of integral membrane proteins (Menezo, 2006). 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 (Bigler et al., 2000).

The human fertilin β is synthesized as a precursor in the testis and cleaved to its mature form in the corpus epididymis (Evans, 1999). Fertilin β is proteolytically processed while spermatozoa are in transit through the epididymis helping in acquisition of fertilization competence in spermatozoa and exposes an epitope that has a role in sperm-egg interactions. Also it triggers relocalization of fertilin from the whole sperm head to the posterior head where this is important for successful fertilization (Wolkowicz et al., 2008).

The aim of this study was to highlight the association between fertilin β mRNA expression and fertilization ability of human sperm as determined by sperm function.

MATERIALS AND METHODS

A total of 141 subjects were included in the current study. Semen samples were obtained from men attending the Andrology Outpatient Clinic, Mansoura University. Informed consent was obtained from all participants. They were grouped into fertile men as control group (n=60) and infertile men (n=81). Infertility is defined as the inability of a sexually active, non-contracepting couple to achieve pregnancy in one year. The male partner can be evaluated for infertility or subfertility using a variety of clinical interventions, and also from a laboratory evaluation of semen.” according to the recommendation of WHO (WHO, 2010). After complete liquefaction, Computer assisted semen analysis (CASA) (Autosperm Fertipro, Belgium) (Hinting et al., 1998) and acrosin activity test of spermatozoa by gelatinolysis were performed (Henkel et al., 1995). Sperm morphology was evaluated on stained smear by phase contrast microscope and Sperm Mac stain (Fertipro, Belgium) according to World Health Organization. Sil-select gradient (Autosperm Fertipro, Belgium) has been performed on the pellet to select spermatozoa only and to avoid the contamination either with spermatogenic or pus cells.

Reverse transcription-PCR for fertilin β mRNA expression:

Semen samples were collected, and total RNA was extracted using the TriFast reagent (Peqlab, Germany) as described in the manufacturer's instructions. The purity of RNA was checked at 260 nm using the Nanodrop spectrophotometer, NanoPhotometer® P-Class (Implen, Germany). The absence of RNA degradation was confirmed by electrophoresis on a 1.5% agarose gel electrophoresis containing ethidium bromide. First-strand cDNA was generated by using Maxima First Strand cDNA Synthesis Kit for RT-PCR (Thermo Scientific, U.S.A) according to manufacturer's instructions. Fertilin β and GAPDH genes were amplified using a PTC-200 thermal cycler (MJ Research, Essex, UK). Primer sequences were as follows:

Primer	Sequence	Product length (bp)	Reference
Fertilin β	Forward: 5 –AAC CAC CAA CTG CCA TTA TTC-3 Reverse: 5-TTA TTG ACC GTA TTT TCT CCG -3	155	(Depa-Martynów et al., 2007)
Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH)	Forward: 5'- AAT CCC ATC ACC ATC TTC C-3' Reverse: 5'-CAT CAC GCC ACA GTT TCC -3'	382	(Yang et al., 2007)

The cycle profile was as follows: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 52°C for 1 minute and elongation at 72°C for 1 minute, and a final incubation at 72°C for 10 minutes.

The amplified RT-PCR products were electrophoresed on 2% agarose gel and DNA was visualized with ethidium bromide staining via light UV transilluminator and photographed. The results were analyzed with scion image R release Alpha 4.0.3.2. Software for windows which performs bands detection and conversion to peaks. Area under each peak was calculated in square pixels for quantification. Fertilin β mRNA expression levels were determined by calculating the ratio between the square pixel values of the target bands in relation to the control bands (GAPDH, housekeeping gene for RT-PCR).

Alpha- glucosidase activity in seminal plasma was measured according to the method described by Guerin et al. (1986) to evaluate the function of the epididymis. The kit supplied from Ferti Pro N.V. (Industriepark Noord, Beerneme, Belgium).

Acrosin activity index determination was assessed by gelatin-covered slides which prepared by 5% gelatin (Merck, Darmstadt, Germany) onto microscope slides. Purified spermatozoa were smeared on the prepared slides and incubated in a moist chamber at 37°C for 2 hours. The halo diameter, halo formation rate and acrosin activity index were calculated (Henkel et al., 1995).

