Cytoplasmic droplets are normal structures of human sperm but are not well preserved by routine procedures for assessing sperm morphology

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BACKGROUND: There is a discrepancy between the use of terminology employed by clinicians and basic scientists concerning the cytoplasmic droplets of sperm. Most clinicians consider their presence on sperm to be indicative of abnormal sperm, whereas basic scientists consider them to be attributes of normal sperm. METHODS: The presence of cytoplasmic droplets on human sperm was examined using conventional air-dried, fixed and stained sperm smears and in living and fixed wet preparations. RESULTS: Cytoplasmic droplets were found on the majority of motile sperm and in fixed preparations but only half of them were found in air-dried smears. There was no relationship between the presence of abnormally large cytoplasmic droplets, indicative of abnormal sperm, and the droplets found on living cells. CONCLUSION: The term ‘cytoplasmic droplet’ is confusingly used to describe two different sperm structures: large amounts of retained, excessive cytoplasmic remnants, that survive the air-drying procedure and are observed on abnormal sperm in conventionally stained sperm smears, and osmotically sensitive vesicles that are present on normal living sperm. A plea is made to retain the term ‘cytoplasmic droplet’ for the latter structure of normal sperm and to use the term ‘excess residual cytoplasm’ to describe the abnormally retained cytoplasm observed on abnormal sperm in smears.

Key words: artefacts/cytoplasmic droplets/human sperm/morphology/nomenclature

Introduction

Cytoplasmic droplets are a normal component of mammalian sperm, found at the neck of immature caput sperm and at the end of the midpiece in mature cauda sperm. Their movement along the midpiece during migration through the caput epididymidis is a characteristic feature of sperm maturation and they are retained on the majority of mammalian sperm stored in the cauda epididymidis (Cooper and Yeung, 2003). There are fewer sperm with droplets in the bovine ampulla (Branton and Salisbury, 1947; Rao and Hart, 1948) and few on ejaculated sperm from bulls (O’Donnell, 1969), rams (White et al., 1959) and boars (Lasley and Bogart, 1944a,b). Recent studies suggest that droplets may play an important role in fertility, since sperm are subjected to a hypotonic challenge upon ejaculation into the female tract, and water would initially enter the sperm’s cytoplasm, the bulk of which is in this organelle. In transgenic mice, sperm that cannot maintain their volume upon osmotic challenge exhibit flagellar angulation occurring at the site of the cytoplasmic droplet, and the mice are infertile (Cooper et al., 2004).

Observations on human ejaculated sperm that have been swollen by the channel blocker quinine and that display poor penetration of surrogate mucus suggest that volume regulation may play a role in human fertility (Yeung and Cooper, 2001; Yeung et al., 2003). The vast majority of literature on ‘cytoplasmic droplets’ of human sperm considers them to be indicative of abnormality with sperm being described as of ‘diminished maturity’ (Gergely et al., 1999) or ‘immature sperm’ (Ollero et al., 2000). The different terminology used to describe the cytoplasmic structure of human sperm (‘cytoplasmic droplets’, Rago et al., 2003), ‘cytoplasmic residues’ (Keating et al., 1997), ‘residual sperm cytoplasm’ (Aitken et al., 1994), ‘abnormal retention of cytoplasmic droplets’ (Zini et al., 2000), ‘retention of cytoplasm’ (Mak et al., 2000) attests to the confusion surrounding this organelle.

Such disagreement in terminology is not helped by the different descriptions given by semen analysis manuals. According to the World Health Organization (1999), cytoplasmic droplets observed in air-dried semen smears should only be considered morphological defects when large
(greater than one-third or one-half the sperm head size), implying that smaller droplets are not abnormal. The ESHRE/NAFA handbook (2002) confirms that sperm with retained cytoplasm less than one-third the sperm head size are normal but adds to the confusion by stating that residues larger than this are abnormal ‘and classified as cytoplasmic droplets’, associating that name with an abnormal structure.

