Original Article

Effects of Sperm Acrosomal Integrity and Protamine Deficiency on *In Vitro* Fertilization and Pregnancy Rate

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Abstract

Background: The objective of this study was to evaluate the relationship between protamine deficiency, and acrosomal integrity with fertilization and pregnancy rate in patients undergone in vitro fertilization (IVF).

Material &Methods: Semen samples from 70 infertile couples undergoing IVF at Isfahan Fertility and Infertility center were assessed in this study. Semen analysis was carried out according to WHO criteria. Protamine deficiency, Sperm morphology and acrosin activity were assessed by Chromomycin A3 (CMA3), Papanicolaou staining and Gelatinolysis tests, respectively. Coefficients of correlation and student t-test were carried out using the Statistical Package for the Social Studies (SPSS 11.5) and P-value lower than 0.05 was considered as significant.

Results: Fertilization rate, percentage of halo formation, mean halo diameter and abnormal morphology show a significant correlation with percentage of CMA3 positivity. CMA3 positivity, percentage of halo, mean halo and sperm morphology showed a significant correlation with fertilization rate. Among the aforementioned parameters percentage of halo had the highest correlation. In the present study patients were divided into two groups according to pregnancy status. None of the studied parameters were significantly different between pregnant and non-pregnant patients. However, percentage of halo formation showed a slightly significant difference ($r=0.306$; $P=0.058$).

Conclusion: The results of this study revealed that, even though sperm morphology, sperm protamine content and acrosome formation are events related to spermiogenesis, sperm acrosomal integrity assessed by percentage of halo formation has more profound effect on fertilization rate and pregnancy outcome during IVF procedure.

Keywords: Acrosin Activity, Protamine Deficiency, IVF, Pregnancy

Introduction

During late spermatogenesis, concomitant with the formation of acrosome and perinuclear theca, somatic cell histones are replaced by protamines. Proatmines are small positively charges, testis - specific nuclear proteins, that facilitate chromatin condensation and compaction. This type of compaction has many properties; including, increasing hydro dynamicity of the sperm, protection of sperm from DNA damage and synchronization of the cell cycle between sperm and oocyte. In addition protamine is believed to be involved in the imprinting of the paternal genome during spermatogenesis (1-3).

Studies have shown that an aberration in protamine content results in human male infertility. In particular, men with abnormal expression of protamine gene present with diminished parameters of semen quality, such as sperm count, motility and head morphology (4-9).

Recent studies on the failed fertilized oocytes post ICSI show that, premature chromosomal condensation (PCC) is higher in the semen samples with protamine deficiency relative to semen samples with normal protamine content (10). PCC takes place when chromatin from a cell in G1 phase of cell cycle interacts with cytoplasm of a cell in metaphase. In natural fertilization, sperm is in G1 of cell cycle while the oocyte is in metaphase II; however, in such circumstance PCC does not take place, since the sperm chromatin mainly contains protamines and not histones (11, 12). Meiosis promoting factor (MPF) which induces chromatin condensation can only act on the histones and not on protamines. Upon entrance of sperm into the oocyte, sperm associated oocyte activating factors (SAOAF), is released

from the perinuclear theca associated with the acrosome, and results in inactivation of MPF. During inactivation of MPF, protamines are replaced with histones, oocyte terminates meiosis and enters G1 phase of cell cycle and becomes synchronized with sperm. However, when a protamine deficient sperm or sperm with excessive histones enters the oocyte, due to presence of excessive histones, sperm undergoes PCC and may result in failed fertilization (13-17).

The acrosome reaction (AR) is another essential prerequisite for successful fertilization (18). The AR is a modified exocytotic event in which the outer acrosomal membrane fuses with the plasma membrane of the spermatozoon at discrete points resulting in hybrid membrane vesicles (19). These vesicles then detach from the spermatozoa and finally lead to the complete loss of the acrosome with the release of the acrosomal enzymes which are thought to play a role in the penetration of spermatozoa through out the outer oocyte vestments (19, 20). Acrosin is a trypsin-like serine proteinase that is exclusively found within the acrosome of mammalian spermatozoa (21). It is associated with the zona pellucida binding and zona pellucida penetration of spermatozoa. Moreover, this enzyme has been found to be involved in capacitation, acrosome reaction, and chromatin decondensation during male pronucleus formation (21).

