Original Article

Using Deep Learning Algorithm: The Study of Sperm Head Vacuoles and Its Correlation with Protamine mRNA Ratio

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Abstract

Objective: It is necessary to evaluate fertility effective agents to predict assisted reproduction outcomes. This study was designed to examine sperm vacuole characteristics, and its association with sperm chromatin status and protamine-1 (*PRM1*) to protamine-2 (*PRM2*) ratio, to predict assisted pregnancy outcomes.

Materials and Methods: In this experimental study, ninety eight semen samples from infertile men were classified based on Vanderzwalmen's criteria as follows: grade I: no vacuoles; grade II: ≤2 small vacuoles; grade III: ≥1 large vacuole and grade IV: large vacuole with other abnormalities. The location, frequency and size of vacuoles were assessed using high magnification, a deep learning algorithm, and scanning electron microscopy (SEM). The chromatin integrity, condensation, viability and acrosome integrity, and protamination status were evaluated for vacuolated samples by toluidine blue (TB) staining, aniline blue, triple staining, and CMA3 staining, respectively. Also, *Protamine-1* and *protamine-2* genes expression was analysed by reverse transcription-quantitative polymerase chain reaction (PCR). The assisted reproduction outcomes were also followed for each cycle.

Results: The results show a significant correlation between the vacuole size (III and IV) and abnormal sperm chromatin condensation (P=0.03 and P=0.02, respectively), and also, protamine-deficient (P=0.04 and P=0.03, respectively). The percentage of reacting acrosomes was significantly higher in the grades III and IV spermatozoa in comparison with normal group. The vacuolated spermatozoa with grade IV showed a high protamine mRNA ratio (*PRM-2* was underexpressed, P=0.01). In the IVF cycles, we observed a negative association between sperm head vacuole and fertilization rate (P=0.01). This negative association was also significantly observed in pregnancy and live birth rate in the groups with grade III and IV (P=0.04 and P=0.03, respectively).

Conclusion: The results of our study highlight the importance sperm parameters such as sperm head vacuole characteristics, particularly those parameters with the potency of reflecting protamine-deficiency and *in vitro* fertilization/ intracytoplasmic sperm injection (IVF/ICSI) outcomes predicting.

Keywords: Algorithm, Human Sperm, Pregnancy, Protamines, Vacuole

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Introduction

While normal sperm parameters were found in almost 15% of infertile males, the routine semen analysis is not sufficient to evaluate the male fertility status (1). Nowadays, several assays have been suggested to achieve more details in the male infertility diagnosis (2), which included nucleus assessment (chromatin integrity and condensation, protamination status, aneuploidy), and sperm function assay (1, 2). In addition, the evaluation of the detailed morphology of motile sperm in real-time at a high magnification (up to ×6600) which is called the motile sperm organelle morphology examination (MSOME), is another of these evaluations. In fact, MSOME is seen sperm morphology with more details, which is not provided at ×400 or ×200 magnifications (2).

Although, the origin and nature of vacuole remain unknown, sperm head vacuole has been defined as one of the most important of sperm abnormalities (2). Sperm morphology, particularly head vacuoles, has a major effect on the assisted reproductive techniques (ARTs) outcomes (3). Therefore, the selection of 'good' spermatozoa prior to intracytoplasmic sperm injection (ICSI) may be a powerful step to obtain better outcomes. The presence of morphologically and morphometrically normal head (2, 4, 5) and lack of vacuoles or less than two small vacuoles is determined a 'good' spermatozoa (6). On the other hand, the classification of spermatozoa has been defined as the following four groups according to the presence or size of vacuoles: grade I: no vacuoles; grade II: ≤ 2 small vacuoles (which occupy < 4% of the head's area); grade III: more than two small vacuoles or ≥1 large vacuole (which occupy between 13% to 50% of the head's surface area); and grade IV: large vacuole with other abnormalities (7). The difference in size and location of sperm-head vacuoles may be associated with chromatin condensation failure as well as nuclear DNA damage (8, 9). So that,

the many studies have indicated the negative impact of spermatozoa' nuclear vacuoles on embryo development, blastocyst rate, and pregnancy outcomes (10-13).

