Sperm vacuoles cannot help to differentiate fertile men from infertile men with normal sperm parameter values

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STUDY QUESTION: Can the assessment of sperm vacuoles at high magnification contribute to the explanation of idiopathic infertility?

SUMMARY ANSWER: The characteristics of sperm head vacuoles (number, area, position) are no different between fertile controls and patients with unexplained infertility.

WHAT IS KNOWN ALREADY: Until now, the assessment of sperm head vacuoles has been focused on a therapeutic goal in the intracytoplasmic morphologically selected sperm injection (IMSI) procedure, but it could be pertinent as a new diagnostic tool for the evaluation of male fertility.

STUDY DESIGN, SIZE, DURATION: This diagnostic test study with blind assessment included a population of 50 fertile men and 51 men with idiopathic infertility. They were selected from September 2011 to May 2013.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Fertile men were within couples who had a spontaneous pregnancy in the last 2 years. Infertile men were within couples who had unexplained infertility and were consulting in our centre. After analysis of conventional sperm parameters, we investigated the number, position and area of sperm head vacuoles at high magnification (∗6000) with interference contrast using an image analysis software. We also carried out a nuclear status analysis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling assay (TUNEL), sperm chromatin structure assay (SCSA) and aniline blue staining.

MAIN RESULTS AND THE ROLE OF CHANCE: Concerning the vacuoles data, we did not find any significant difference between the two populations. We found no significant correlation between the vacuolar parameters (mean number of vacuoles, relative vacuole area and percentage of spermatozoa with large vacuoles) and either conventional semen parameters, male age or the data from the aniline blue staining, SCSA assay and TUNEL assay.

LIMITATIONS, REASONS FOR CAUTION: Despite the fact all of the vacuole parameters values were identical in fertile and infertile men, we cannot totally exclude that a very small cause of unexplained infertilities could be related to an excess of sperm vacuoles.

WIDER IMPLICATIONS OF THE FINDINGS: In line with its widely debated use as a therapeutic tool, sperm vacuole assessment for diagnostic purposes does not seem useful.

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Key words: unexplained infertility / fertile men / sperm vacuole / MSOME / IMSI
Introduction

Many publications have shown that the morphology of spermatozoa is an important predictive factor for outcome of natural conception (Jouannet et al., 1988) and intrauterine insemination (Lee et al., 2002) as well as for conventional in vitro fertilization (Kruger et al., 1988). Different classifications of sperm morphology have been used according to the size, shape and appearance of the cell. Recently a new aspect of sperm morphology, the head vacuoles, has shown to be of interest. The vacuoles can be easily observed using Nomarski interference contrast microscopy at high magnification: ×6000 to 10000 (optical magnification ×1000 associated with digital enhancements that achieves a final magnification up to 6000 to 10000) even on motile spermatozoa (MSOME: Motile Sperm Organellar Morphology Examination) (Bartov et al., 2002; Perdrix and Rives, 2013). The origin of these vacuoles raises many questions. Several studies have found a link between increased levels of fragmented DNA and the presence of sperm head vacuoles (Boughali H, 2006; Franco et al., 2008; Garolla et al., 2008; Oliveira et al., 2010; Hammoud et al., 2012). Others publications associate the large vacuoles with chromatin condensation defects (Boitrelle et al., 2011; Perdrix et al., 2011; Franco et al., 2012).

The observation of sperm head vacuoles is used to select spermatozoa for intracytoplasmic sperm injection (ICSI) to perform intracytoplasmic morphologically selected sperm injection (IMSI) (Bartov et al., 2001). The clinical results from randomized studies comparing IMSI and ICSI are conflicting: some authors have reported increased implantation rates (Antinori et al., 2008; Wilding et al., 2010) while others have not found any difference (Balaban et al., 2011; Leandri et al., 2013). However none on the randomized trials reporting live birth rate could show a statistical difference between IMSI and ICSI (Balaban et al., 2011; Wilding et al., 2010; Leandri et al., 2013; Teixeira et al., 2013).

Until now, the assessment of sperm head vacuoles has been focused on a therapeutic goal in the IMSI procedure, but it could be pertinent as a new diagnostic tool for the evaluation of male fertility.