Recent observations on osmotically sensitive ‘midpiece vesicles’ have prompted the view that such vesicles may be a normal component of human sperm (Abraham-Peskir et al., 2002; Chantler and Abraham-Peskir, 2004), although these authors consider them distinct from ‘cytoplasmic droplets’ by admitting that such ‘droplets’ survive air-drying and are characteristic of immature sperm.

The recommended clinical procedure for evaluating human seminal sperm (the production of air-dried smears before fixation: World Health Organization, 1999) can cause morphological artefacts. For instance, it produces severely swollen sperm heads when applied to immature epididymal sperm (Yeung et al., 1997; Soler et al., 2000), yet no swollen sperm heads are observed if the cells are fixed before making the smears. Such a drastic procedure as air-drying has been shown to disrupt fragile, osmotically sensitive midpiece vesicles (Abraham-Peskir et al., 2002). Thus the apparent absence of droplets from a normal spermogram may be an artefact of semen preparation for sperm morphological analysis.

This report describes experiments performed in order to assess the presence of true (not abnormal) cytoplasmic droplets on human sperm in living, in fixed and wet, and in air-dried preparations.

Materials and methods

Preliminary experiments

Three observations on semen from one donor, a healthy father, revealed the presence of cytoplasmic droplets on human sperm. (i) An ejaculate was produced directly in a vessel containing 5% (v/v) glutaraldehyde in phosphate-buffered saline (PBS) (Dulbecco’s, Sigma, Germany) and aliquots of the material were compressed under a cover slip for microscopic evaluation. (ii) Within 30 s of production an ejaculate was incubated at 37 °C and within 1 min, and every 5 min for 30 min before and after liquefaction, 50 μl was transferred to 500 μl 5% (v/v) glutaraldehyde fixative in Biggers–Whitten–Whittingham (BWW) medium (Biggers et al., 1971) and the percentage of sperm bearing droplets was ascertained microscopically. (iii) An ejaculate was brought to the laboratory within 75 s and incubated under 2 ml BWW medium [osmotic pressure (OP) 328 mmol/kg] containing 4 mg/ml bovine serum albumin. Sperm emerging from the liquefying ejaculate were evaluated in 5 μl aliquots for motility and presence of droplets in unfixed, wet preparations. After liquefaction, routine semen smears for morphology were made for evaluation of cytoplasmic droplets.

Ejaculates

Human ejaculates of widely differing quality were obtained with informed consent from 37 patients attending the Institute of Reproductive Medicine and from 12 student volunteers. Ten normozoospermic samples were included and the characteristics of the semen provided in this study, as analysed according to the World Health Organization manual (1999), are presented in Table I.

Measurement of osmotic pressure

The osmotic pressure of 10 μl semen was measured after liquefaction with a vapour pressure osmometer (Wescor Vapro 5510; Kreienfeld Scientific Measuring Systems, Germany), calibrated daily with a 290 mmol/kg standard solution. As the viscosity of semen retards saturation of the detection chamber, a timed delay of 2 min was employed before reading to ensure accuracy, as recommended by the manufacturer. BWW medium with osmotic pressure of 230 mmol/kg (BWW230) was made by reducing the amount of NaCl.

Microscopical observations

After liquefaction, semen samples were incubated for 15 min at 37 °C and 3 μl were examined under a 22 × 22 mm cover slip by phase contrast microscopy (Olympus BH-20, Japan) with a ×40 objective and ×10 ocular on a heated stage (Mini-Tüb, Germany) at 37 °C. The percentage of motile sperm (WHO grades a + b + c) was determined and immotile and motile sperm were separately assessed for the presence of (i) cytoplasmic droplets (small, regular distensions at the neck or midpiece: Figure 1a, c, e), (II) abnormal residual cytoplasm (large, irregular material along the mid-piece: Figure 1d, h, i), (III) coiled or looped tails (Figure 1g, j, k) or (IV) none of the above categories. To 10–20 μl of these samples was added an equal volume of 7% (v/v) glutaraldehyde and after 60 min at room temperature the fixed cells were washed by addition of 1 ml PBS, and centrifugation at 500 g for 5 min. The pellet was examined as a wet preparation at ×400 magnification for the presence of categories I–IV above. Similar preparations were examined in which semen was mixed with appropriate volumes of BWW230 to a final osmolality of 290 mmol/kg, the osmotic pressure of cervical mucus (Rossato et al., 1996).

