J Assist Reprod Genet (2014) 31:307–312
DOI 10.1007/s10815-013-0157-0

GAMETE BIOLOGY

The prevalence of sperm with large nuclear vacuoles is a prognostic tool in the prediction of ICSI success

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Received: 5 November 2013 / Accepted: 9 December 2013 / Published online: 20 December 2013

Abstract
Purpose To investigate if there is a correlation between the prevalence of sperm with large nuclear vacuoles (LNV) and intracytoplasmic sperm injection (ICSI) outcomes.
Methods Two hundred male patients undergoing ICSI had their sperm morphology evaluated through motile sperm organelle morphology examination (MSOME) and the percentage of LNV sperm was recorded and correlated to the ICSI outcomes.
Results The percentage of sperm with LNV negatively influenced the blastocyst formation (S: 16.9, R^2: 20.5 %, p=0.004) and implantation (S: 34.7, R^2: 26.2 %, p=0.001). There were significant differences in the percentage of sperm with LNV between patients in which pregnancy was achieved or not (22.2 % vs. 28.4 %, p<0.001) and in patients with ongoing pregnancy or not (22.4 % vs. 28.5 %, p<0.001). The incidence of sperm with LNV was determinant to the decreased odds of pregnancy (OR: 0.74, p<0.001) and increased odds of miscarriage (OR: 1.46, p<0.001). The area under the curve (AUC) was sufficient to distinguish between couples which did achieve pregnancy or not (AUC: 0.922, p<0.001).
Conclusions The MSOME is a prognostic tool in the prediction of ICSI success and could be used to select patients that should have their sperm selected by MSOME for ICSI.

Keywords MSOME · IMSI · Sperm · Vacuole · Semen analysis

Capsule The evaluation of sperm morphology under high-magnification is a prognostic tool in the prediction of ICSI success in couples undergoing ICSI as a result of male factor.

Clinical trial ID: ClinicalTrials.gov NCT01727947

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Introduction

Current research on sperm morphology has been directed towards the sperm head. The accurate morphological assessment of spermatozoa for intracytoplasmic sperm injection (ICSI) depends on the resolution power of the optical magnification system. Since 2001, motile sperm organelle morphology examination (MSOME) has been used to select spermatozoa under high-magnification (DIC/Nomarski) for ICSI [1].

A morphological normal spermatozoon analysed by MSOME is defined as having a normal head with no vacuoles or fewer than two small vacuoles that occupy for less than 4 % of the head's cross-sectional area [2–4].

The sperm vacuole is a concavity extending from the surface of the sperm head to the nucleus through the acrosome [5]. It has been suggested that sperm vacuoles should be regarded as normal features of the sperm head that occur naturally during the process of condensation of sperm nuclei, and should not be regarded as degeneration but as physiological changes that do not compromise the sperm quality [5, 6]. Meanwhile, studies suggest that the presence of sperm vacuoles is related to male sub fertility [7], lower mitochondrial membrane potential [8], higher incidence of chromosomal abnormalities [8, 9], and sperm chromatin packaging/DNA abnormalities [1, 4, 6, 10–16]. It has also been suggested that sperm vacuoles reflect non-reacted acrosome [17, 18].

One particular sperm alteration is the presence of large nuclear vacuoles (LNV); their occurrence has a

negative association with natural male fertility potential [19, 20] and has been related to reduced ICSI outcomes [11], increased DNA fragmentation [8] and failure of chromatin condensation [16].

It has been suggested that the sperm vacuole mirrors the spermatozoon's 'birth' and 'maturation' and that the spermatozoon may bear the mark of the (epigenetic) events that it has experienced [16]. Moreover, a global impairment of the spermatogenesis process has been proposed as a common origin of the LNV [9]. Therefore, we hypothesized that the outcomes of ICSI cycles would correlate with the incidence of sperm with LNV in the semen sample prepared for injection.

Therefore the objective of this study was to investigate if the incidence of sperm with LNV in a semen sample analysed by MSOME is associated with sperm parameters and ICSI outcomes.

