Sperm Vacuoles: Origin and Implications

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Introduction

Since the beginning of the 1990s and the establishment of intracytoplasmic sperm injection (ICSI) [1] it is nowadays possible to help infertile couples due to male factors, e.g., severe oligoasthenoteratozoospermia or azoospermia by injecting single spermatozoa from ejaculate, [2], epididymal or testicular sperm [3].

Under in vivo conditions or conventional in vitro fertilization (IVF), there is continuous natural selection against inherited factors which reduce fertility. Natural barriers occur within the male and female tract to remove faulty gametes. If we keep in mind that ICSI bypasses the natural barriers of spermatozoa selection, fertilization with abnormal spermatozoon bears the danger of potential genome enrichment with pathological alleles for the future generations [4].

With such an conceivable scenario that genetic infertility factors may be propagated via subfertile males, it might be reasonable to develop specific techniques for more accurate spermatozoa selection. As still few possibilities are available for a "positive" selection of spermatozoa, which can be later on used for injection of oocytes, particularly refined morphology assessment would be eligible.

The assessment of sperm morphology by Kruger´s strict criteria (spermocytogramme) is routinely applied and widely accepted as one of the most important predictor that correlated with a reduction of the fertilizing potential [4, 5]. This highlights the notion that sperm morphology evaluation is a very important task in the treatment of infertile couples.

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Bartoov et al. [6] reported that quantitative ultramorphological sperm analysis using scanning electron microscopy (SEM) and transmission electron microscopy (TEM) is clinically informative, and is recommended when the male infertility factor cannot be clearly diagnosed by routine tests prior to first assisted reproductive technique (ART) trial. However, such as for classical spermocytogramme, morphological assessment is performed after fixation and staining processes.

In order to counteract the problem of morphological evaluation on stained spermatozoa, Bartoov et al. [7] introduced the MSOME. With the use of Nomarski differential interference contrast optics (DIC), a better three-dimensional view of the head became available. It is possible to observe in real time details, such as the so-called vacuoles, on the surface of motile sperm head.

Cephalic vacuoles are the subject of debates and controversies [8] and raised several issues regarding their origin, the reason for the occurrence of vacuoles and their pathological character with potential implications in infertility.

In Vivo Formation of Vacuole-Like Structures

When Are They Produced?

Using DIC optic, vacuoles appear as depressions at the cell surface, like lunar craters that are visible with the tangent sunlight. This observations shows that the terminology "vacuole" for these structures is misleading. With different microscopic approaches, the vacuole-like structures on the sperm head were termed craters [9] concavities [10], hollows [11] or lacunae [12]. Boitrelle et al. [10] observed that the sperm plasma membrane was intact and invaginated nearby the vacuole and that the sperm-head's thickness falls to 300 nm at the site of the large vacuole. They concluded that vacuole-like structures are nuclear depressions which correspond to a concavity in the plasma membrane rather than a hole.

The origin of vacuoles is still not fully elucidated. Literature referring to animal models [13, 14] as well as to human spermatozoa [15–18] describes the formation of nuclear vacuoles during the spermiogenesis. The same hypothesis was set up for human spermatozoa, in 1989 Baccetti et al. suggested that the nuclear and acrosomal invaginations are formed during spermiogenesis [15–18] According to these findings, the presence of vacuoles is already noticeable in elongated spermatids after testicular retrieval. The presence of vacuoles in round spermatids was demonstrated recently by Tanaka et al. [18]. Based on the classification of Clermont et al. [19], Tanaka et al. [18] and Mansour et al. [20] there are low rates (18 %) of vacuoles in spermatids entering cap phase (stage Sb1), their occurrence increases during stage Sb2 to reach a high level of 93.8 % when they are at the acrosomal phase (stage Sc; these stages of spermiogenesis).

If it is obvious that small and large vacuoles are observed in the majority of ejaculated spermatozoa and their frequency differs according to the severity of the male infertility. If it is often difficult to observe vacuole-free sperm cells in ejaculates from infertile men, in contrast to semen derived from proven fertile men. Tresholds were established for fertility, for example, Falagario et al. [21] identified a cut off of 20 % for sperm nuclear vacuolization on the total of sperm in a seminal sample. According to De Vos et al. [22] the prevalence of vacuoles in normal shaped spermatozoa seems to be low. Under high magnification, they analyzed the frequency of vacuoles in 330 male infertility semen. They reported that almost 33.3 % of the spermatozoa were morphologically normal and exhibited less than two small vacuoles. Normal shape spermatozoa with more than two small vacuoles or at least one large vacuole represent 12.3 % of the population. Finally 54.4 % showed abnormal head shapes with or without large vacuoles in conjunction with other abnormalities.