Moreover, replacement of DNA-binding histones by protamines is a most important parameter in the fertilization success (14). Recently, an association between improper protamine mRNA/protein ratio and male infertility has been found. This ratio is known as a suitable biomarker for fertilization success. The relative ratio of protamine-1 to protamine-2 is almost at a 1:1 ratio (15). This relative ratio of protamine-1 to protamine-2 has been reported as a range of 0.5 to 1.4 for normozoospermia specimens (16). The correlation between male infertility and abnormal protamine mRNA ratio has been found (15). However, it should be further investigated whether the protamine mRNA ratio is impacted by vacuolated spermatozoa percentage, and correlated with fertilization, embryo development and pregnancy rates during *in vitro* fertilization (IVF) or ICSI cycles. Therefore, the sperm genomic quality and its association with assisted reproductive outcomes has been located as a one of the most important goals in recent years.

However, it is not clear whether vacuoles influence assisted reproductive outcomes (e.g., fertilization rate, embryo development, and pregnancy rate). Therefore, we used deep learning algorithm (17) to select the 'good' spermatozoa. And also, we tried to investigate relations among vacuole(s) and the protamine-1 to protamine-2 mRNA ratio and assisted reproductive outcomes. In this study, we used a novel deep learning algorithm in combined with high magnification and SEM images to visualize sperm's vacuoles and its association to chromatin status, protamine mRNA ratio, and the sperm fertility potential. Also, we compared the protamine-1 to protamine-2 mRNA ratio among spermatozoa with different vacuole grades. In addition, the acrosome reaction, chromatin condensation and integrity, protamination status, and fertilizing capacity of semen samples with different degrees of vacuolated spermatozoa were studied during ICSI/IVF cycles. To the best of our knowledge, this is the first study to provide empirical evidence for this issue.

Materials and Methods

Participants

A total of 309 specimens was collected from fertile and infertile men (age 22-38 years) who visited in Alzahra hospital (IVF center), Rasht, Iran, between May 2018 to September 2019. This experimental study was approved by the Guilan University of Medical Sciences committee (IR.GUMS.REC.1397.154). In addition, the informed consent was obtained from all the volunteer participants in the present study. The couples who received ICSI or IVF services with an ICSI or IVF failure history were invited to this study. They were excluded based on their spermocytogram, and woman age. The semen samples were collected via masturbation after three to four days of sexual abstinence. The semen samples were analyzed according to the World Health Organization (WHO) criteria (18). The semen parameters such as pH, volume, motility, morphology, concentration, viability were assessed. The vacuolated semen samples were included in this study (n=98). Also, female factor infertility, maternal age >40 years, and less than three oocytes made our exclusion criteria. The couples with male factor infertility (e.g., severe teratozoospermia, asthenozoospermia, and oligoasthenoteratozoospermia) were also excluded from this study to remove effects of other sperm parameters on sperm quality and ART outcomes.

Totally, ninety-eight semen samples were included in this study. The vacuolated sperm categorization and selection during ICSI cycles were performed with both high magnification (×1000) and a novel deep learning algorithm (17) as real-time (× 400). Also, a part of semen sample (~100 µl) was prepared for scanning electron microscopy (SEM) to view and determine the percentage of sperm's vacuoles in more detail. Based on information obtained from evaluations, the semen samples were categorized into four groups according to Vanderzwalmen's criteria: grade I: no vacuoles (normal/control group); grade II: ≤ 2 small vacuoles (which occupy < 4% of the head's area); grade III: more than two small vacuoles or ≥1 large vacuole (which occupy between 13 to 50% of the head's surface area); and grade IV: large vacuole with other abnormalities (7).

Assay using a novel deep learning algorithm

Using deep learning algorithm, sperm morphology, especially vacuole was analyzed. This algorithm was performed with a high accuracy (94.65%) to detect sperm's vacuoles. In addition, this method worked very fast and categorized sperm images in real-time. Therefore, the classification of spermatozoa was done using this algorithm in line with the results of high magnification (×1000) and SEM images.