Unexplained infertility represents ~8% of infertility (Thonneau et al., 1991). Diagnostic tools currently available are sometimes not able to explain the origin of infertility and conventional semen examination cannot always differentiate fertile and infertile men (Guzick et al., 2001). It is well recognized that the male infertility is present in more than half of infertile couples (Thonneau et al., 1991; Oehninger, 2001). Damages to the nuclear component of spermatozoa may play a major role in some cases of unexplained infertility where current approaches have failed to reveal any cause (Rybar et al., 2009).

The aim of the present study was (i) to accurately define vacuole criteria (number, position and area) in a population of fertile men, (ii) to compare these data with that obtained from men of couples with unexplained infertility (and therefore to evaluate sperm vacuoles as a diagnostic tool for male fertility) and (iii) to study the relationships between vacuole criteria and conventional semen parameters or data from sperm nucleus assessment (TUNEL assay, SCSA, aniline blue staining).

Materials and Methods

Study design

A total of 50 fertile men with proven spontaneous fertility and 51 men within a couple with unexplained infertility entered the study. Semen samples were collected by masturbation after 2–6 days of sexual abstinence and were processed for analysis after liquefaction for 20 min at 37°C on the unselected fresh sperm, we carried out a sperm count, and motility, vitality and conventional morphology analyses as well as a detailed morphometric analysis of the vacuoles at high magnification using image analysis software as previously described (Gatimel et al., 2013a). Measurements using the software were carried out by a single operator who was blinded to the origin (fertile or infertile men) of the sample. An aliquot of fresh sperm was used for assessment of chromatin condensation by aniline blue staining and other aliquots were cryopreserved for further analysis of DNA fragmentation by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labelling assay (TUNEL) and for assessment of chromatin quality by sperm chromatin structure assay (SCSA). The spermogram, sperm freezing for nuclear assessment and sperm preparation for high-magnification analysis were all performed within 1 h after ejaculation.

Study population

Fertile men were aged between 18 and 45 years. They were within a couple with a spontaneous pregnancy in progress at the time of inclusion or in the past 2 years. Volunteers were all recruited through the maternity department of the University Hospital of Toulouse during the pregnancy or after delivery posters and flyers in the hospital. The volunteers had no medical, andrologic or surgical history since the beginning of pregnancy and there was no history of recurrent miscarriage. On the day of inclusion, men were asked about their medical, surgical and andrologic history. These subjects were selected from September 2011 to September 2012.

Infertile men were within a couple with unexplained infertility and who consulted in our centre from September 2011 to May 2013. The women were <38 years old. They all had regular menstrual cycles between 27 and 30 days, an anti-Mullerian hormone (AMH) levels >1 ng/ml (AMH Gen II ELISA, Beckman Coulter), an FSH level <10 mIU/ml, a normal hysterosalpingography showing bilateral tubal patency and absence of severe endometriosis (grade 3 and 4, AFrS score). All men had normal semen analysis (spermogram and conventional morphology) according to the WHO 2010 reference limits.

All subjects gave their informed consent to participate to the study. The protocol was approved by the Comité de Protection des Personnes, reference 1117503.

Conventional sperm parameters

Semen evaluation (spermiogram) was performed according to the standard methodology proposed by the WHO guidelines (WHO, 2010) and sperm morphology was evaluated using a computer-assisted sperm analysis on slides of fresh semen. We had chosen assessment of sperm morphology by CASA since we have previously shown that it is more objective and reproducible than manual assessment (Marnet et al., 2000). The smears were air-dried for 10 min then stained using Kit RAL 555 (RAL Diagnostics, Martillac, France). A Hamilton Thorn version 12.3 HTM-IVOS analyzer was used. The spermatozoon was identified and then analysed by computerized software according to strict criteria morphology (Kruger et al., 1995; Marnet et al., 2000; Menkveld et al., 2001).