Hormonal profile including: follicle stimulating hormone (FSH), lutenizing hormone (LH), testosterone & prolactin were measured on sera collected from blood samples by enzyme linked immuno-sorbant assay (ELISA).

STATISTICAL ANALYSIS

Statistical analysis was done by using MedCalcR program version 10.0.1 (Schoonjans et al., 1995).

RESULTS

Table (1) showed semen parameters in both fertile and infertile groups expressed as median and range. The present study showed decreased motility, velocity and alpha-glucosidase activity in infertile group compared with fertile group.

Table (1): Semen parameters of fertile and infertile groups (median and range)

Parameters	Fertile n=60	Infertile n=81
Volume (ml)	4.0 (2.0-7.0)	4.0(1.0-9.2)
Concentration (million /ml)	70.4 (44.8-96.0)	8.15* (0.85-18.84)
Grade A motility (%)	54.0 (41.0-60.0)	4.0* (0.0-25.0)
Grade A+B motility (%)	61.0 (51.0-67.0)	12.0* (2.0-39.0)
Velocity (µm/sec)	79.65 (63.4-86.6)	36.2* (8.8-75.5)
Linear velocity (µm/Sec)	62.3 (42.9-70.7)	20.8* (5.7-52.6)
Linearity index (%)	82.0 (67.5-86.7)	60.3* (33.7-97.4)
Normal morphology (%)	64.0 (58.0-66.0)	2.0* (0.0-18.0)
WBCs (million/ml)	0.6 (0.4-0.6)	0.8 (0.3-5.4)
Alpha-glucosidase activity (U/ml)	69.3 (45.4-90.7)	26.4* (18.4-44.6)

n = number of cases

* significant at P<0.05 comparison between the fertile and infertile groups

Analysis of variance revealed that sperm fertilin β mRNA expression is significantly decreased in infertile samples as compared with fertile group (table 2, fig 1). Sperm function tests including Acrosin index levels, halo, and halo percent were all high statistically significant in fertile men when compared with infertile group as shown in (table 2).

Table (2): Fertilin β mRNA expression and acrosin activity test in the fertile and infertile groups (median-range)

Parameters	Fertile n=60	Infertile n=81
Acrosin activity index	12.25 (8.86-19.49)	1.17* (0.55-9.57)
Halo diameter (nm)	17.4 (12.8-23.7)	10.3* (9.0-14.6)
Halo formation rate %	76.0 (63.0-90.0)	12.0* (6.0-66.0)
Fertilin mRNA expression	1.76 (0.96-2.38)	0.61* (0.21-1.28)

n = number of cases

* significant at $P < 0.05$ comparison between the fertile and infertile groups

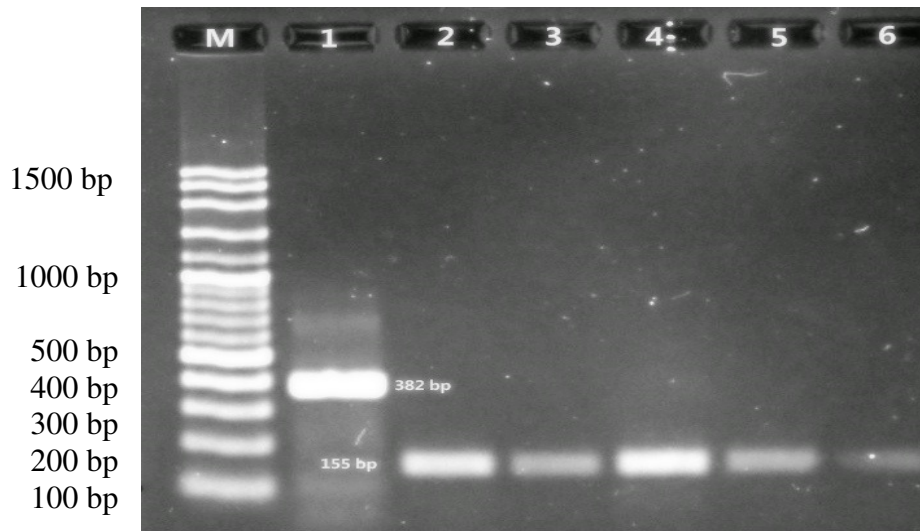


Figure 1 Expression analysis of fertilin β mRNA from semen samples by RT-PCR. M= 100 bp DNA ladder. The upper 382 bp band represents the internal control GAPDH (lane 1); the lower 155 bp bands represent amplification of fertilin β from fertile samples (lane 2 & 4) and infertile samples (lane 3, 5 & 6).