In this way, for detecting abnormalities in the vacuole, Javadi and Mirroshandel (17) have proposed a novel deep learning approach. They have trained a deep convolutional neural network on mini-batches generated from the training set. The size of a mini-batches in this study is 64, which is a common value. Mini-batch means you only take a subset of all your training data during model construction. Also, they have proposed oversampling and data augmentation in order to overcome the problem of low count of training samples and class imbalance (i.e., the sperms number with abnormal vacuole in our training data is smaller than the number of normal sperms). This network consists of 24 convolutions, three pooling, and two fully-connected layers. The overall trainable parameters of the model are 5,637,649. The implementation of the model was done using TensorFlow and Keras.

Scanning electron microscopy

For correct measurement of sperm vacuole, each semen samples were evaluated by SEM to observe the smallest

details. The semen samples were washed by using sucrose density gradient centrifugation at 3000 rpm for 10 minutes at RT temperature, and then, fixed in Karnovsky solution for 30 minutes at 4˚C. Then, the samples were treated with 1% osmium tetroxide (OT20816-12-0, Merck, Germany) for 30 minutes as a post fixation step. Afterwards, the ascending degrees of ethanol (50, 70, 80, 90, 96%, and absolute alcohol) (E64-17-5, Hamontebmarkazi, Iran) were used to dehydrate. The drying was performed at a critical point (Balzers CPD-010). The specimens coated with gold (MED-010, BALZERS, USA) were examined in a Philips FEM 515 scanning electron microscope (Philips SEM 515, F.E.I. Company, Netherlands).

Sperm chromatin assays

Toluidine blue stain

The abnormality in the sperm chromatin structure was distinguished using toluidine blue (TB) staining. In this way, the air-dried smears (~100 µl semen samples) on silane-coated slides (SL002-72, BioMarq, India) were fixed in 96% ethanol-acetone solution (CAS 67-64-1, Merk, Germany) (1:1) at 4˚C for 1 hour. To hydrolysis, slides were put in 0.1 N HCl (109060, Merck Millipore, Chine) at 4˚C for 5 minutes, then were washed. The staining was done with 0.05% TB (in 50% Mcllvaine's citrate phosphate buffer, pH=3.5) (T92-31-9, Merck, Germany) for 5 minutes at room temperature (RT). On average, 100 sperms were evaluated in each slide using a light microscope. The observation of light blue or deep violet/purple heads is the sign of existence normal or abnormal chromatin structure, respectively (Fig.1A).

Aniline blue stain

The adhesion between lysine residues of histones and aniline blue (AB) stain were detected the abnormal condensation of sperm chromatin. Briefly, the smears (~100 µl semen samples) were fixed in the 4% formalin (50-00-0, Junsei Chemical, Tokyo, Japan). After washing, the slides were stained with 5% AB (AB 229660250, Sigma-Aldrich Co., St. Louis, MO, USA) in a solution of 4% acetic acid (pH=3.5) (A64-19-7, Merk, Germany) for 5 minutes at RT. On average, 100 spermatozoa in each slide were observed under a light microscope. The sperms with dark-blue or colorless heads were considered as abnormal and normal chromatin condensation, respectively (Fig.1B).

Acrosome reaction assessment

The acrosome status (reacted acrosome and intact acrosome) was evaluated using triple staining. In brief, sperms (~100 µl semen samples) were put in 2% trypan blue (1:1) (T10282, Sigma, Germany), incubated at 37˚C for 15 minutes, and centrifuged at 600 × g for 5-10 minutes. Then, the pellet was washed and diluted in the Ham's F10 solution to obtain a clear/ pale blue mixture. In the next step, the washed sperms were fixed using glutaraldehyde (3% glutaraldehyde in 0.1 M cacodylate buffer at pH=7.4) (G111-30-8, Sigma, China) for 30 to 60 minutes, and were centrifuged at 6000×g for 5 minutes. The pellet was stained with Bismark brown Y (10114-58-6 , Sigma, Germany) at 40˚C for 5 minutes. Then, Rose Bengal stain (100467, Merck, Germany) was added at 24˚C for 20-45 minutes. The smears were prepared from stained sperms, washed (in water), dehydrated (in an ascending degree of alcohol), and cleared in xylene (108633, Merck Millipore, China). At the end, almost 100 sperms in each slide were examined under a light microscope. Four staining templates were seen as follows: i. Dead sperm and intact acrosome as dark-blue post-acrosomal regions and pink acrosomes, respectively, ii. Dead sperm and degenerated acrosome as dark-blue post-acrosomal regions and blue/ white acrosomes, respectively, iii. Alive sperm and intact acrosome as light brown post-acrosomal regions and pink acrosomes, respectively, and iv. Alive sperm and degenerated acrosome as light brown post-acrosomal regions and blue/white acrosomes, respectively (Fig.1C).