Silva et al. [23] investigated the influence of paternal age on sperm quality by MSOME. The frequency of large nuclear vacuoles was significantly higher in the older group (>41 years age) compared to the younger age groups. Such observation corroborated the study of De Almeida Ferreira Braga et al. [24].

How to Consider Vacuole-Like Structures: As a Sign of Nuclear Dysfunction or as a Normal Stage in the Acrosomial Process?

Vacuole-Like Structure and Nuclear Dysfunction

The most interesting question in connection with vacuoles is, whether these large intranuclear lacunae or structure like vacuoles are the morphological manifestation of nuclear dysfunction. Assuming that they seem to appear during the last maturation step of round spermatids, do they originate from a natural process or, more likely, from pathological (stress) situations during spermiogenesis or even early in the first stage of the spermatogenesis? In other words, what hides behind spermatozoa with large nuclear vacuoles?

The literature is controversial, while some studies report that there is no relationship between the presence of sperm head vacuoles and sperm function suggesting that sperm vacuoles should be regarded as a normal feature of the sperm head [11, 18], others mentioned that it is related to male subfertility [25]. However, Tanaka et al. [18] highlighted that the size of the vacuoles is of importance and suggested that spermatozoa with large vacuoles should not be used for injection.

A multitude of studies concluded that vacuoles reveal indirectly nuclear dysfunction in terms of lower mitochondrial potential [26], DNA integrity, aneuploidy rate and problems related with chromatin condensation.

Out of ten studies [10, 11, 26–31] determining the degree of DNA fragmentation usually with Tunnel assay, five [24, 27, 29, 31, 32] reported that vacuole-free spermatozoa yields lower rates of DNA fragmentation as compared with vacuolated spermatozoa. Perdrix et al. [27] observed for vacuolated spermatozoa a significant increase in the rates of aneuploidy and diploidy. However, for Boitrelle et al.[30] and de Almeida Ferreira Braga et al. [24], the presence of sperm aneuploidy was not correlated with the presence of nuclear vacuoles. Assuming that DNA fragmentation is mostly due to oxidative attack, and that sperm DNA condensation is a protection against ROS (reactive oxygen species), it may result that the apparent divergences between these papers could be explained by different levels of oxidative stress in patients, leading to different levels of DNA fragmentation [33].

Several DNA and chromatin staining assays including aniline blue and chromomycin A3 (CMA3) were applied in order to assess more precisely information about integrity of DNA in vacuolated spermatozoa. A negative correlation between the incidence of vacuoles and abnormally condensed chromatin was observed in all the nine conducted studies [10, 11, 26–32]. In these manuscripts, spermatozoa with large vacuoles were selected by micromanipulation before being studied by different microscopy and immunocytochemistry techniques. All the conducted studies concluded that vacuoles did not take their origin in the acrosome but that they are linked to areas of chromatin decondensation [10].

The presence of craters most likely reflect molecular defects responsible for anomalies of sperm chromatin packaging and abnormal chromatin remodeling during sperm maturation [34–36]. Boitrelle et al. [10, 30] observed chromatin condensation at the site of the vacuole and concluded that a large vacuole appears to be a nuclear "thumbprint" linked to failure of chromatin condensation. This was also confirmed in another study of Boitrelle and colleagues for small head vacuoles [10] Perdrix et al. [37] recently published their observations of the correlation between the presence of large nuclear vacuoles and chromosome architecture modifications, adding a new argument for the association between nuclear vacuole-like structure and chromatin disorganization.

According to the growing body of literature adding new arguments for the association between vacuoles and chromatin disorganization, an association between the two becomes more and more obvious. With the disorganization of the chromatin and the vacuoles in the sperm head, the spermatozoa and its DNA becomes more assailable to attacks by ROS [38–40]. Thereby, DNA fragmentation would depend on two steps, the occurrence of vacuoles in connection with insufficient chromatin condensation and on the presence of ROS. This could explain why the correlation between the presence of

vacuoles on the rate of DNA fragmentation is not observed unanimously.

Chemes and Alvarez Sedo [41] studied the morphology of the sperm head by TEM. They proposed that the small lacunae observed in spermatozoa nucleus characterize the site of a normal proteolytic activity linked to histone to protamine transition. However, Haraguchi et al. [42] suggested that larger lacunae may be the result of a deregulated histone-protamine transition during spermiogenesis due to an overactive or disregulated ubiquitin proteasome system.