Different studies have shown that failed fertilization is related to reduce acrosin activity in the acrosome and/or reduced protamine content in the DNA (21-25). Therefore, the aim of this study was to evaluate the protamine deficiency through assessment of global protamine deficiency by Chromomycin A3 and acrosome integrity by gelatinolysis test in order to define which of these factors have a more profound effect on fertilization and pregnancy rate during in vitro fertilization procedure.

Material and Methods
Sperm preparation
This study was initially approved by the ethical and scientific committee of Isfahan Fertility and Infertility center and Royan institute. Semen samples were obtained from 70 couples referred to Isfahan Fertility and Infertility Center for IVF treatment. The

semen samples were collected by masturbation after 3–4 days of abstinence on the day of oocyte recovery. Routine semen analysis was carried out by light microscopy according to WHO criteria (26). Media used for IVF procedures were obtained from Vitrolife, GIII series (Gothenburg, Sweden).

Semen samples were prepared for routine IVF using discontinuous Pure Sperm gradients (80: 40) (Nidacon, Gothenburg, Sweden) and then washed using G-Rinse. After insemination of oocytes, the remaining processed samples were used for evaluation of protamine deficiency, acrosin activity and sperm morphology using chromomycin A3 staining, gelatinolysis test and modified Papanicolaou staining, respectively.

In vitro fertilization protocol
Ovarian stimulation and ovulation induction were induced using, Buserelin, human menopausal gonadotropin (hMG, Menogon,) in combination with recombinant FSH (Gonal-F) and hCG injections daily (Organon, Holland). Oocyte cumulus complexes (COCs) were retrieval by trans-vaginal needle-guided ultrasound at 36 h post hCG. COCs were collected into G-Mops and then transferred into G-Fert, where they were inseminated by 100,000 sperm per oocyte. 16 to 18 hours post insemination oocytes were dissected and fertilized oocytes were defined by presence of pronuclei. Fertilized oocytes were then further cultured in G1 for the next 48 hours. Embryos were scored daily. Embryos for transfer were selected on basis of morphology and cell stage and were transferred using Labotect (Germany) transfer catheters (24). Pregnancy was initially assessed by ß-hCG and was further confirmed by ultrasound based on fetal heart beat. Fertilization rate was calculated by dividing number of 2 pronuclei zygotes with number of metaphase II oocytes. Pregnancy rate was calculated by dividing the number of pregnancies over the number of embryo transfer cycles.

Assessment of protamine deficiency (Chromomycin A3 staining)
Processed semen samples were fixed in Carnoy's solution [methanol: glacial acetic acid 3:1 (Merck, Germany)] at 4 °C for 5 min. Smears were prepared and each slide was treated for 20 min with 100 µl of CMA3

solution (Sigma, USA) [0.25 mg/ml in McIlvaine buffer (7 ml citric acid 0.1 M + 32.9 ml $Na_2HPO_4 \times 7H_2O$, 0.2 M, pH 7.0, containing 10 mM MgCl2)]. The slides were then rinsed in buffer and mounted with buffered glycerol (1:1). Microscopic analysis of the slides was performed using an Olympus fluorescent microscope (BX51, Tokyo, Japan) with the appropriate filters (460–470 nm). On each slide, 100 sperm cells were evaluated. Evaluation of CMA3 positivity was carried out using Olysia software. Pixel intensity of each sperm was recorded. Sperm with pixel intensity of higher than 100 were considered as CMA3 positive or protamine deficient, while those with pixel intensity of lower than 100 were considered as CMA3 negative or with normal amount of protamine (22).

Gelatinolysis Test

Acrosome integrity was evaluated by the gelatin slide test. The test is based on the ability of acrosomal enzymes to hydrolyze a high molecular weight protease like gelatin. Gelatinolysis test was carried out according to Henkel *et al* 1995 (21).

Twenty microliters of processed semen sample were diluted for evaluation of acrosin activity 1:10 in phosphate buffered saline (34.22 mM Nacl, 20.8mM $Na_2HPO_4 \times 2H_2O$, 1.42 mM KH_2PO_4) containing 15.72 mM α-D glucose(anhydrous), (Osmolarity: 280 mosml/kg).