Chromomycin A3 stain

The degree of sperm protamination was determined by chromomycin A3 (CMA3) staining (89158-860, Sigma, Germany) (Fig.1D) as a detector of guanosine-cytosine-rich sequence. All air-dried smears (~100 µl semen samples) were fixed in the methanol/glacial acetic acid (3:1) for 20 minutes at 4˚C. Then, the slides were treated for 20 minutes with 100 µl of CMA3 solution (0.25 mg/ml CMA3 in McIlvaine's buffer, containing 10 µm $MgCl_2$). The sperms with dull yellow staining (CMA3 negative) and bright yellow fluorescence (CMA3 positive) were considered as normal and abnormal chromatin protamination, respectively.

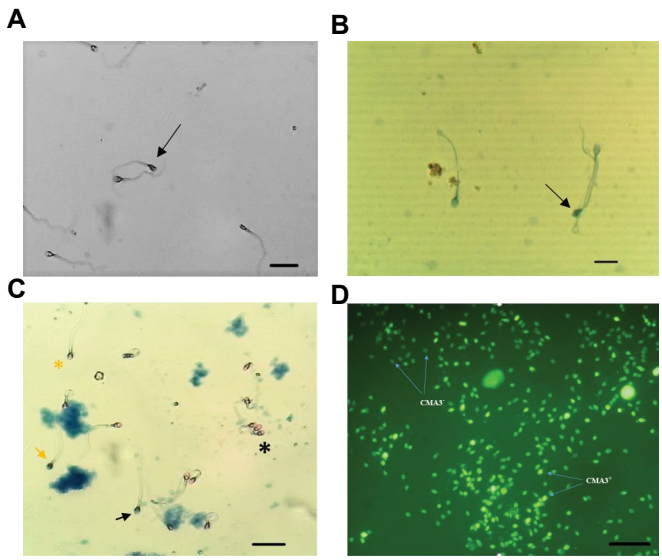

Fig.1: Sperm chromatin assays. **A.** Sperm cell heads with abnormal chromatin structure were deep violet (arrow) following toluidine blue staining. **B.** Sperm cell heads with abnormal chromatin condensation were dark blue (arrow) following aniline blue staining. **C.** The status of sperm acrosome reaction and viability was observed as following: dead sperm with an intact acrosome (black arrow), dead sperm without an acrosome (yellow arrow), live sperm with an intact acrosome (black star), and live sperm without an acrosome (yellow star) (scale bar: 10 µm). **D.** Spermatozoa stained with CMA3 was with dull yellow/ normal chromatin ($CMA3^-$) and bright yellow/abnormal chromatin ($CMA3^+$) (scale bar: 100 µm).

Vacuolization and sperm parameters

The results of AB staining showed significant abnormal condensation rate of chromatin in the grade IV (P=0.02) in comparison with the control group (grade I). There was no significant difference in the viability and abnormality of the DNA structure of vacuolated spermatozoa among different grades (P=0.15, Table 1).

The presence of bright yellow fluorescence (CMA3-positive) was observed more frequently in the spermatozoa with a large nuclear vacuole (LNV) (grade III: 2336⁄4200; 55.6% vs. grade IV: 621⁄1500; 41.4%) than other groups. Therefore, a higher percentage of sperm protamine deficiencies in the vacuolated spermatozoa with grade III (P=0.04) and IV (P=0.03) was detected. Also, the presence of more than one small nuclear vacuole showed more abnormal chromatin protamination in comparison with large non-nuclear vacuole (P=0.03). The percentage of reacted acrosomes (blue/white) was significantly higher in the non-nuclear vacuoles spermatozoa (grade III and IV) in comparison with other groups (P=0.04 and P=0.03, respectively). In addition, there was no significant difference in the sperms viability rate among these different groups (P=0.25).