Could vacuoles be a selective mechanism for defective sperm to be removed in the natural selection-process? We know that in sperm "incomplete apoptosis" is a common phenomenon [43]. Spermatozoa which do not pass the "quality control" due to, e.g., DNA-defects or other aberrations during spermatogenesis undergo the normal pathway towards apoptosis but are not removed by phagocytes. Maybe the formation of vacuoles is a mechanism for abnormal spermatozoa to be attacked by ROS during storage and thereby being discarded.

In the light of these studies, we know that during spermiogenesis, spermatids undergo a complex restructuring program in which, in addition to acrosome and sperm tail formation, DNA is tightly packed leading to a drastic reduction in the size of the nucleus. These unique cellular reconstruction process requires spermatid-specific genes to execute their regulatory roles. It is estimated that 600–1.000 germ cell-specific genes participate in spermiogenesis, and specific genes such as Prm1, Prm2, Tnp1, Tnp2, and H1t2 are involved in chromosomal packaging [44].

Chromatin condensation takes place during spermiogenesis allowing protection of the paternal genome during the transit from the male to the oocyte prior to fertilization. The chromatin is radically reorganized and undergoes an extreme condensation resulting in a shift from a nucleosome-based genome organization to the sperm-specific, highly compacted nucleoprotamine structure [45]. About 85 % of human sperm histones is replaced with protamines, whereas only 15 % of the DNA remain organized by histones or is attached to the nuclear matrix [46]. Recently, Rousseaux et al. [47] demonstrated a new key stone in DNA compaction in humans and murines. They found that a testis-specific protein called bromodomain testis-specific protein (BRDT), which possesses two bromodomains capable of interactions with hyperacetylated histones, is likely to be at least partially responsible for the replacement of histones by protamines. The genome-wide incorporation of a new histone variant called testis-specific histone 2B (TH2B) might also play an important role in this histone to protamine transition as shown in murine models [48].

Prior to histone replacement by protamines, the nucleosomes are destabilized by hyperacetylation and by DNA methylation [49, 50]. Moreover the distribution of the remaining 15 % nucleosomes after the 85 % nucleosomes to nucleoprotamine replacement is not random but concerns gene regions involved in the epigenetic control and the early embryonic development [46, 50–52]. On the other side, the ratio between the two protamine subtypes protamine 1 and 2, which should normally be close to 1, can have a significant negative impact on fertility when disturbed [53]. Taken together, these data supports the idea that bad condensation of sperm DNA has a great impact on male fertility. All these potential epigenetic pattern disturbances may represent the basis of numerous human disorders.

Beside that epigenetic role of sperm chromatin condensation, particular organization of sperm DNA is also important for its protection, especially against fragmentation, during spermatozoa journey through male and female genital tracts [43].

Vacuole-Like Structure: A Receptacle of Acrosomal Enzymes?

As vacuoles are mostly localized in the anterior part of sperm head, in the region of the acrosome, one of the hypothesis on the origin of vacuoles was that they were mostly of acrosomal origin [54]. Kacem et al. stated that sperm nuclear vacuoles are mainly associated with the presence of acrosomal enzymes such as trypsin-like acrosin that may induce a harmful effect after oocyte injection. As consequence, they concluded that a

large majority of normal, regularly shaped spermatozoa showing no vacuoles have already undergone their acrosome reaction and should be selected for injection.

Montjean et al. [55] tested the effect of inducers of the acrosome reaction. After incubation of sperm in either hyaluronic acid or follicular fluid for 90 min, they observed a highly significant decrease in the presence of vacuoles as a consequence of the acrosome reaction.

The study of Neyer et al. [56] did not corroborate those of Kacem and Montjean [54, 55]. In a time-lapse set-up they monitored single spermatozoa in sperm capture channel during 24 h and observed that the induction of the acrosome reaction using calcium ionophore A23587 did not lead to any modifications in pre-existing vacuole appearance, disappearance or formation [56].

In a recent paper, Gatimel et al. [57] described the MSOME performed on the semen of two men suffering from globozoospermia. In these two patients, all the spermatozoa totally lacked acrosomal structures, as confirmed by TEM and SEM, but vacuoles were present in the majority of cells (92 and 76 %), at a rate comparable to that observed in fertile controls. From those studies, we may conclude that there is a negative relation between the presence of vacuoles and the sperm capacity to undergo acrosome reaction. For Boitrelle et al. [10, 30], sperm membrane and acrosome cap are intact at the site of these depressions.