Semen samples were smeared on pre-coated gelatin slides and incubated in a moist chamber at 37°C for 2 hours. The halo diameter around each 100 spermatozoa which was representative of sperm present in the ejaculate was measured in phase contrast (Olympus, CKX41, Tokyo, Japan) with Olysia software. The halo formation rate was calculated per slide as the percentage of spermatozoa showing a halo.

Statistical Analysis

Coefficients of correlation and Student *t-test* were carried out using the Statistical Package for the Social Studies (SPSS 11.5) software to compare results between different groups.

Results

Semen samples were obtained from 70 IVF candidates. The mean age of the female and male partner was 30 ± 4.7 and 34.4 ± 4.5 respectively. The result of semen parameters, Gelatinolysis test, protamine deficiency, fertilization, and pregnancy rate are shown in table 1.

The results of the correlation analysis of aforementioned parameters with fertilization rate, percentage CMA3 positivity, percentage of halo formation and mean halo diameters are presented in table 2.

Fertilization rates, percentage of halo formation, mean halo diameters and sperm abnormal morphology show significant correlation with percentage CMA3 positivity.

Moreover, fertilization rate shows a significant correlation with percentage of halo, mean halo diameters and sperm abnormal morphology.

Percentage of halo also shows a significant negative correlation with sperm abnormal morphology. In this study, mean halo diameter shows a significant correlation with sperm abnormal morphology, motility and density.

Pregnancy results were obtainable from 39 out of 70 IVF candidates. 20 out of 39 patients were pregnant and had positive ß-hCG and fetal heart beat. Patients were divided according to pregnancy status.

Age of female partner, semen parameters, percentage of halo formation, mean diameter of halo, percentage CMA3 positivity, number of oocytes, fertilization rate, total number of embryos on day3, and total number of good quality 8-16 cell embryo were compared in the two groups (table 3).

The results reveal that among the aforementioned parameters, only percentage of halo formation was close to be significantly (P= 0.058) different between the pregnant and non-pregnant couples.

Previous studies revealed that patients with higher than 60% halo may have higher fertilization rate, therefore, patients were also sub grouped according to lower and higher than 60% halo.

Percentage of fertilization and the mean number of good quality embryos between patients with lower and higher than 60% halo were compared.

Percentage of fertilization and mean number of good quality embryos were significantly lower in the patients with lower than 60% halo (P<0.05).

Table 1: Descriptive analysis of semen parameters, acrosome integrity, %CMA3 positivity and fertilization rate

Parameters	Number of cases	Minimum	Maximum	Mean	Std. Deviation
Concentration (million/ml)	68	5	120	55.33	25.70
% Motility	68	5	70	46.57	13.76
% Abnormal morphology	68	38	100	65.07	12.84
% Halo	70	42	100	77.67	12.92
Mean halo diameter	70	5.53	17.90	12.31	2.94
% CMA3 positivity	60	11	100	42.10	17.24
% Fertilization post IVF	50	20	100	71.62	23.41

Table 2: The correlation between different sperm parameters with fertilization rate, percentage CMA3 positivity, percentage of halo formation and mean halo diameter

parameters	Fertilization rate r (P-Value)	% CMA3 positivity r (P-Value)	% Halo formation r (P-Value)	Mean halo diameter r (P-Value)
Concentration (million/ml)	0.007 (0.964)	-0.099 (0.451)	0.103 (0.401)	0.259 (0.033)*
% Motility	0.337 (0.142)	-0.223 (0.087)*	0.203 (0.097)	0.308 (0.011)*
% Abnormal morphology	-0.330 (0.022) *	0.274 (0.034) *	-0.339(0.005) **	0.282 (0.020)*
% Halo	0.445 (0.001) **	-0.290(0.025) *	---	0.789(0.000) **
Mean halo diameter	0.322 (0.023) *	-0.363 (0.004)**	0.789 (0.000)**	----
% CMA3 positivity	-0.340(0.022)*	---	-0.290(0.025)*	-0.363(0.006)* *

*: Correlation is Significant at the 0.05 level   **: Correlation is Significant at the 0.01 level

Table 3: Comparison of different male and female parameters between pregnant and non-pregnant couples.

