

Human sperm centrin levels & outcome of intracytoplasmic sperm injection (ICSI) - A pilot study

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Background & objectives: The objective of the present study was to compare the levels of sperm centrin a centrosomal protein that influences cell migration, in normal fertile donors and in oligoasthenozoospermic males (count 5 million/ml and motility <40%, grade c+d) undergoing intracytoplasmic sperm injection (ICSI) and to correlate with the outcome of ICSI.

Methods: The prospective study carried out at Inkus IVF Centre, Mumbai, India, during (January-December 2003). It included 20 normal fertile donor males (group I) and 20 oligoasthenozoospermic (OA) males (group II). Group II was further divided in II a and II b according to the centrin levels. Centrin levels were measured by using enzyme linked immunosorbent assay (ELISA) in both groups. All participants underwent an ICSI procedure and the levels of centrin and outcome of ICSI were correlated.

Results: Centrin levels were significantly lower ($P<0.001$) in group II (0.39) as compared with group I (1.34). With centrin levels <0.45 optical density (OD) (group II a) the pregnancy rate was further reduced, with only 2 pregnancies (out of 14) both of which, ended in abortion. Cases in group II showed levels of centrin much lower than in the fertile group. Further lowered centrin levels were associated with lowered pregnancy rates in OA males, but statistically was not significant.

Interpretation & conclusions: The study revealed that lower centrin levels in OA males resulted in lower pregnancy percentage in this group after ICSI. Disturbances in centrosomal protein could be one of the possible causes of ICSI failure.

Key words Centrin - human - ICSI - oligoasthenozoospermia - pregnancy outcome - sperm

Centrosome is the microtubule organizing centre (MTOC) paternally inherited by oocyte during fertilization^{1,2}. Highly organized organelles, the mammalian centrosomes consists of a pair of centriole and pericentriolar matrix that surrounds the individual centriole and the microtubules^{3,4}. Centrosomes are involved in the nucleation and organization of the microtubule in the oocyte and leads to union of sperm

and oocyte as well as for formation of mitotic spindle⁵. In human spermatogenesis characteristic double centrioles are seen. The proximal centriole remains associated with the dense fibers of the flagellum and the distal forms the axoneme¹. Following entry into the oocyte, the sperm centrosomes form the MTOC essential for the movement and fusion of male and female pronuclei^{6,7}. The sperm centrosome is preserved

where there is paternal inheritance, while in the oocyte, centrosome is reduced/inactivated during oogenesis.

Direct assessment of sperm centrosome function is difficult though scientists have used heterologous intracytoplasmic sperm injection (ICSI) technique⁸⁻¹¹. These studies indicate the importance of sperm centrosomal function and their dysfunction leading to infertility. However, the precise molecular and cellular mechanism following the entry of sperm into the cytoplasm is limited.

The centrosome serves as a scaffold for anchoring an extensive number of regulatory proteins. Apart from various other proteins centrin has been reported to be associated with centrosome duplication and to sever axonemal microtubules from their associated basal bodies¹²⁻¹⁶.

Centrin is a 20 KDa calcium binding protein. The deficiency of which causes defects in basal body replication, segregation and maturation¹⁷. The expression of centrin was reported to be reduced in human sperm with dysplasia of fibrous sheath, a rare form of teratozoospermia¹⁸. The present study was undertaken to assess the role of centrin on the outcome of ICSI. The levels of centrin was measured in two groups of males: normozoospermic and oligoasthenozoospermic (OA) males. In this study we present an analysis of ICSI outcome, with reference to centrin levels in these two groups.