However, the hormonal profile presented significant increase in FSH levels and significant decrease in testosterone levels in infertile than fertile group. While there was insignificant difference between both groups regarding LH and prolactin (table 3).

Table (3): serum hormonal levels in the fertile and the infertile groups

Parameters	Fertile n=60	Infertilen=81
FSH (mIU/ml)	6.8 (3.9-9.6)	10.1 * (5.2-22.0)
LH (mIU/ml)	6.4 (4.5-9.3)	6.4 (3.0-12.4)
Prolactin (ng/ml)	5.4 (3.5-8.6)	6.5 (2.4-12.5)
Estradiol (pg/ml)	32.0 (24.0-45.0)	36.0* (20.0-123.0)
Testosterone (ng/dl)	790.0 (506.0-1106)	530.0* (301.0-1200.0)

n = number of cases

* significant at $P < 0.05$ comparison between the fertile and infertile groups

Spearman rank correlation test showed that fertilin β mRNA expression was found to be significantly positively correlated with all studied seminal parameters (concentration, normal morphology, velocity, linearity index, grade A&B motility, and alpha-glucosidase ($p < 0.0001$)), sperm functions (acrosine activity index, Halo diameter, and Halo formation rate %, ($p < 0.0001$)) and testosterone level ($r = 0.493$, $p < 0.0001$), while there was significant negative correlation with FSH level ($r = -0.427$, $p < 0.0001$).

Stepwise multiple regression analysis was done between different studied variables to detect the factors which may influence changes of fertilin β mRNA expression, where sperm morphology was found to be the possible determinant one (F-ratio = 322.9, $p < 0.001$).

ROC curve analysis was done to discriminate between fertile group and infertile group using fertilin β mRNA expression, grade A motility, acrosin activity as discriminative markers (fig 2, table 4).

Table (4): ROC curve analysis of fertilin β mRNA expression, acrosin activity index and grade A motility

Variable	AUC	SE	95% CI
Fertilin β mRNA expression	0.961	0.0176	0.914 to 0.987
Acrosin activity index	0.986	0.0111	0.950 to 0.999
Grade A motility%	0.991	0.00927	0.957 to 1.000

AUC: Area Under Curve SE: Standard Error CI: Confidence Interval

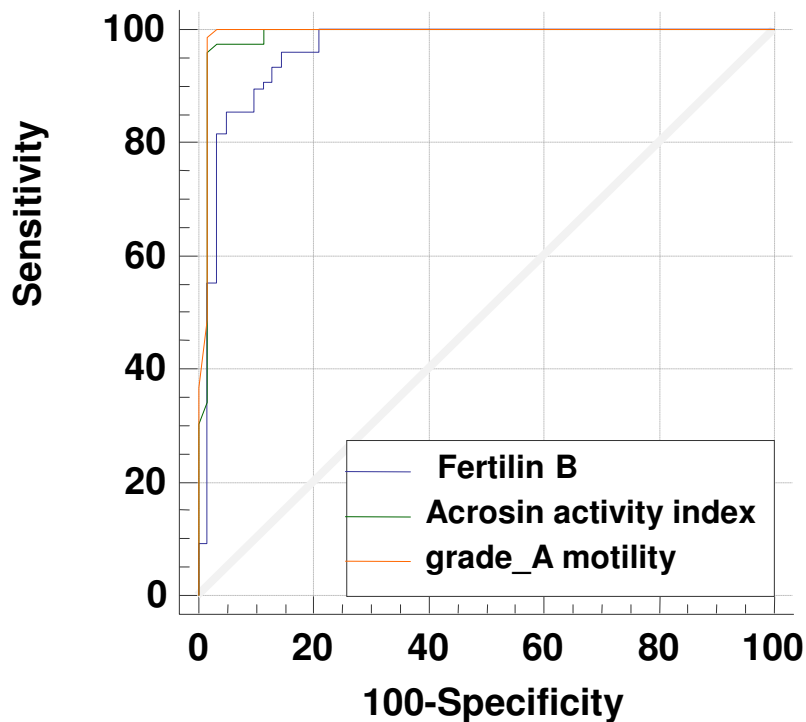


Fig 2: ROC curve analysis of fertilin β mRNA expression in fertile and infertile groups