Aniline blue staining

A 50 μl sample of fresh sperm was washed in 10 ml of phosphate buffer saline (PBS) by centrifugation for 10 min at 400 × g, and then 10 μl of the pellet was spread on a glass slide and left to dry. The smears were then fixed in 3% buffered glutaraldehyde in PBS for 5 min. Slides are dipped in a 300 ml solution which was a mixture of aniline blue solution and acetic acid solution. The final concentration of aniline blue was 0.07 M and the final concentration of acetic acid was 0.67 M (Auger et al., 1990). A total of 100 spermatozoa per slide
were evaluated (precision of 10%). The threshold of nuclear immaturity was 20% (Hammadeh et al., 1998).

SCSA and TUNEL assays

Sperm DNA Fragmentation Index (DFI) and high DNA stainability (HDS) were measured by the sperm chromatin structure (SCSA) technique (Evenson et al., 2002) routinely used in our laboratory (Pecou et al., 2009; Ahmad et al., 2012). The sperm chromatin structure assay defines abnormal chromatin structure as an increased susceptibility of sperm DNA to acid-induced denaturation in situ. For each sample, two aliquots were processed and assessed separately. The value taken was a mean of the two aliquots, and data from the cytometer were analysed by WinMDI 2.8 (Scripps Institute, La Jolla, CA, USA). The extent of DNA denaturation was expressed as a DNA Fragmentation Index (DFI), which is the ratio of red to total (red plus green) fluorescence intensity. We also calculated the fraction of highly DNA stainable cells by setting an appropriate gate on the scattergram and considering fluorescence intensity higher than the upper border of the main cluster which represents the sperm population with nondetectable fluorescence intensity. We also calculated the fraction of highly DNA stainable cells by setting an appropriate gate on the scattergram and considering fluorescence intensity higher than the upper border of the main cluster which represents the sperm population with nondetectable fluorescence intensity.

The detailed protocol for TUNEL analysis has been described previously (Sergerie et al., 2007). Analysis by TUNEL consisted of subtracting control (no terminal deoxynucleotidyl transferase enzyme) green fluorescence histograms from terminal deoxynucleotidyl transferase-positive green fluorescence histograms, yielding the percentage of cells showing DNA strand breaks.

High magnification morphological examination

Each 50 μl sample of fresh sperm was washed in 2.5 ml G-IVF washing solution (Vitrolife, Göteborg, Sweden) by centrifugation for 5 min at 400 × g. The pellet was resuspended in 100 μl G-IVF and the spermatozoa were fixed by addition of 100 μl PBS-formaldehyde 3.7%. A 2 μl aliquot of this suspension was placed in a glass-bottomed dish (WillCo-dish, WillCo Wells BV, The Netherlands) and examined by Nomarski interference contrast microscopy with a Leica DFC-290 camera mounted on a Leica DMI 6000× microscope with an immersion objective lens ×100 and camera magnification ×1. For each subject, sperm head vacuoles were analysed on 80–100 spermatozoa (mean 92) which represents an error of 10.4% by sample. They were randomly photographed and separately analysed using digital imaging system software (Leica Application Suite Interactive Measurement version 3.6; Leica). The Leica LAS Interactive Measurement module allows measurement of sperm head areas and vacuole areas by manually depicting their outline. It has been previously used for this goal (Perdrix et al., 2011, 2012; Gatimel et al., 2013a,b). The area and position of each vacuole were recorded. To indicate the location of the vacuoles, the sperm head was divided into three equal parts along its length (anterior, median and basal) and the position of the vacuole was given by its centre as previously published (Gatimel et al., 2013a). We have chosen to use values with two decimals places because in a preliminary study (data not shown) we evaluated the imprecision of the measurement of the same object: the standard deviations measured were 0.290 for an area of 13 μm² (CV: 2.5%) and 0.033 for area of 0.30 μm² (CV: 10.5%). Measurements were carried out by a single operator who was blinded to the origin (fertile or infertile men) of the sample.

Relative vacuole area is the ratio of the area of all the vacuoles of a spermatozoon to the area of its head. In the present study, we assessed the percentage of small and large vacuoles with the thresholds used by Perdrix et al. (2011) because this is the only team that has employed an image analysis software and that defined their thresholds based on ROC curves analysis in order to discriminate different clinical phenotypes: normal sperm and men with severe sperm alterations (Saidi et al., 2008).