The routine air-dried, Papanicolaou-stained semen smears of the same ejaculates were examined at both ×400 and ×1000 magnification by the same observer of the wet, fixed preparations and scored according to the same criteria for defining droplets. These stained smears were also examined for the presence of normal cytoplasmic droplets, defined as being smaller than one-third to one-half the sperm head size, and also for the presence of abnormal cytoplasmic residues by experienced andrology technicians.

Statistics

Differences between populations were assessed by paired or unpaired t-tests, and relationships by linear regression. P < 0.05 was accepted as statistically significant.

Table I. Characteristics of semen used in this study

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SEM (range)</th>
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<tbody>
<tr>
<td>Semen volume (ml)</td>
<td>4.3 ± 0.3 (1.6–7.6)</td>
</tr>
<tr>
<td>Sperm concentration (10^6/ml)</td>
<td>50.1 ± 7.6 (12–201)</td>
</tr>
<tr>
<td>Sperm count (10^6/jaculate)</td>
<td>200.2 ± 28.0 (24–828)</td>
</tr>
<tr>
<td>Motility (%)</td>
<td></td>
</tr>
<tr>
<td>Grade a</td>
<td>30.8 ± 2.0 (5–47)</td>
</tr>
<tr>
<td>Grade b</td>
<td>19.3 ± 1.4 (8–39)</td>
</tr>
<tr>
<td>Grade c</td>
<td>8.0 ± 0.6 (1–16)</td>
</tr>
<tr>
<td>Grade d</td>
<td>41.9 ± 1.2 (25–68)</td>
</tr>
<tr>
<td>Normal morphology (%)</td>
<td>12.0 ± 1.0 (2–27)</td>
</tr>
<tr>
<td>Abnormal residual cytoplasm</td>
<td></td>
</tr>
<tr>
<td>Osmolality (mmol/kg)</td>
<td>336.1 ± 3.2 (283–395)</td>
</tr>
</tbody>
</table>

Grade d 41.9
Grade c 8.0
Grade b 19.3
Grade a 30.8

Results

Preliminary observations

In order to determine if cytoplasmic droplets, as seen in other mammalian species and considered as normal structures, were present on human sperm, one ejaculate was fixed immediately upon ejaculation and two were fixed within 75 s of sample production. Observations on the sample delivered into fixative revealed the presence of droplets on the majority of sperm within the unliquefied coagulum. When aliquots of an ejaculate were fixed at intervals before and after liquefaction, 47–65% of sperm had visible droplets over the 30 min examined. When sperm taken from the medium surrounding a liquefying ejaculate were examined in wet preparations, 49–57% of immotile sperm bore droplets, whereas 68–92% of motile sperm did. The mean percentages of droplets observed in conventional Papanicolaou-stained smears of the two liquefied samples (14.5, 10.0%) were far lower than those found in the glutaraldehyde-fixed samples examined at 1 min (65%) or 20 min (67%).

Sperm morphology and motility

The majority of live sperm viewed in wet preparations in semen (mean 336 mmol/kg at the time of processing) and in media of female tract tonicity (290 mmol/kg) were observed to have cytoplasmic droplets at the neck regions, sometimes extending along the length of the midpiece (Figure 1b, f). The percentage of motile sperm with droplets significantly exceeded that of immotile cells in both semen and at the osmolality of cervical mucus (290 mmol/kg) and there was no difference in motility of sperm in these fluids (Table II). The same sperm suspensions fixed in glutaraldehyde also revealed cytoplasmic droplets on the majority of them.