Cut off point of fertilin β mRNA expression at value of ≤ 1.13 can distinguish between fertile and infertile groups with specificity 85.5 %, and sensitivity 96.1%, and the area under the curve (AUC) was 0.961. Also Grade A motility can be used as a discriminative marker between fertile and infertile groups at ≤ 24 with specificity 98.4 %, and sensitivity 96.1 %, and AUC was 0.991. Acrosin activity index is another marker at ≤ 5.1 with specificity 98.4 %, and sensitivity 98.7 %, and AUC was 0.986. By comparing AUC of fertilin β mRNA expression was significantly different from that of acrosin activity index ($p=0.02$) and grade A motility ($p=0.01$) while there was insignificant difference of AUC between that of acrosin activity index and grade A motility ($p=0.13$).

DISCUSSION

Fertilin α and β form a heterodimer and belong to the ADAM family of integral membrane proteins (Menezo, 2006). In fact, ADAM domain-containing protein family, were regulated during epididymal transit and showed changes in the N-linked sialoglycoprotein profile which is a major hallmark of sperm maturation (Villaverde et al., 2016).

The studied infertile samples showed reduced levels of fertilin- β mRNA expression compared to the fertile group. These results were consistent with the hypothesis of possible direct role of fertilin- β in sperm-egg plasma membrane interaction and fertilization (Sukhikh et al., 2012). Sperm from mice lacking fertilin β were shown to be deficient in migration from the uterus into the oviduct, sperm-egg membrane adhesion and fusion and binding to the egg zona pellucida resulting in infertile males (Cho et al., 1998). Furthermore, a monoclonal antibody against fertilin potently inhibits sperm-egg fusion, providing an evidence that fertilin has a critical role in fertilization (Primakoff et al., 1987).

One possible hypothesis that explains decreased fertilin β expression is deficiency of inositol polyphosphate 5-phosphatase (Inpp5b) that regulates the levels of 5-phosphorylated phosphoinositides on cellular membranes. It is expressed in the Sertoli cells and epididymis and has been involved in protein trafficking and secretion (De Camilli et al., 1996). Infertility in Inpp5b deficient animals could be explained by improper targeting and/or secretion of the protease that acts on fertilin β processing in the epididymal epithelial cells. Incomplete processing of fertilin β with defective maturation, translocation or folding of sperm protein resulted in reduced motility and reduced ability to fertilize eggs due to abnormalities in sperm function and maturation (Hellsten et al., 2001). This was in line with the current work results that showed reduced motility of infertile sperms.

Moreover in globozoospermic round headed infertile sperm there is down regulation of cytoskeleton proteins involved in cell movement, signal transduction and membrane shape maintenance as cytoplasmic actin and tubulin- α -2 chains (Liao et al., 2009). Actin polymerization and protein tyrosine phosphorylation are related processes occurring in bovine sperm capacitation (Brener et al.,

2003). Actin polymerization is Ca^{2+} -dependent process, as it was almost completely blocked by chelating intracellular Ca^{2+} (**Rivlin et al., 2004**).

This could explain the correlation between sperm morphology and fertilin β mRNA expression as shown by stepwise regression analysis in the present study. Decreased fertilin β mRNA expression that was possibly due to Inpp5 deficiency would decrease levels of 5-phosphorylated phosphoinositides and consequently intracellular Ca^{2+} . It has been postulated that the increase in sperm intracellular Ca^{2+} induce turning swimming with active flagellar bending (**Yoshida and Yoshida, 2011**). Thus decreased intracellular Ca^{2+} leads to lack of activation of the mammalian sperm that affects actin polymerization and hence sperm capacitation.

Another theory for association between decreased fertilin β and infertility is calmeglin abnormality. Calmeglin is a Ca^{2+} binding protein localized on endoplasmic reticulum (ER) membranes acts as a testis-specific molecular chaperone and specifically complexed with both fertilin α & β . In calmeglin deficient-mice, there was loss of heterodimerization of fertilin α , β and fertilin β was not detectable in mature sperm. Furthermore, Calmeglin is essential for spermatozoa to migrate into oviduct (**Cho et al., 2000, Ikawa et al., 2001**). Thus Calmeglin and fertilin β knockout mouse lines share a common infertile phenotype, indicating the importance of calmeglin as ER chaperone for sperm membrane protein maturation as well as its importance for spermatozoa migration into oviduct (**Bass et al., 1998, Yoshinaga et al., 1999**).