Statistical analysis

The descriptive statistics and correlation tests were performed with Statview for Windows (Abacus Concepts, Inc., Berkeley, CA, USA). A P-value of 0.05 was retained as significant. Pearson correlation test was performed for assessment of correlations between vacuolar parameters (mean number of vacuoles, relative vacuole area and percentage of spermatozoa with large vacuoles) and data from the analysis of the nuclear material and the conventional semen parameters, as well as age and smoking. Data are means ± SD. Student’s test or Mann–Whitney’s test was used for comparison of means of the two groups depending on the normality of distribution of variables.

Results

The control population consisted of 50 healthy fertile men aged from 21 to 41 years (31.6 ± 4.7 years) with proven fertility (time to spontaneous conception 4 ± 3 months). The conventional sperm parameters are presented in Table I. Of the 50 fertile men, 14 had at least one altered sperm parameter according to WHO 2010 criteria: 6 had an oligozoospermia with a sperm concentration between 2 × 10⁹/ml and 11 × 10⁹/ml, 12 had an asthenozoospermia with 20–30% progressive motility and 4 of the fertile men had a necrozoospermia with 41–57% vitality. Concerning the assessment of DNA fragmentation by TUNEL assay, of chromatin quality by SCSA and of chromatin condensation by aniline blue staining, data are presented in Table I. No fertile men showed a percentage of fragmented sperm DNA measured by the TUNEL assay above the accepted threshold value of 20% with this technique (Sergerie et al., 2005). Assessment of chromatin quality using the SCSA showed DNA fragmentation indexes (DFI) below the threshold of 30% (Evenson et al., 2002). An increase in immature nuclei after aniline blue staining was observed for two fertile men (22 and 26%). All the descriptive parameters of the vacuoles are presented in Table II. The mean percentage of spermatozoa displaying at least one vacuole in our fertile men population was 95.8%. The vast majority (91.0%) of those vacuolated spermatozoa showed small vacuoles (<13% of the head area).

The population of infertile patients consisted of 51 infertile men experiencing unexplained infertility and aged from 27 to 52 years (34.5 ± 4.9 years). The infertile men were older than the fertile men (P < 0.005). All of the 51 infertile men had conventional sperm parameters within the normal ranges according to WHO 2010 criteria since it was a selection criteria. As with the fertile men, none of the infertile men showed a percentage of fragmented sperm DNA (TUNEL assay) above the threshold value of 20%. Data from SCSA assay in infertile men showed a DFI below the threshold of 30% except in three infertile patients who had a DFI of 43.0, 31.7 and 36.8%. An increase in immature nuclei after aniline blue staining was observed in two infertile patients (24% and 27%).

No significant difference was found between fertile and infertile men for the conventional semen parameters as well as for all of the vacuoles data (Tables I and II).

Therefore, in absence of any statistical difference, we combined the two populations in order to look for correlations. We found no significant correlation between the vacuolar parameters (mean number of vacuoles, relative vacuole area and percentage of spermatozoa with large vacuoles) and the conventional semen parameters or the data from aniline blue staining, the SCSA assay and the TUNEL assay. In the same way, we did not find any correlation between vacuole parameters and age of the men (Table III), nor between vacuole parameters and...
### Table I
Clinical data, conventional semen parameters, and analysis of nuclear material by aniline blue staining, TUNEL and SCSA in a population of 50 fertile men and 51 infertile men with idiopathic infertility.