The protamine mRNA ratio in the vacuolated spermatozoa

As seen in Figure 3, the assessment of *PRM1* gene expression showed a significant difference in the grade III (median 0.4457 ± 0.03, P=0.03) in comparison with the control group (group I, normal semen samples). In addition, comparison between grade IV and control group showed a significant difference in the *PRM1* gene expression (P=0.0001). While, there is no significant difference in the *PRM1* gene expression in the grade II (median 0.83184) in compared to the control group (median 1.0201 ± 0.06, P=0.35, Fig.3). In addition, the analysis of *PRM2* gene expression showed significant differences among grade II (0.6623, P=0.01), grade III (median 0.60262 ± 0.007, P=0.0001), and grade IV (median 0.2772 ± 0.012, P=0.0001) in comparison with the control group (median 1.001 ± 0.04, grade I).

The protamine mRNA ratio was evaluated among different vacuolization grades in the fertile and infertile men. Vacuolated spermatozoa from infertile men with grade IV (median 3.40006 ± 1.81, P=0.008) displayed a significant difference in the protamine mRNA ratio in comparison with the control group (median 1.02 ± 0.81 m, P=0.004).

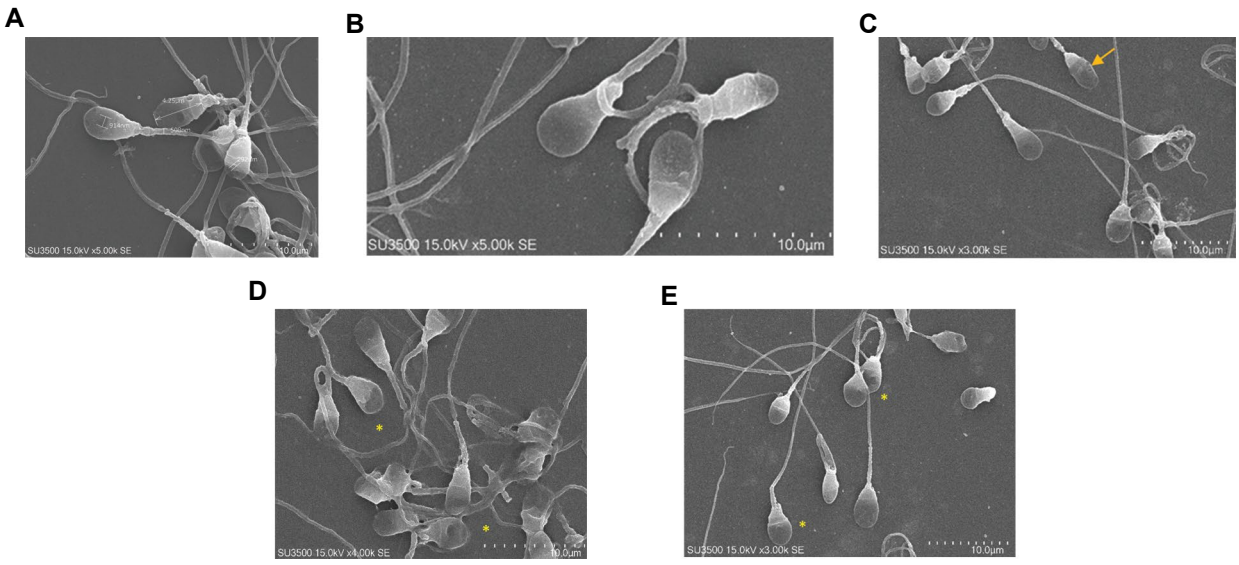

Fig.2: Evaluation of sperm morphology using the scanning electron microscope. **A.** The presence of small and large vacuoles and its location in the nuclear or non-nuclear position is clear. **B.** Grade I: without vacuoles, **C.** Grade II: with ≤ 2 small vacuoles (arrows), **D.** Grade III: more than two small vacuoles or ≥1 large vacuole (Stars), and **E.** Grade IV: with large vacuole (stars).