ROC curve analysis showed that sperm fertilin β mRNA expression, together with acrosin activity index and grade A motility could be used as markers to assess infertility with relatively high specificity and sensitivity. Hormonal changes are believed to be important in sperm production.

This study showed elevated FSH levels together with low testosterone levels that were in agreement with Wei et al. (**2013**) who reported similar results in patients with oligo-astheno-teratospermia (OAT) which is a common phenotype for infertile men.

In this work, fertilin β mRNA expression showed positive correlation with testosterone and negative correlation with FSH. Both correlations satisfied statistical significance. The main role of testosterone action in spermatogenesis is during meiosis and spermiogenesis

(**Ruwanpura et al., 2010**). Therefore its adequate supply is crucial for Sertoli cell survival and differentiation in early postnatal life (**Sharpe et al., 2003**). Testosterone and FSH are considered the major hormonal regulators for male germ cell development at different stages of spermatogenesis by controlling their survival from apoptosis, rather than by stimulating their proliferation (**Ruwanpura et al., 2010**). Thus, these results of deranged hormonal profile could exert its effect on sperm survival through enhancing extrinsic or intrinsic apoptotic pathways. In conclusion, the present work showed reduced fertilin β mRNA expression in infertile men which denotes that fertilin β is important to identify the intermolecular relationships among zona binding related proteins and may be necessary to understand the basis of sperm associated fertility. Taken together with the observed hormonal imbalance in the infertile group, this may affect the sperm morphology and motility and hence be a cause of infertility.

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الملخص العربي

العلاقة بين التعبير الجيني للحمض النووي الريبوزي للفرنتيلين بيتا والقدرة الإخصابية للحيوانات المنوية

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الفرنتيلين بيتا هو بروتين يوجد على سطح الحيوان المنوي و ينتمي إلى عائلة البروتينات السطحية حيث يعتقد أن هذا العامل هو الوسيط للاتصاق والاندماج بين الحيوانات المنوية والبويضة والذي يلعب دورا هاما في الإخصاب عند الثدييات. وتهدف هذه الدراسة إلى فحص العلاقة بين هذا العامل وقدرة الحيوانات المنوية على الإخصاب على النحو الذي يحدده تفاعله مع وظائف الحيوانات المنوية عن طريق مقارنة التعبير الجيني للحمض النووي الريبوزي للفرنتيلين بيتا في مجموعة الرجال الذين يعانون من العقم مع المجموعة ذات الخصوبة العادية، وربط هذا بالقياسات الهرمونية لهذه المجموعات.

وقد أجريت هذه الدراسة على عينات السائل المنوي من ١٤١ رجلا (٨١ رجلا يعانون من العقم من المترددين على عيادة أمراض الذكورة في مستشفى المنصورة الجامعي وكذلك ٦٠ رجلا ذو خصوبة طبيعية للمقارنة). وقد تم تحليل عينات السائل المنوي طبقا لتوصيات منظمة الصحة العالمية باستخدام جهاز يعمل بالكمبيوتر وقياس نشاط الاكروزين، وقد تم قياس التعبير الجيني للفرنتيلين بيتا بطريقة النسخ العكسي لتفاعل البلمرة المتسلسل (RT-PCR).

وقد تم قياس هرمون FSH ، LH، وهرمون التستوستيرون وهرمون البرولاكتين بطريقة ELISA. وقد أظهرت الدراسة انخفاض مستوى التعبير الجيني للحمض النووي الريبوزي للفرنتيلين بيتا في مجموعة العقم بالإضافة إلى القدرة على الحركة وأيضا انخفضت الوظائف الأخرى. كما اتضح أن التغيرات في الفرنتيلين بيتا يمكن أن تؤثر في التشكل الطبيعي للحيوانات المنوية وبالتالي قدرتها على الإخصاب. ويرتبط العقم مع انخفاض التعبير الجيني للحمض النووي الريبوزي للفرنتيلين بيتا في الحيوانات المنوية الذي قد يؤدي إلى فشل الهجرة إلى قناة فالوب نتيجة الخلل في تجهيز الفرنتيلين بيتا وعدم نضج الحيوانات المنوية.