<table>
<thead>
<tr>
<th></th>
<th>Fertile men</th>
<th>Patients with unexplained infertility</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>Mean 31.6</td>
<td>Mean 34.5</td>
</tr>
<tr>
<td></td>
<td>SD 4.7</td>
<td>SD 4.9</td>
</tr>
<tr>
<td><strong>Time to pregnancy/ duration of infertility (months)</strong></td>
<td>3.7</td>
<td>51.4</td>
</tr>
<tr>
<td></td>
<td>Median 31</td>
<td>Median 34</td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>26.2</td>
<td>24.7</td>
</tr>
<tr>
<td></td>
<td>Min 21</td>
<td>Min 27</td>
</tr>
<tr>
<td><strong>Tabacco (number of cigarettes per day)</strong></td>
<td>3.4</td>
<td>4.2</td>
</tr>
<tr>
<td></td>
<td>Max 41</td>
<td>Max 52</td>
</tr>
<tr>
<td><strong>Abstinence duration (days)</strong></td>
<td>3.3</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Median 1.0</td>
<td>Median 27</td>
</tr>
<tr>
<td><strong>Volume (ml)</strong></td>
<td>3.7</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Min 20.0</td>
<td>Min 33.6</td>
</tr>
<tr>
<td><strong>Sperm concentration (10^6/ml)</strong></td>
<td>100.8</td>
<td>41.8</td>
</tr>
<tr>
<td></td>
<td>Max 380.0</td>
<td>Max 254.0</td>
</tr>
<tr>
<td><strong>Total sperm number (10^6 per ejaculate)</strong></td>
<td>405.4</td>
<td>268.0</td>
</tr>
<tr>
<td></td>
<td>Median 297.0</td>
<td>Median 235.0</td>
</tr>
<tr>
<td><strong>Progressive motility (%)</strong></td>
<td>45.1</td>
<td>41.6</td>
</tr>
<tr>
<td></td>
<td>Min 4.4</td>
<td>Min 15.0</td>
</tr>
<tr>
<td><strong>Vitality (%)</strong></td>
<td>73.1</td>
<td>72.2</td>
</tr>
<tr>
<td></td>
<td>Max 90.0</td>
<td>Max 72.2</td>
</tr>
<tr>
<td><strong>Normal forms (strict criteria %)</strong></td>
<td>17.1</td>
<td>15.7</td>
</tr>
<tr>
<td></td>
<td>Median 69.0</td>
<td>Median 63.0</td>
</tr>
<tr>
<td><strong>Aniline blue staining immature nuclei %</strong></td>
<td>4.1</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td>Min 0.9</td>
<td>Min 1.5</td>
</tr>
<tr>
<td><strong>SCSA DFI %</strong></td>
<td>7.8</td>
<td>7.9</td>
</tr>
<tr>
<td></td>
<td>Max 12.1</td>
<td>Max 15.1</td>
</tr>
<tr>
<td><strong>SCSA HDS %</strong></td>
<td>5.7</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td>Median 5.2</td>
<td>Median 3.3</td>
</tr>
<tr>
<td><strong>TUNEL DNA fragmented %</strong></td>
<td>6.6</td>
<td>7.1</td>
</tr>
<tr>
<td></td>
<td>Min 3.8</td>
<td>Min 3.3</td>
</tr>
</tbody>
</table>

BMI, body mass index; NS, non-significant.

Comparison (P): <0.005, <0.005, NS, NS, 0.001, NS, NS, NS, NS, NS, NS, NS, NS, NS, NS.
Table II: Head vacuolar parameters values per men (n = 50 for fertile men and n = 51 for infertile men).