Table 1: Vacuolization and sperm parameters

Vacuole grade	N	Protamination status (%)	Spont. A.R. (%)	Condensation status (%)	Chromatin integrity (%)	Viability (%)	*PRM1 : PRM2*
I	27	28.3 ± 2.9	15.1 ± 1.9	22.7 ± 2.1	23.7 ± 2.4	65.45 ± 10.08	1.01917
II	24	32.4 ± 2.6	19.8 ± 2.1	29.5± 2.2	26.8± 2.5	59.9 ± 9.81	1.1149
III	23	36.1 ± 3.2*	27.16 ± 2.4*	32.4 ± 3.2	28.9 ± 3.1	59.02 ± 9.07	0.7397
IV	24	41.3 ± 3.4**	29.9 ± 2.7*	38.6 ± 3.6*	29.7 ± 3.2	55.14 ± 9.14	3.400**

There is a significant difference between chromatin protamine-deficient (CMA3), spontaneously reacted acrosomes and abnormal chromatin condensation (AB staining) in the spermatozoa with grade III and IV in comparison with the control group (grade I). The χ2 test was used to analysis differences among the groups. Data are expressed as mean ± SD and percentage (%). *; P=0.04, **; P=0.03, and Spont. A.R.; Spontaneously acrosome reaction.

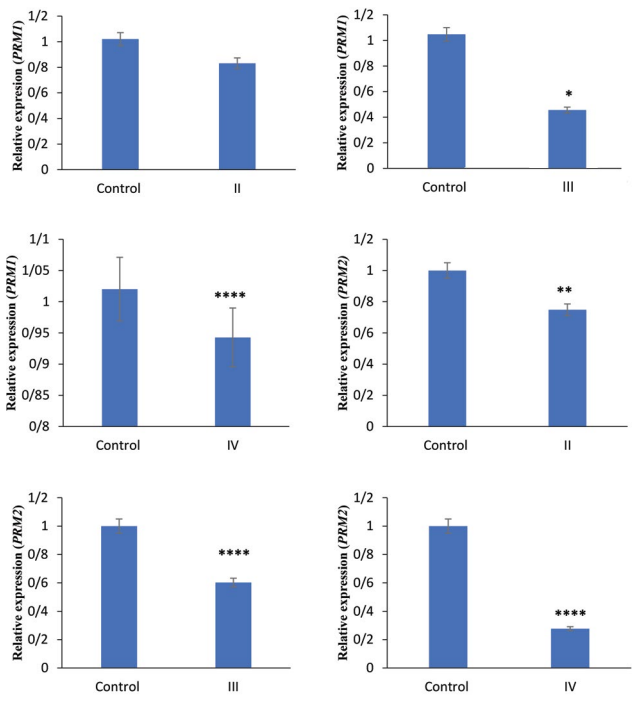

Fig.3: The *PRM1* and *PRM2* gene expression of vacuolated spermatozoa. A significant difference was seen in the *PRM1* gene expression (underexpression) of grade III (P=0.03) and grade IV (P=0.0001) of spermatozoa in comparison with the control group (grade I). Also, there is a significant difference in the *PRM2* gene expression (over-expression) among different grades of spermatozoa in comparison to the control group (grade I): II (P=0.01), III (P=0.0001), and IV (P=0.0001), respectively. *; P<0.05, **; P<0.01, and ****; P<0.0001.