Materials and methods

Experimental design, patients and inclusion criteria

This prospective non-randomised study analyzed semen samples of 200 couples, undergoing their first ICSI attempt, as a result of male factor infertility (oligo/terato/astenozoospermia or any combination of those abnormalities). Immediately after ICSI, a total of 200 spermatozoa of each sample were analyzed under high magnification (x6,600). The incidence of sperm with LNV (sperm cells presenting at least one vacuole that occupied >13 % of nuclear area) in each sample was assessed. Linear and binary regression analyses, controlled for maternal age, were used to investigate the influence of LNV on ICSI outcomes. The percentage of sperm with LNV was compared between the groups of patients who experience pregnancy and miscarriage. Receiver operating characteristic (ROC) curve analysis was performed to assess the predictive value of LNV spermatozoa on the achievement of pregnancy.

Inclusion criteria were as follows: women with age ≤37 years, undergoing ICSI as result of male factor, with regular menstrual cycles of 25–35 days, normal basal FSH and LH levels, BMI less than 30 kg/m^2, presence of both ovaries and intact uterus, absence of polycystic ovaries, endometriosis, or gynaecological/medical disorders and a negative result in a screening for sexually transmitted diseases. No patient had received any hormone therapy for at least 60 days preceding the study.

A written informed consent was obtained, in which patients agreed to share the outcomes of their own cycles for research purposes, and the study was approved by the local institutional review board.

Controlled ovarian stimulation

Ovarian stimulation was achieved by the administration of recombinant follicle-stimulating hormone and gonadotropin-releasing hormone antagonist, as previously described [21].

Semen sample collection and preparation

All semen samples were collected in the laboratory by masturbation after ejaculatory abstinence. After liquefaction for 30 min at room temperature, the semen samples were evaluated according to the threshold values established by the WHO in 2010 [22]. A 2-layered density gradient centrifugation technique was used for the sperm preparation (50 % and 90 % Isolate, Irvine Scientific, Santa Ana, CA, USA). Sperm count and motility were estimated in the recovered fractions.

MSOME

ICSI was performed according to Palermo et al. [23]. Immediately after ICSI, the MSOME was conducted. A total of 200 spermatozoa of each sample were analysed at high magnification using an inverted Nikon Diaphot microscope equipped with high-power differential interference contrast optics (DIC/Nomarski). The total calculated magnification was x6,600. An aliquot of the sperm cell suspension was transferred to a microdroplet of modified human tubal fluid medium containing 8 % polyvinyl pyrrolidone (PVP; Irvine Scientific, Santa Ana, CA) in a sterile glass dish (FluoroDish; World Precision Instrument, Sarasota, FL). The dish was placed on a microscope stage above an Uplan Apo x 100 oil/1.35 objective lens previously covered by a droplet of immersion oil.

LNV spermatozoa were defined by the presence of one or more vacuoles occupying >13 % of the sperm nuclear area [9, 24]. Figure 1 shows a LNV spermatozoa analyzed by MSOME. The same technician performed all sperm analysis. The incidence of sperm with LNV in each sample was recorded.

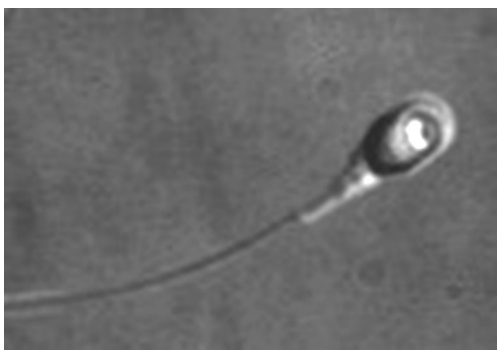

Fig. 1 Spermatozoa presenting a large nuclear vacuole analysed by MSOME

Fertilisation, embryo quality and embryo transfer

Approximately 16 h after ICSI, fertilisation was confirmed by the presence of two pronuclei and the extrusion of the second polar body. Embryos were maintained in a 50 μL drop of culture medium (Global®, LifeGlobal, Connecticut, USA) supplemented with 10 % protein supplement covered with paraffin oil in a humidified atmosphere under 6 % CO2 at 37ºC for 5 days.

High-quality embryos were defined as those showing 8–10 cells on the third day of development, less than 15 % fragmentation, symmetric blastomeres, absence of multinucleation and absence of zona pellucida dysmorphisms.

High-quality blastocysts were defined as full or expanded or hatching or hatched blastocysts, presenting a normal inner cell mass (ICM) and trophectoderm (TE), according to Gardner and Schoolcraft [25].