<table>
<thead>
<tr>
<th></th>
<th>Head area (μm²)</th>
<th>Vacuoles number per spermatozoon</th>
<th>Distribution of vacuoles</th>
<th>Vacuoles area (μm²)</th>
<th>Relative vacuole area (%RVA)</th>
<th>Anterior vacuoles area (μm²)</th>
<th>Median vacuoles area (μm²)</th>
<th>Basal vacuoles area (μm²)</th>
<th>% of sperm without vacuoles</th>
<th>% of sperm with RVA = [0;6.5] %</th>
<th>% of sperm with RVA = [6.5;13] %</th>
<th>% of sperm with large vacuoles (RVA &gt; 13%)</th>
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</thead>
<tbody>
<tr>
<td><strong>Fertile men</strong></td>
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<td></td>
</tr>
<tr>
<td>Mean</td>
<td>12.53</td>
<td>1.5</td>
<td>20.2</td>
<td>72.9</td>
<td>6.9</td>
<td>0.79</td>
<td>6.5</td>
<td>0.19</td>
<td>0.57</td>
<td>0.04</td>
<td>4.2</td>
<td>56.6</td>
</tr>
<tr>
<td>SD</td>
<td>0.86</td>
<td>0.2</td>
<td>6.2</td>
<td>7.3</td>
<td>3.4</td>
<td>0.15</td>
<td>1.3</td>
<td>0.09</td>
<td>0.12</td>
<td>0.02</td>
<td>3.0</td>
<td>12.5</td>
</tr>
<tr>
<td>Median</td>
<td>12.56</td>
<td>1.5</td>
<td>18.9</td>
<td>74.6</td>
<td>6.5</td>
<td>0.78</td>
<td>6.3</td>
<td>0.16</td>
<td>0.58</td>
<td>0.04</td>
<td>4.0</td>
<td>58.5</td>
</tr>
<tr>
<td>Min</td>
<td>10.67</td>
<td>1.1</td>
<td>11.8</td>
<td>52.8</td>
<td>1.3</td>
<td>0.46</td>
<td>3.7</td>
<td>0.07</td>
<td>0.32</td>
<td>0.00</td>
<td>0.0</td>
<td>22.0</td>
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<tr>
<td>Max</td>
<td>14.36</td>
<td>1.9</td>
<td>35.0</td>
<td>84.8</td>
<td>14.6</td>
<td>1.14</td>
<td>9.3</td>
<td>0.45</td>
<td>0.82</td>
<td>0.08</td>
<td>12.0</td>
<td>82.0</td>
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<td><strong>Patients with unexplained infertility</strong></td>
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<tr>
<td>Mean</td>
<td>12.44</td>
<td>1.5</td>
<td>20.7</td>
<td>72.0</td>
<td>7.3</td>
<td>0.83</td>
<td>6.8</td>
<td>0.20</td>
<td>0.59</td>
<td>0.04</td>
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<td>56.4</td>
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<tr>
<td>SD</td>
<td>0.86</td>
<td>0.2</td>
<td>7.4</td>
<td>7.8</td>
<td>4.0</td>
<td>0.26</td>
<td>2.2</td>
<td>0.14</td>
<td>0.16</td>
<td>0.03</td>
<td>3.0</td>
<td>15.6</td>
</tr>
<tr>
<td>Median</td>
<td>12.49</td>
<td>1.5</td>
<td>19.7</td>
<td>74.3</td>
<td>6.3</td>
<td>0.82</td>
<td>6.6</td>
<td>0.17</td>
<td>0.56</td>
<td>0.04</td>
<td>2.0</td>
<td>58.0</td>
</tr>
<tr>
<td>Min</td>
<td>9.42</td>
<td>1.1</td>
<td>6.4</td>
<td>50.4</td>
<td>1.4</td>
<td>0.41</td>
<td>3.4</td>
<td>0.05</td>
<td>0.27</td>
<td>0.00</td>
<td>0.0</td>
<td>18.0</td>
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<tr>
<td>Max</td>
<td>14.01</td>
<td>2.1</td>
<td>43.4</td>
<td>90.9</td>
<td>18.2</td>
<td>2.07</td>
<td>16.9</td>
<td>0.78</td>
<td>1.14</td>
<td>0.14</td>
<td>15.0</td>
<td>90.0</td>
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<td><strong>Comparison</strong></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</table>

For each man, high-magnification parameters have been calculated after analysis of 100 sperm. By dividing the vacuoles area by the number of vacuoles (data not shown) we obtain the mean area of one vacuole: it is 0.62 μm² in anterior position, 0.54 μm² in median position and 0.42 μm² in basal position.

SD, standard deviation; RVA: relative vacuole area (%) = [vacuoles area (μm²)/head area (μm²)] × 100.
tobacco consumption. The only correlation we obtained were positive ones between age and percentage of fragmented (TUNEL) and between age and DFI (SCSA). The subjects were divided into three groups according to age: group I, <30 years; group II, 30–37 years; and group III, ≥37 years. The SCSA analysis showed that the percentage of DFI in the older group (III) was significantly higher than that in the younger (I) group (P < 0.05). The TUNEL assay also showed that the percentage of fragmented DNA in the older group (III) was significantly higher than that in the group I and II (P < 0.05). Regression analysis demonstrated a significant positive correlation between the DFI and male age (P < 0.005; r = 0.32) and between the percentage of fragmented DNA by TUNEL assay and male age (P < 0.005; r = 0.29).