<p>| Table II. Sperm motility in semen and Biggers–Whitten–Whittingham (BWW290) and the percentage of motile and immotile sperm each displaying cytoplasmic droplets |
|---|---|
| Motility (%) | Droplet-bearing sperm (%) |</p>
<table>
<thead>
<tr>
<th>Motile sperm</th>
<th>Immotile sperm</th>
</tr>
</thead>
<tbody>
<tr>
<td>In semen</td>
<td>49.3 ± 2.6 (10–87)</td>
</tr>
<tr>
<td>In BWW290</td>
<td>49.0 ± 2.8 (11–82)</td>
</tr>
</tbody>
</table>

Values are mean ± SEM (range) from 49 samples.

²Significantly different between motile and immotile fractions, P < 0.05.
In order to compare this value with that obtained from the live wet preparations, a weighted mean was obtained by multiplying the percentage of motile and immotile sperm by their respective percentages of droplets. This percentage (40.5 ± 1.5, n = 50, mean ± SEM) was not different (paired t-test) from that of the glutaraldehyde-fixed sperm (38.4 ± 1.7).

Sperm morphology in fixed and smeared preparations
The percentage of conventionally air-dried and Papanicolaou-stained sperm revealed significantly fewer droplets than observed in fixed wet preparations, whether observed at ×400 (magnification used for the fixed and wet preparations; 52%) or ×1000 (magnification used for routine semen analysis: 51%), although there was a significant correlation between the percentages of droplets observed in the dried and wet preparations by the same observer [Figure 2: $r = 0.500$ (×400), $r = 0.432$ (×1000)]. When abnormal cytoplasmic residues (defined as greater than one-third to one-half the sperm head size; World Health Organization, 1999) were assessed by experienced andrology technicians, the percentage was low (~9.6 ± 0.6) and bore no relation to the percentage of true droplets found in wet and fixed preparations (Figure 2). There was a statistically significant linear relationship between the percentage of what the andrology technicians assessed as abnormal ‘cytoplasmic droplets’ and what were assessed as residual cytoplasm (category II) in 47 wet preparations at ×100 ($r = 0.584$; data not shown).

Discussion
The results of this study indicate that ejaculated sperm from men possess true cytoplasmic droplets, which are normal structures of sperm in all other mammalian species, as judged from wet preparations on living gametes and in rapidly fixed preparations. The percentage of motile sperm with droplets in both semen and medium of 290 mmol/kg being higher than that of immotile cells suggests that they are not deleterious to vitality or motility and are clearly not a marker for poor sperm quality. That the percentage of sperm bearing droplets in live preparations agreed with estimates obtained from glutaraldehyde-fixed preparations confirmed that such droplets were not artefacts of cell fixation. Thus human sperm resemble other mammalian sperm in having a mid-piece cytoplasmic droplet; what differs is their position, at the neck rather than the end of the annulus, and that they remain attached to the spermatozoon in the ejaculate. Ultrastructural micrographs of well-fixed, human ejaculated sperm also demonstrate a cytoplasmic droplet at the neck (Holstein and Roosen-Runge, 1981; Johnson, 1982; Neugebauer et al., 1990) as they do in the epididymis (Ånberg, 1957).

A related and important observation for the morphological assessment of human ejaculates was the far lower percentage of droplets on sperm observed in air-dried, Papanicolaou-stained smears. Indeed, the same observer counted far fewer droplets in stained smears than in the live or fixed wet preparations of the same ejaculates. This attests to the general inadequacy of the routine method for preserving structures sensitive to the stresses accompanying air-drying before
fixing and staining. As even human sperm heads may expand under these conditions (Yeung et al., 1997; Soler et al., 2000), it should not be surprising that far less rigid and osmotically sensitive vesicles would collapse during preparation.