Embryo transfer was performed on day 5 of development using a soft catheter with transabdominal ultrasound guidance. One to three embryos were transferred per patient, depending on embryo quality and maternal age.

Clinical Follow-up

A pregnancy test was performed 12 days after embryo transfer. All women with a positive test had a transvaginal ultrasound scan 2 weeks after the positive test. A clinical pregnancy was diagnosed when the foetal heartbeat was detected. Pregnancy rates were calculated per transfer. Miscarriage was defined as pregnancy loss before 20 weeks.

Data analysis and statistics

Data are expressed as mean ± standard deviation for continuous variables, while percentages were used for categorical variables. Mean values were compared by Student's *t* parametric test or Mann–Whitney non-parametric test. Percentages were compared by the Chi-squared or Fisher exact test, only when the expected frequency was five or fewer.

Linear and binary regression analyses, controlled for maternal age, number of retrieved oocytes and number of transferred embryos, were performed to study the influence of LNV spermatozoa on ICSI outcomes. Receiver operating characteristic (ROC) curve analysis was performed to assess the predictive value of LNV spermatozoa on the achievement of pregnancy. The results are expressed as correlation coefficients (R) or regression coefficients (RC) or odds ratios (OR) with 95 % confidence intervals (CI) and p values. The ROC curve results are expressed as area under curve (AUC) with 95 % CI. The results were considered to be significant at the 5 % critical level ($p<0.05$). Data analyses were carried out using the Minitab® and MedCalc® statistical programs (Table 1).

Table 1 Correlation results between the incidence of LNV and semen parameters

Variable	Incidence of LNV sperm	
	R	*p*-value
Paternal age	0.09	0.502
Native sperm sample		
Total sperm count	−0.273	<0.001
Percentage of progressive sperm	−0.360	<0.001
Percentage of immotile sperm	0.354	<0.001
Prepared sperm sample		
Total sperm count	−0.423	<0.001
Percentage of progressive sperm	−0.255	<0.001
Percentage of immotile sperm	0.322	<0.001

LNV large nuclear vacuoles

Results

Mean male age was 37.7±8.5 years, total sperm count was 23.8±14.8 million, percentage of progressive spermatozoa was 35.3±4.4 %, non-progressive spermatozoa was 22.3±8.4 % and percentage of normal forms was 2.6±0.6 %. The mean incidence of LNV spermatozoa was 24.2±10.9 % (9,675/40,000).

Paternal age was not correlated with the incidence of LNV. Total sperm count and percentage of progressive motility before and after sperm preparation were inversely correlated with the incidence of LNV. In addition, a direct correlation between the percentages of immotile sperm before and after sperm preparation and the incidence of LNV were observed (Table 1).

Mean female age was 34.2±2.0 years. The fertilization rate was 83.6 %, high quality embryos rate on day 3 of development was 77.3 %, blastocyst formation rate was 38.5 %, mean number of transferred embryos was 1.8±0.4, implantation rate was 33.3 %, pregnancy rate was 37.0 % (68/184) and miscarriage rate was 23.5 % (16/68).

There were no correlations between the incidence of LNV and fertilization rate and high quality embryos rate on day 3. However, the incidence of LNV was negatively correlated with blastocyst formation and implantation rates (Table 2).

Table 2 Correlation results between the incidence of LNV and ICSI outcomes

Variables	Incidence of LNV	
	R	*p*-value
Fertilization rate	0.321	0.250
High quality embryos on D3	0.285	0.500
Blastocyst formation rate	–0.453	0.004
Implantation rates	–0.512	0.001

LNV large nuclear vacuoles, *D3* day 3 of embryo development

Table 3 Linear and binary regression analysis results of LNV's influence on ICSI outcomes

Outcomes	Incidence of LNV		
	Slope	R^2 (%)	*p*-value
Blastocyst formation rate	16.9	20.5	0.004
Implantation rate	34.7	26.2	0.001
Outcomes	OR	CI	*p*-value
Odds of Pregnancy	0.74	0.58–0.95	<0.001
Odds of miscarriage	1.46	1.16–2.27	<0.001

LNV large nuclear vacuoles

Regression analyses results are shown in Table 3. The percentage of LNV spermatozoa negatively influenced the blastocyst formation and implantation rates. Additionally, the incidence of LNV spermatozoa was determinant to the decreased odds of pregnancy and increased odds of miscarriage.