Discussion

In order to investigate the potential diagnostic value of sperm head vacuoles, we have begun by studying fertile men. To our knowledge, this is the first study that has investigated sperm morphology at high magnification on samples from so many fertile men. Some authors have studied normozoospermic men (Perdrix et al., 2012; Tanaka et al., 2012) but they were all infertile patients. The values provided here are from men within a couple with a spontaneous pregnancy in progress at the time of inclusion or in the last 2 years and with a time to pregnancy ≤12 months. It has been shown that such men have larger semen volumes, higher sperm concentrations, and higher total numbers of motile and morphologically normal cells per ejaculate than fathers without any known time-to-pregnancy, men in the general population or men screened for normozoospermia (Cooper et al., 2010). Another feature of this study is the use of an image analysis software; as for any morphological evaluation, it is necessary to use the most objective and precise tool as possible, especially when quantitative parameters are considered (such as the head and vacuolar areas). One consequence of the lack of a precise image analysis tool when using eye-based observations of the sperm morphology is the absence of a continuum of values for vacuole areas. This leads to the existence in the literature of many definitions of the large vacuoles and the arbitrary threshold can range from 25% (Boitrete et al., 2011) to 50% (Franco et al., 2008; Mauri et al., 2010; Oliveira et al., 2010) of sperm head area. In contrast, authors who have used an image analysis software could define precisely what a large vacuole is by considering the distribution of the vacuole sizes in the studied population (Saidi et al., 2008; Perdrix et al., 2011). The vacuoles that occupy >13% of the area of the sperm head are significantly more observed in spermatozooa from males presenting severe sperm alterations and are associated with chromatin alterations (Perdrix et al., 2011).

In our population of fertile men, the vacuoles area by spermatozoon was 0.79 μm² and the relative vacuole area is 6.5%. Anterior vacuoles are a little less common than the median vacuoles but they are larger. Basal vacuoles are rarer and smaller (see legend of Table II). This location of vacuoles is consistent with that found by Watanabe et al. (2011) in their group of three fertile donors. The prevalence of small vacuoles found in fertile men was extremely high, leading us to conclude that most of them should be considered as a common feature in normal human sperm and not associated with any pathology.

Regarding the vacuole parameters (Table II), we did not find any significant difference between the fertile and infertile men. Perdrix et al. (2011) have demonstrated (using ROC curves analysis) a higher discriminative power of the relative vacuole area to distinguish semen samples with normal and abnormal parameters over the other MSOME criteria. In our study, the relative vacuole area did not discriminate our two populations. In addition, we found no difference between fertile and infertile men in the percentage of spermatozoa without vacuoles or in the percentage of spermatozoa with large vacuoles. Thus, sperm vacuole assessment by high magnification (>6000) with interference contrast microscopy does not detect, by itself, some abnormalities involved in unexplained infertility.

The reason we studied vacuole parameters was that damage to the genetic component of spermatozoa (including chromatin condensation disorders) has been reported to be involved in idiopathic infertility (Rybar et al., 2009). Sperm chromatin alterations can result in poor embryonic development, decreased implantation rates, lower pregnancy rates and higher pregnancy loss (Evenson et al., 1999; Virro et al., 2004; Bungum et al., 2007; Carrell, 2008). Conventional sperm analyses poorly address these aspects of sperm quality and function. During the last decades, several methods have been developed to explore the nucleus status (Sperm Chromatin Dispersion test, TUNEL assay, SCSA, COMET assay) and it has been proposed that sperm DNA integrity could be a fertility predictor to be used as a supplement to the traditional sperm parameters (Evenson et al., 2002; Bungum et al., 2007). However most of these methods are not easily used in routine situations.