Material & Methods

Collection of semen samples: Human sperms were obtained from fertile donors (n=20) and OA patients (n=20) at Inkus IVF Centre, Mumbai, during January - December 2003 participating in an ICSI programme. The approval of institutional review board was obtained. Informed consent was obtained from all patients and donors. The collection of semen was done by masturbation after 3-4 days of sexual abstinence. The semen analysis was performed according to the World Health Organization manual¹⁹. The normozoospermic males (group I) had normal semen parameters count > 20 million/ml, motility > 50 per cent, grade a + b, morphology > 15 per cent normal forms. The OA males (group II) had semen parameters count < 5 million/ml, motility < 40 per cent, grade c + d morphology > 15 per cent normal forms. Individual sperm morphology was assessed according to the WHO criteria for normality and abnormality. Malformation of sperm head, midpiece and tail led to

the classification "abnormal". The cases for this study were selected based on semen parameters. For ICSI morphologically normal spermatozoa was selected for injection into the oocyte, from the pool of washed sperm sample.

Ovarian stimulation and ICSI procedures: These were done according to the methods described earlier²⁰. Pregnancy was confirmed by measuring β -hCG concentration, two weeks after embryo transfer.

Levels of centrin by enzyme linked immunosorbent assay (ELISA): The cell ELISA was done by a modified method of Malviya *et al*²¹. The semen samples from both the groups were washed thrice in phosphate buffer saline (PBS, pH 7.5). The concentration of washed sperm cell was adjusted to 1 million with 0.01M PBS (pH 7.5). The cells were coated on to the ELISA wells and incubated overnight at 37^o C. To each well was added 0.25 per cent glutaraldehyde to fix the cells and incubated for 1 h at 37^o C. Three washings with 0.1 per cent Tween PBS were given each for 5 min. The cells were then blocked with 1 per cent bovine serum albumin (BSA) - PBS for 1 h at 37^o C. The cells were then incubated with primary antibodies (anti centrin 20H5, 1:100 prepared in 0.01 M PBS), overnight at 4^o C. Anticentrin 20 H5 primary antibody was a gift from Dr. Salisbury (Mayo Clinic College of Medicine, Mayo Clinic, Rochester, Minnesota 55905, USA). Three washes with 0.1 per cent Tween-PBS were given for five minutes each. Further, the cells were incubated with secondary antibodies conjugated with horseradish peroxidase- anti-mouse IgG (1:200) for 1 h at 37^o C.

After incubation in the dark for 20 min in 200 μ l of orthophenyl diamine (OPD) substrate solution (8 mg OPD + 0.03% H₂O₂ in 0.1 M citric acid and 0.2 M sodium hydrogen orthophosphate) the bound peroxidase was visualized.

The reaction was terminated by adding 100 μ l of 4 N H₂SO₄ to each well and colorimetric readings were taken at 490 nm in a titertek multiscan plate reader (Titertek, USA). Negative control was with PBS (0.01 M).

Cell smears often need to have the lipid components of the cell membranes broken down to improve penetration of the antibodies. This breakdown may be affected by soaking the preparations in a buffer containing detergent such as triton X-100 or tween 20.

Statistical analysis: t test was used to compare group I and group II. Between groups I, II a and group b, one way analysis of variance was used to test the significance.

Chi square test was performed for comparison between groups I and II.

Results

The level of centrin in group I was significantly higher ($P<0.001$) compared to group II (Table I). Group II was further classified arbitrarily into two subgroups - II a (n=14) (centrin levels <0.45 OD) and II b (n=6) (centrin levels >0.45 & <1.0 OD). Significant decrease was also observed in group IIa as compared with group II b and group I (Table I).

There were 10 pregnancies in group I (50%), of which 2 aborted and others delivered full term normal babies whereas in group II with low centrin levels, the pregnancies occurred only in 5 cases (25%) of which two (40%) aborted. In group II a, where the pregnancy occurred in only two cases (14.3%), both aborted. In group IIa the centrin level was > 0.45 OD and < 1.00 OD, there were 3 pregnancies (Table II). The lowered centrin levels was seen in OA males and resulted in lower pregnancy percentage in this group after ICSI. The levels of centrin seem to be critical not only for sperm motility but also for the initiation and continuation of pregnancy specially in cases where levels are < 0.45 OD. Although the percentage-wise data showed a 50 per cent reduction in pregnancy rate in group II, statistically it was not significant.