Vacuolated spermatozoa and *in vitro* fertilization/ intracytoplasmic sperm injection outcomes

The results of the influences of different grading of sperm vacuolization and normal sperm on the clinical outcomes are shown in the Table 2. In the IVF cycles, a decrease in the fertilization rate was observed in the cases who received sperms of grade III (39.6%, P=0.018) and IV (32.4%, P=0.012) in compared to the control group (70.3%). However, no significant difference was seen in the fertilization rate in the ICSI cycles (III, 60.52 and IV, 57.2%) in comparison with the control group (65.1%, P=0.12). Increased levels of sperm vacuoles were also associated with a decreased rate of embryo development in comparison with the control group. So that, development rate in the ICSI cycles was significantly decreased in the grades of III=51.33% (P=0.04), and IV=49.17% (P=0.02), in compared to grade I=57%. While, declined embryo development rate was recorded in the IVF cycles as following: grades III=55.01 and IV=56% (P=0.04). The increased percentage of vacuolated sperms was correlated with the decreased chance of an embryo developing to the blastocysts stage. In this way, the rate of successful pregnancy was significantly decreased in the groups with vacuolated sperms (III and IV) under IVF treatment (28.57 and 21.42%, P=0.04 and P=0.019, respectively) in compared to the control (grade I) group, while this outcome was also significant in the ICSI group (III and IV grades: 33.3 and 20%, P=0.02 and P=0.011, respectively).

Factors ranking

Another important experiment was measuring of the effect of different aspects of vacuole on the male fertility (Table 3). The effect of vacuole location (nuclear) weighed more than the effect of other parameters on pregnancy.

Table 2: The effect of different vacuolization grade on the ART outcomes

Vacuole grade of sperm	ART technique	Fertilization rate (%)	Cleavage rate (%)	Clinical pregnancy rate P/ET (%)	Live birth LB/IE (%)
I	ICSI	65.1	57	5/12 (41.66)	3/5 (60)
	IVF	70.3	68.1	7/15 (46.66)	4/7 (57.1)
II	ICSI	53.26	55.31	4/11 (36.36)	2/4 (50)
	IVF	60.03	62.32	5/13 (38.46)	3/5 (60)
III	ICSI	60.52	51.33*	3/9 (33.33)*	1/3 (33.3)*
	IVF	39.6**	55.01*	4/14 (28.57)*	1/4 (25)*
IV	ICSI	57.2	49.17*	3/10 (30)**	1/3 (33.3)*
	IVF	32.4**	56*	3/14 (21.42)**	1/3 (33.3)*

There is a significant difference between ART outcomes and grades of spermatozoa (III and IV). The χ2 test was used to analysis differences among the groups. Data are expressed as mean ± SD and percentage (%). *; P<0.05, **; P<0.01, ART; Assisted reproductive technique, ICSI; Intracytoplasmic sperm injection, IVF; *In vitro* fertilization, P; Positive cycle, ET; Embryo transfer, LB; Live birth, and IE; Implanted embryo.

Table 3: The effect of different features of vacuoles on male fertility potential

Feature	Protamination status	Protamine ratio	Chromatin condensation	Acrosome reaction	Fertilization	Pregnancy
Nuclear location	0.0812	0.0752	0.0537	0.0529	0.0832	0.0875*
Number	0.0689	0.0543	0.0312	0.0241	0.0776	0.07601
Size	0.0567	0.0487	0.0192	0.0138	0.0617	0.0651

*; The effects of nuclear location weighed more than the effects of other features on pregnancy. The WEKA test was used to analysis differences among the groups.

Discussion

A novel insight was provided in this study that how vacuolization affects sperm fertility potential. It is a better predictor of IVF/ICSI outcomes following evaluation of sperm by using high magnification, deep learning algorithm, and SEM images. The results of this study show that variations in vacuole parameters including higher size, greater frequency, and nuclear location were associated with protamine-deficient sperms as well as CMA3 positive and aberrant *PRM1* and *PRM2* gene expression. In addition, the presence of non-nuclear vacuole leads to increased immature acrosome reaction and decreases the fertilization rate under IVF cycles.