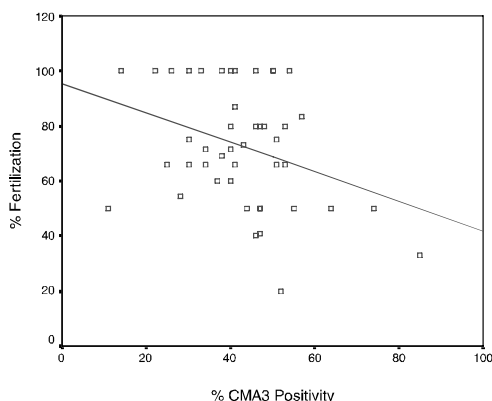| Parameters	Non-pregnant Mean± SD	pregnant Mean± SD	P-Value
Concentration (million/ml)	51.94±26.97	57.21±26.52	0.548
% Motility	45.89±17.11	46.05±11.37	0.973
% Abnormal morphology	64.00±15.27	61.78±10.32	0.605
% Halo formation	74.10±16.84	82.50±8.95	0.058
Mean halo diameter	11.89±3.43	13.29±2.42	0.153
% CMA3 positivity	37.18±16.34	40.61±13.59	0.519
Fertilization rate	70.29±24.28	75.30±22.24	0.581
Age of female partner	32.69±5.10	30.11±3.59	0.133
No. of oocytes	8.57±5.12	8.27±6.56	0.888
total number of embryos on day3	4.76±3.41	4.00±2.26	0.484
total number of 8-16 cell embryo	3.76±2.48	2.86±1.72	0.284

Figure 1: Correlation between percentage of sperm with protamine deficiency and fertilization rate (r = -0.340, P = 0.022)
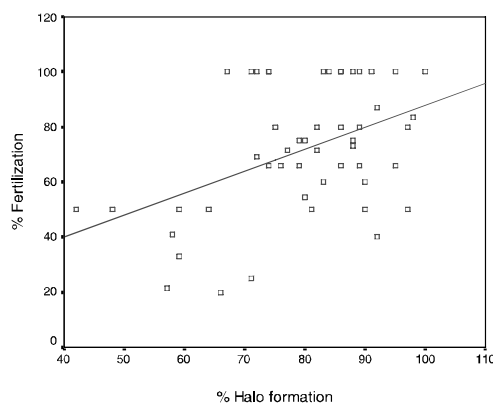

Figure 2: Correlation between % halo formation and fertilization rate (r = 0.445, P = 0.001)

Discussion

For fertilization to be successful, sperm and egg components must mutually initiate the cascade of events required for full embryonic development. The subset of these events that immediately follow sperm-egg fusion and that lead up to mingling of sperm and oocyte chromosomes is collectively termed "oocyte activation". The literature reveals that fertilization failure may be explained by different factors related to oocyte or/and semen characteristics.

Such factors include the number of oocytes, quality of oocytes, viability, motility, morphology, acrosome integrity and chromatin status of the semen sample (27-29). Sperm protamines are ubiquitous in the sperm of mammals, which is an indication of their importance in reproduction (1, 30-32). Protamines are necessary for proper packaging of DNA during spermiogenesis and may have other significant functions (31). In mice, protamine haplo insufficiency causes severe infertility and results in abnormal embryo development (33, 34). Previous studies in human beings have noted that protamine deficiency may also be relate to male infertility (4-9, 35-40).In this study, also a significant negative correlation was observed between protamine deficiency and fertilization rate. Like previous studies, this may indicate that protamine deficiency is associated with failed fertilization (22, 41).

Relationship between protamine deficiency and fertilization rate could be evaluated from different aspects. A significant correlation has been reported between protamine deficiency and semen parameters (5, 8). The results of this study also showed a positive correlation between abnormal morphology assessed according to WHO criteria with CMA3 positivity, indicating that CMA3 may implement its effect through sperm morphology. However, fertilization is a multifactorial process and, logistic regression analysis has been used to determine which of the semen parameters or nuclear maturity tests affect fertilization independently (22). The results of these types of analysis reveal that abnormal morphology and protamine deficiency affect fertilization independently (22). Therefore, one may conclude that defect in protamination renders spermatozoa functionally immature and thus protamine

deficiency may limit fertilization of oocyte (22). In this study the significant negative correlation observed between protamine deficiency and abnormal morphology with fertilization rate further confirms the above possibility (table2).