Table I. Centrin levels in groups I, IIa and IIb

Groups	Levels of centrin, mean \pm SD	Range	
		Minimum	Maximum
Group I (n = 20)	1.34 \pm 0.13	1.12	1.60
Group II	0.39 \pm 0.09		
Group II a (n =14)	0.3543 \pm 0.02	0.19	0.45
Group II b (n = 6)	0.4817 \pm 0.01	0.46	0.51

* $P<0.001$ (ANOVA)

Table II. Analysis of outcome of ICSI in groups I, II, (II a and II b)

	Pregnancy % (number)	Abortions% (number)
Groups I (n=20)	50 (10)	20 (2)
Group II (n=20)	25 (5)	40 (2)
Group II a (n=14)	14.3 (2)	100 (2)
Group II b (n=6)	50 (3)	Nil

Discussion

The centrosome specially the proximal centriole is considered to be the kinetic centre of sperm motility. Any disturbance in the molecular content and arrangement may lead to low motility. It has been reported that proximal sperm centriole is carried into the oocyte at fertilization⁶. Therefore it is connected with process of fertilization and cell division of fertilized oocyte. The human sperm centrosome is responsible for normal syngamy and early embryonic development²². It is possible that immotile and non-progressive motile spermatozoa may possess abnormalities or absence of centrioles. Hence, we studied the expression of centrin in OA males and compared it with normozoospermic fertile males.

We found a decrease in centrin expression in OA males as compared with normal fertile males suggesting a role for centrin in motility of spermatozoa. None of the OA patients had centrin levels in the fertile range. Since centrin is a calcium binding protein involved in microtubule severing during flagellar excision¹³, it might play a vital role during fertilization. Zoran *et al*²³ have proposed that the calcium, transient at fertilization, triggers the excision of sperm axoneme from the centrosome. Studies have demonstrated that fertilization is accompanied by an increase in the intracellular calcium. This increased calcium might be a centrin induced uncoupling of sperm tail axoneme from the basal body also helping in the separation of centrioles at anaphase²⁴. Hence centrin has a function in the increase of intracellular calcium levels during acrosome reaction. The decreased levels of centrin in OA cases may hamper the acrosome reaction which requires an intracellular calcium levels, thus leading to failed fertilization.

Abnormal centrin expression has been shown in teratozoospermic males¹⁸ where the sperm exhibited abnormal alignment of head tail function, indicating severe centrosomal disturbance. The present study pointed to disturbances in centrosomal protein centrin as a possible cause of failure with ICSI. Lowered centrin levels may lead to lowered intracellular calcium leading to malfunctioning of the centrosome and spindle formation. This in turn may lead to chromosomal aberrations²⁵. The pregnancy rate in group I was similar to the earlier reported studies with ICSI²⁶. The pregnancy percentage was significantly lower when the centrin values were below 0.45 OD. There were only two pregnancies both of which resulted in abortion indicating that probably centrin alterations may

compromise not only fertilization but also embryo quality and progression of pregnancy. As with any physiological measure there is a range of values, some fall in the grey zone and some values are so low that fertilization may be hampered. These results act as pointers to the cause of ICSI failures and there is a need to undertake in depth study on a larger number of cases. A number of causes in males have been shown to result in reproductive failures. To mention a few: male age, paternal exposure to toxic material, occupation²⁷⁻²⁹, anomalies of chromatin organization, reduced decondensation of chromatin, DNA strand breaks DNA fragmentation³⁰⁻³⁶ and elevated levels of sperm aneuploidy^{20,37}. Apart from these causes our study points to the possibility of centriolar defects resulting in failed fertilization and embryo development specially in men with compromised fertility status. Understanding of this aspect may be important for designing novel approaches for diagnosis and treatment of infertility.

In conclusion, there was a decrease in centrin levels in asthenozoospermic cases. The percentage pregnancies after ICSI was significantly reduced in the group with lowered centrin levels. Our results point to disturbances in centrosomal protein centrin as a possible cause of failure of ICSI. Since the number of cases studied was small, further studies with more number of patients will be necessary to confirm this finding.

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