In the present study, sperm vacuole assessment failed to distinguish fertile men and men with idiopathic infertility. We also found no difference in nuclear integrity between fertile and infertile men. This could be explained by the fact that the vast majority of vacuoles should not

### Table III Vacuole and nuclear parameters according to male age of fertile and infertile men.

<table>
<thead>
<tr>
<th></th>
<th>Group I Age &lt; 30 years</th>
<th>Group II 30 &lt; Age &lt; 37 years</th>
<th>Group III Age ≥ 37 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>24</td>
<td>50</td>
<td>27</td>
</tr>
<tr>
<td>Vacuoles number per spermatozoon</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Vacuoles area (μm²)</td>
<td>0.81</td>
<td>0.80</td>
<td>0.84</td>
</tr>
<tr>
<td>Relative vacuole area (RVA) (%)</td>
<td>6.5</td>
<td>6.6</td>
<td>6.8</td>
</tr>
<tr>
<td>% of sperm without vacuoles</td>
<td>3.5</td>
<td>4.4</td>
<td>3.2</td>
</tr>
<tr>
<td>% of sperm with large vacuoles (RVA &gt; 13%)</td>
<td>8.2</td>
<td>9.3</td>
<td>10.0</td>
</tr>
<tr>
<td>Aniline blue staining immature nuclei %</td>
<td>7.4</td>
<td>8.0</td>
<td>8.0</td>
</tr>
<tr>
<td>SCSA DFI %</td>
<td>10.8</td>
<td>12.9</td>
<td>17.5*</td>
</tr>
<tr>
<td>TUNEL DNA fragmented %</td>
<td>5.8</td>
<td>6.3</td>
<td>9.0*hc</td>
</tr>
</tbody>
</table>

All the difference between the different groups are non-significant except for comparisons a, b, c.

*SCSA DFI between group I and III; P < 0.05.
*TUNEL DNA fragmented % between group II and III; P < 0.05.
**TUNEL DNA fragmented % between group I and II; P < 0.05.
be considered as chromatin alterations but as physiological changes. Their high frequency whatever their size in our two populations (including fertile men), highlights the physiological nature of some of these vacuoles as shown by Tanaka (Tanaka et al., 2012). In some old studies, transmission electron microscopy (TEM) showed that the vacuoles are structures specific to the human sperm head (Bedford, 1967; Zamboni et al., 1971) and that a human sperm nucleus usually contained one or more vacuoles at different locations (Schnall, 1952). According to the data about MSOME in the literature, only large vacuoles are associated with impairment of chromatin organization and the subpopulation of spermatozoa with large vacuoles was quite low in our two populations.

Unlike some authors (Oliveira et al., 2009; Perdrix et al., 2012), we did not find any correlation between the vacuolar parameters (mean number of vacuoles, relative vacuole area and percentage of spermatozoa with large vacuoles) and conventional sperm parameters. These results are in agreement with Komiya et al. (2013). We must consider that in our two populations the dispersion of values was quite low, which could explain why we did not find any correlation between vacuolar parameters and analysis of sperm nuclear material. Indeed, even though correlations between morphology at high magnification and DNA integrity have been described in infertile men (Oliveira et al., 2010), Maettner et al. have shown that in normal samples, the rate of spermatozoa with fragmented DNA is low and independent from MSOME morphology in contrast to oligo-astheno-teratozoospermic samples (Maettner et al., 2013). Our results showed a correlation between male age and sperm DNA defects assessed by SCSA and TUNEL assay as previously shown (Singh et al., 2003; Moskovtsev et al., 2006; Bungum et al., 2011; Fang et al., 2011; Horta et al., 2011; Belloc et al., 2014). These data demonstrated a decline in sperm DNA quality, as reflected by TUNEL and SCSA analysis, with increased age, which is likely to be due to oxidative stress (Singh et al., 2003).

In conclusion, this study is the first to describe, in a population of fertile men, the morphological parameters of sperm head vacuoles using high magnification with interference contrast microscopy. Furthermore, after comparing vacuole parameters (number, area, position) between fertile controls and patients with unexplained infertility, we did not find any significant difference. We can conclude that sperm vacuole assessment should not be recommended as a tool for investigation of the fertility status.

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Authors’ roles

N.G. recruited volunteers, performed experiments, collected and analysed data, and drafted the manuscript. L.M. performed experiments. C.E.-L. performed the TUNEL and SCSA analyses. R.D.L. and J.P. designed the study and critically revised the manuscript.

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Conflict of interest

None declared.

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Sperm vacuoles in men with normal sperm parameters