Although, limitations of routine semen analysis have been reported, this is performed as a common evaluation in many clinical practices (19). This conventional semen analysis does not recognize the subtle abnormalities in the male genome, DNA structure and condensation (2). The abnormalities in chromatin structure and condensation is known to be correlated with numerous indicators of assisted reproductive outcomes, including fertilization rate, embryo development rate and quality, pregnancy and spontaneous miscarriage (19-21). Although, it has been determined that human sperms have a highly dynamic and key roles in the embryonic development, the utility of more detail analysis of sperm is still a matter of debate (22, 23). In the present study, the predictive value of vacuolated sperm testing was distinguished between potentially pregnant and not potentially pregnant couples who were undertaken IVF or ICSI cycles. As mentioned above, these poor outcomes may have related to abnormalities of chromatin condensation and sperm protamination (CMA3 positive) with aberrant *PRM1* and *PRM2* gene expression. While, the cause of abnormal sperm chromatin condensation is still unclear. The results of this study suggest a direct correlation between sperm nuclear vacuolization and abnormalities in the sperm chromatin packaging. It seems that the contribution of the immune seminal cells, mature sperms and immature germ cells lead to the production of reactive oxygen species that can cause vacuolated head in the sperms (22). It has been also reported that poor chromatin condensation and aneuploidy could be observed in the spermatozoa with large vacuoles (24).

In this study, the protamine mRNA ratio of infertile men was 0.739 ± 0.212 and 3.400 ± 1.281 in the vacuolated spermatozoa with grades III and IV, respectively. While, the protamine mRNA ratio has been reported in the previous studies as follows: 0.83 ± 0.05 (n=50) (25), 1.3 ± 0.1 (n=12) (26) and 0.98 ± 0.02 (n=77) (16), a range of 0.54 to 1.43 of the protamine ratio has been reported in normozoospermic men (16). The ratio of 1.06 ± 0.60 versus 10.68 ± 33.72 was also seen in the normozoospermia semen samples versus teratozoospermia ones (15). Therefore, present study outcomes indicated that vacuolization affects negatively the protamine ratio in the infertile men. So that, a low protamine ratio was seen in the vacuolated spermatozoa with grade III (protamine-1 was underexpressed). Also, a high protamine ratio was observed in the vacuolated spermatozoa with grade IV (normal expression of protamine-1 and underexpression of protamine-2). Aoki et al. (27) reported *PRM1* under expression and *PRM2* overexpression in the infertile patients with a low protamine ratio. On the other hand, in the patients with a high protamine ratio, *PRM2* was underexpressed and *PRM1* has a normal expression. Numerous studies also indicated a significant aberrant of protamine ratio in infertile men (28, 29) and our result is in line with them.

Moreover, it is widely accepted that there is a correlation between sperm quality and infertility (22). In addition, our study results indicate this correlation between sperm quality and fertility potential. In this way, the embryos resulting from morphologically abnormal sperm lead to significantly lower pregnancy rates (2). The correlation between spermatozoa with large nuclear vacuoles and ICSI outcomes has been reported (24). While the origin and consequences of vacuoles of sperm head are also a problem of controversy. Therefore, the association among different sizes, locations, and frequencies of vacuole with chromatin status, IVF/ICSI outcomes, and weight of each feature (size, location, and frequency of vacuoles) on pregnancy rate are essential that this study considered them.

Kacem et al. (30) showed that a large sperm head vacuole could originate from spermatogenesis damaging, abnormal maturation or modifications during the acrosome reaction. Our results are consistent with the results of this study. So that, the immature acrosome reaction was greater in the spermatozoa of grade III and grade IV, therefore, the fertilization rate was decreased in these groups.

Conclusion

The results of this study indicate that the semen samples from infertile men are characterized by a higher ratio of vacuolization grades, although, there are categorized in normozoospermia samples. This frequency of vacuolization may be correlated to abnormal chromatin condensation, greater sperm protamine deficiencies, declined *PRM1* and *PRM2* gene expression, and a high protamine mRNA ratio. Also, tracing the IVF/ICSI outcomes showed that the poor fertilization rate during (IVF cycles), embryo quality, and declined clinical pregnancy rate may have related to the abnormal maturation and sperm head vacuoles. Therefore, it seems the evaluation of the semen sample vacuole status, as a definite parameter before starting treatment cycles, may be a useful tool for selecting the best treatment cycle (IVF or ICSI) in ART plan.

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Authors' Contribution

F.G., M.H.B.; The design of the study, experiment conductors, interpretation, and manuscript drafting and reviewing. S.Z.H.K, M.E.; *In vitro* experiment performance, critical reagents provider, statistical analysis and contribution of patient data. All authors read and approved the final manuscript.

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