Comparisons between pregnant and non-pregnant couples and between couples with ongoing pregnancy and miscarriages are shown in Tables 4 and 5, respectively. The groups were similar with regards to cycles' characteristics. However, blastocyst formation rates were significantly higher in pregnant couples and couples with ongoing pregnancy, as compared to non-pregnant couples and couples that miscarried. There were significant differences in the percentage of LNV spermatozoa between patients in which pregnancy was achieved or not (22.2 % vs. 28.4 %, $p<0.001$) and in patients with ongoing pregnancy or not (22.4 % vs. 28.5 %, $p<0.001$).

A ROC curve analysis was performed to assess the predictive value of the incidence of LNV spermatozoa on the achievement of pregnancy. The calculated area under the ROC curve (AUC) was sufficient to distinguish between couples which did achieve pregnancy or not (AUC: 0,922; CI: 0,871–0,958, $p<0,001$) (Fig. 2).

Table 4 Comparison of cycles' outcomes between pregnant and non-pregnant couples

Variable	Pregnant group (*n*=68)	Non-pregnant group (*n*=116)	*p*-value
Female age (y-old)	33.73±2.11	33.47±2.13	0.420
FSH (IU)	1,902±397	1,916±394	0.822
Oocytes	11.64±4.03	10.69±6.27	0.211
Male age (y-old)	35.18±9.80	36.13±4.56	0.432
Sperm count (million)	30.5±4.9	32.1±5.7	0.060
Sperm progressive motility (%)	33.5±5.2	31.9±7.0	0.107
Sperm morphology (%)	2.1±1.1	2.3±0.9	0.201
Fertilization rate (%)	86.1±12.5	85.5±13.9	0.766
High quality embryos rate D3 (%)	69.5±17.3	67.3±22.5	0.464
Blastocyst formation rate (%)	55.4±23.8	50.9±23.2	0.002
Transferred embryos	1.76±0.7	1.71±0.7	0.703

Table 5 Comparison of cycles' outcomes between couples with ongoing pregnancy and couples that miscarried

Variable	Ongoing pregnancy group (*n*=52)	Miscarriage group (*n*=16)	*p*-value
Female age (y-old)	33.60±1.0	34.00±1.4	0.280
FSH (IU)	1,988±115	1,811±565	0.212
Oocytes	11.20±4.1	12.60±3.0	0.140
Male age (y-old)	35.50±5.5	36.80±8.5	0.571
Sperm count (million)	30.1±3.5	30.5±4.3	0.732
Sperm motility (%)	33.4±4.1	33.8±3.9	0.721
Sperm morphology (%)	2.1±0.9	1.9±1.3	0.580
Fertilization rate (%)	88.5±10.1	86.0±8.0	0.314
High quality embryos rate D3 (%)	70.1±17.3	68.0±18.2	0.680
Blastocyst formation rate (%)	65.5±14.9	45.4±14.6	<0.001
Transferred embryos	1.5±0.3	1.7±1.1	0.477

Discussion

The impact of sperm morphology on ICSI outcomes has been under debate. With the introduction of the MSOME, it is now possible to examine the sperm morphological normalcy at the sub-cellular level [1]. The objective of this study was to identify if there is a connection between the incidence of