Protamine deficiency may affect fertilization, independent of sperm morphology, through inducing PCC. Protamine deficient sperm with normal morphology may pass through zona pellucida and oocyte, however, when such sperm enters oocyte it undergoes PCC due to active MPF present in the MII oocyte and therefore causes failed fertilization (23, 42-43). In this study acrosomal integrity was evaluated by gelatinolysis test. This test is base on the ability of acrosomal enzyme to hydrolyze high molecular weight proteins like gelatin. This test has been considered as an index for acrosomal integrity (21). In this study a negative correlation was observed between protamine deficiency and acrosomal integrity (mean halo and percentage halo), suggesting that protamine deficient sperm may also have acrosomal defect and therefore are unable to penetrate the oocyte. Such correlation indicates simultaneous occurrence of sperm chromatin remodeling with acrosome formation. Further more, the data in table 2 indicate that the correlation coefficient between fertilization with percentage of halo formation is higher than the correlation between fertilization rate and protamine deficiency in IVF patients (figure 1, 2), thus suggesting that acrosomal integrity has a more profound effect on fertilization rate than protamine deficiency. This is likely explained by the fact that sperm with normal acrosome not only have higher ability to bind and penetrate the zona pellucida but also their potential to induce oocyte activation is higher. Unlike in IVF, the results of previous study suggest that in ICSI cases, the significance of sperm protamine deficiency is higher than sperm abnormality. This could be explained by the fact that protamine deficient sperm are not only prone to sperm PCC but also such sperm have lower potential to activate the oocyte.

Acrosin activity has been shown to be predictive of the fertilizing capacity of human spermatozoa (21). Normal acrosin activity indices are observed in men with high fertilization rates, whereas the halo diameters and halo formation rates are smaller in most

cases of poor fertilization (halo formation<60%, halo diameter<10µm) (21, 44). The result of this study, also show a significant correlation between acrosome integrity indices and fertilization rate (figure 1). By looking at this scatter the results verify those of Henkel et al (1995, 2004) which showed that halo formation rate of less than 60% is indicative of lower fertilization rate. Patients with normal acrosin activity indices and low fertilization, probably have defects other than impaired acrosin activity (44). In this study, patients were divided into two groups according to pregnancy status (table 3) and semen parameters, protamine deficiency, and acrosome indices were compared between pregnant and non-pregnant couples. Except percentage halo formation which showed a closely significant difference (r=0.306; P=0.058), none of the aforementioned parameters were significantly different between the two groups. Patients were also grouped according to percentage of halo formation lower and higher than 60% and percentage fertilization. Mean number of good quality embryos were analyzed between these two subgroups. Percentage fertilization and mean number of good quality embryos were significantly lower in the patients with lower than 60% halo, indicating that high percentage halo results in higher fertilization rate and presence of higher good quality embryo for transfer, thus leading to higher pregnancy rate.

 Furthermore, Tomlinson et al compared semen parameters, DNA damage, and percentage of CMA3 positivity in the pregnant and non-pregnant groups.

The outcomes revealed that, among the aforementioned parameters, only percentage of DNA- damaged spermatozoa was significantly different between pregnant and non - pregnant couples (45).

This could be explained by the fact that semen parameters and chromatin remodeling influence fertilization or early events post fertilization and not after genomic activation and subsequent development which are more related to pregnancy.

In addition, Aoki et al assessed P1/P2 ratio via gel electrophoresis. They observed a significant correlation between P1/P2 ratio and pregnancy rate, whereas no significant correlation was reported between P1, P2 and total protamine concentration with pregnancy rate, which further confirms our result regarding insignificant correlation between global protamine deficiency and pregnancy (41).

Conclusion
The results of this study reveal that, even though, sperm morphology, sperm protamine content and acrosome formation are events related to spermiogenesis, but sperm acrosomal integrity assessed by percentage of halo formation have a more profound effect on fertilization rate which may indirectly affect pregnancy outcome in IVF procedure.

Acknowledgments

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