These findings confirm and extend the observations of osmotically sensitive 'midpiece vesicles' (MPV) extending along the length of the midpiece of human sperm in semen and cervical mucus (Abraham-Peskir et al., 2002; Chantler and Abraham-Peskir, 2004), which were also considered not detrimental to sperm and were not found in air-dried preparations. These authors considered the so-called MPV to be distinguishable from 'cytoplasmic droplets' by the absence of visible content and their higher incidence than conventional 'cytoplasmic droplets'. MPV were found to have no content as rendered visible by X-ray and differential contrast microscopy of wet preparations, whereas 'cytoplasmic droplets' assessed in air-dried preparations were expected to contain a proteinaceous content which is stained green with Papanicolaou dye. Others have considered swollen cytoplasmic droplets to be artefacts of Percoll preparations (Arcidiacono et al., 1983), although they clearly can be observed in the absence of Percoll, and their detection by confocal microscopy (Sofikitis et al., 1994) was also considered abnormal, presumably also because they resembled large cytoplasmic remnants observed in air-dried preparations.

We consider that MPV are 'true' cytoplasmic droplets as observed here (see Figure 1b, f), as they are present on living gametes and neither survive air-drying well. Thus we disagree with Chantler and Abraham-Peskir (2004) that the two organelles are distinct and believe that this difference reflects one of terminology: the abnormal (large, protein-rich, green-stained) 'cytoplasmic droplets' that survive the air-drying procedure are considered by many to be the equivalent of cytoplasmic droplets found on well-fixed sperm, whereas we argue that they are excess residual cytoplasm retained by abnormal sperm produced by imperfect spermiogenesis. This view is supported by electron micrographs of well-fixed semen that reveal the membranous components typical of a true cytoplasmic droplet within a vesicle extending the entire length of the human sperm midpiece (see Figure 3 in Smith et al., 1988). The presence of droplets on living sperm within cervical mucus (Abraham-Peskir et al., 2002) and on fixed sperm recovered from the Fallopian tube after artificial insemination by husband (Mortimer et al., 1982) suggest that they may be markers for functionally superior cells. Whether the presence of true cytoplasmic droplets observed in semen smears is related to fertility deserves examination.

The present observations on the cytoplasmic droplet on the midpiece of the majority of living human sperm highlights the inconsistent and confusing nomenclature whereby 'cytoplasmic droplets' are considered normal components of healthy mammalian sperm by basic scientists, but to be a structure associated with abnormal sperm by clinicians. This difference may well have to do with the preparation methods employed by each; generally well-fixed epididymal sperm versus air-dried smears of ejaculated sperm, respectively.

Midpiece structures surviving the latter, drastic treatment would include abnormally large amounts of excess cytoplasm not removed at spermatization, adhering to the midpiece and staining green with Papanicolaou. As such excess residual cytoplasm has been associated with sperm from smokers (Mak et al., 2000) and men with varicocele (Zini et al., 2000) and with deficiencies in sperm DNA (Fischer et al., 2003) and phospholipid-bound docosahexaenoic acid (Zini et al., 2000), its presence is indeed indicative of abnormal spermiogenesis. Such sperm should not be described as of 'diminished maturity' (Gergely et al., 1999) or as immature (Ollero et al., 2000), but merely abnormal, since they cannot and do not undergo maturation in the epididymis.

A plea is made to adhere to a common nomenclature and reserve the term 'cytoplasmic droplet' of human sperm to that defined for sperm from all other Eutherian species, namely an anatomically normal, osmotically sensitive component of a well-formed spermatozoon, produced by a functional testicular tubule, and which does not survive well the air-drying of semen smears. Conversely, the large, irregular 'abnormal cytoplasmic droplet' (World Health Organization, 1999), that does survive air-drying and is stained in routine semen smears, should not be termed a cytoplasmic droplet; the term 'excess residual cytoplasm' (Aitken et al., 1994) is suggested to indicate the nature of this organelle, found on abnormally formed sperm liberated from a testis displaying damage to the seminiferous epithelium. As both true cytoplasmic droplets and residual cytoplasm could be said to be forms of midpiece vesicles, this term is rejected in favour of the long-existing term cytoplasmic droplet. Acceptance of this terminology should obviate the confusion that has surrounded this topic in the past and reduce confusion arising when the same term is used for two completely different sperm structures.

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