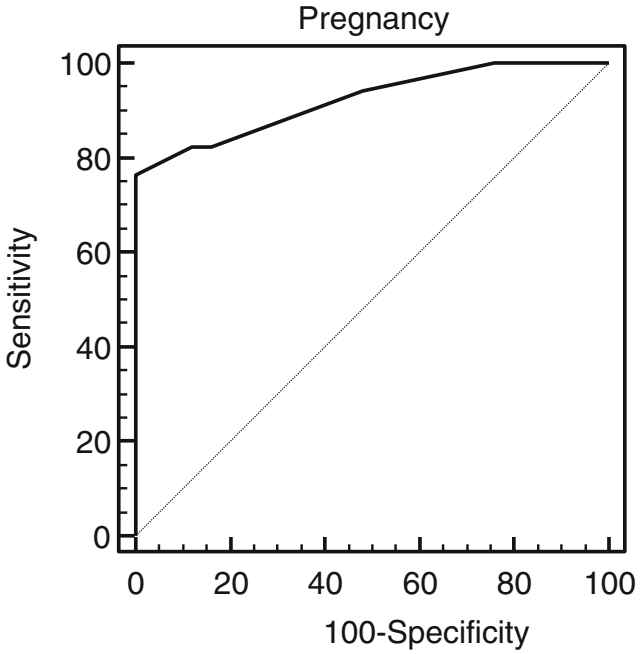

Fig. 2 ROC curve for AUC of incidence of LNV, with dependent parameter the achievement of pregnancy

LNV ana and ICSI outcomes. Our results showed that the incidence of LNV spermatozoa predicts blastocyst formation, implantation, pregnancy and miscarriage rates in couples undergoing ICSI.

Our findings corroborate with several studies that have demonstrated that when spermatozoa showing LNV are injected there is a reduction on the proportion of embryos that develop to the blastocyst stage [2, 3, 26]. We have observed that the incidence of LNV predicts implantation, pregnancy and miscarriage rates in couples undergoing ICSI. These findings are in accordance with previous reports on improved implantation, pregnancy and miscarriages rates using MSOME [10, 11, 27–30], supporting the hypothesis that a late adverse paternal effect on embryo development can be avoided through better selection of spermatozoids under high-magnification.

The origin of sperm vacuoles is disappointingly unknown. Nonetheless, there are many reports suggesting that sperm vacuoles reflect sperm chromatin packaging/DNA abnormalities [1, 4, 6, 10–16].

Franco et al. [12] observed an association between LNV and DNA damage in spermatozoa. We could speculate that the higher the incidence of LNV, the lower the quality of each spermatozoa of the ejaculate. Similarly, a close relationship between the incidence of DNA fragmentation and post-implantation development in ICSI patients has been demonstrated. The pregnancy and miscarriage rates significantly differed between patients with high and low incidence of sperm DNA fragmentation [31].

One possible drawback of this study is related to the absence of consensus about the definition of LNV. In this study, a LNV was defined as a vacuole area occupying >13.0 % of sperm head. This definition has been previously adopted in a study by Perdrix et al. [9]. However, other studies considered large those vacuoles occupying 25 % [16] and 50 % [12] of sperm head area, thus introducing a confounding factor that may account for the lack of agreement regarding the effects of LNV on the ICSI outcomes.

It is important to mention that the majority of patients have a higher percentage of sperm without LNV than sperm with LNV; therefore, one might argue that the incidence of LNV has little clinical utility. However, the MSOME represents a stricter evaluation criterion for sperm morphology and enables the identification of abnormalities more accurately than the analysis of stained samples under 1,000× [32].

The analysis of a single spermatozoon, performed after MSOME, showed significantly better mitochondrial function, chromatin status and aneuploidy rate than observed in unselected cells. Additionally, these parameters were further improved when nuclear vacuoles were lacking [8]. These results suggest a strong relationship between high-magnification morphology and the status of spermatozoa, and may explain the better results of ICSI obtained using spermatozoa selected by MSOME.

The advent of the MSOME has allowed the introduction of a modified microinjection procedure named intracytoplasmic morphologically selected sperm injection (IMSI). Since its development, several studies have investigated the efficacy of this technique, however, the extra time necessary for sperm selection are limitation to a more widespread use of IMSI [30, 33].

The results of the present study may be useful in the selection of patients who should be treated with IMSI. Conventional semen analysis has been used to aid in the selection of the most appropriate treatment for infertile couples: artificial insemination, conventional IVF or ICSI. Our results suggest that the evaluation of sperm morphology under high magnification can be used as a tool to select couples who would benefit from conventional ICSI or IMSI. For that to happen it is necessary the standardization of the definition of LNV and the establishment of a threshold for the incidence of LNV beyond which the cycles' outcomes would be compromised.

In conclusion, our results suggest that the evaluation of sperm morphology under high-magnification is a prognostic tool in the prediction of ICSI success in couples undergoing ICSI as a result of male factor. Because the LNV is not identified in conventional ICSI, the MSOME could be a tool for the selection of patients that should have their sperm selected by MSOME for ICSI.

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