Likewise, Perdrix et al. [27] demonstrated an exclusive nuclear origin of these large head surface depressions using TEM supporting their severe impact on sperm quality.

In Vitro Formation of Vacuole-Like Structures: A Reality?

Peer et al. [58] compared the impact of incubating prepared sperm at 37 °C or at 21 °C. They concluded that after 2 h of incubation at 37 °C in culture media, the incidence of spermatozoa with vacuolated nuclei was significantly higher, so that prolonged sperm manipulations for assisted reproduction therapy should be performed at 21 °C rather than 37 °C. Schwarz et al. [59] reported a significant increase in sperm nuclear vacuolization in washed sperm but not in swim-up sperm. They concluded that the method used for sperm preparation influences sperm nuclear vacuolization and that vacuolization is unaffected by temperature in motile sperm isolated by swim-up.

Neyer et al. [56] developed a system called sperm-microcapture channels that permits an accurate observation of the same population of living spermatozoa over a period of 24 h. They analyzed whether incubation temperature (20 or 37 °C) or oxidative stress stimulates the formation of nuclear vacuoles. They observed that neither incubation at 37 °C nor induction of oxidative stress induce de-novo formation of nuclear vacuoles. According to these observations, they concluded that nuclear vacuoles on the sperm head are already produced at earlier stages of sperm maturation and are not induced or modulated by routine laboratory procedures.

However, Boitrelle et al. [60] observed that cryopreservation of human spermatozoa induces nuclear vacuolization and increases the proportion of spermatozoa with noncondensed chromatin, while Gatimel et al. [61] did not corroborate this conclusion.

Vacuole-Like Structure and Embryo Development

If vacuoles are associated with impaired chromatin packaging and with DNA fragmentation, one crucial question to investigate concerns the significance of vacuoles on the outcome in terms of fertilization, embryo development, pregnancy, miscarriage and health babies born.

This question was studied and reported by a few recent papers. It has been demonstrated that sperm nuclear vacuole size and number, as seen with DIC Nomarski optics, negatively affects blastocyst development. In four successive papers [62–65] it was shown that the occurrence of large nuclear vacuoles and/or abnormal shape reduces the percentage of good-quality embryos reaching the blastocyst stage after culture until day 5. Following the outcome of each embryo after

injection of spermatozoa, they clearly demonstrated that the use of spermatozoa with no vacuoles or less than two small vacuoles can be associated with significantly higher blastocyst rates than injection with spermatozoa showing more than two small vacuoles or one large vacuole with or without abnormal shape. These studies support the previously issued hypothesis that the impact of male infertility may be at an early stage (early paternal effect), when spermatozoa is not able to attain, penetrate and/or activate the oocyte, or at a late stage (late paternal effect) when it could not support embryo development, implantation and pregnancy to term. Late paternal effects are observed after paternal genome activation and blastocyst development failure is one of their first manifestations [66–68].

The link between sperm head vacuoles and impaired chromatin condensation, and the occurrence of DNA fragmentation in the presence of ROS may explain why vacuoles can be related with impaired human embryo development [39, 65, 69] and pregnancy outcomes [67, 70–72].

Vacuole-Like Structures, Pregnancies, and Miscarriages

A more specific analysis of the impact of sperm cells with normal nuclear shape but with large vacuoles was first carried out by Berkovitz et al. [73] on two matched IMSI groups of 28 patients each. Spermatozoa with strictly defined normal nuclear shape but large vacuoles were selected for injection and compared to a control group that included normal nuclear shape spermatozoa lacking vacuoles.

No difference in the fertilization and early embryo development up to day 3 were reported. However, injection of spermatozoa with strictly normal nuclear shape but large vacuoles appeared to significantly reduce pregnancy outcomes (18 % versus 50 %) and seemed to be associated with early abortions (80 % versus 7 %).

Other studies showed also that selection of normal shape spermatozoa with a vacuole-free head was positively associated with pregnancy and lower abortion rates after day 3 or day 5 embryo transfers in couples with previous and repeated implantation failures [62, 74–82], in patients with an elevated degree of DNA fragmented spermatozoa [36] and in patients with a high degree of teratozoospermia [83]. In a recent prospective randomized study, Setti et al. [84] show the beneficial effect of performing IMSI in cases of advanced maternal age (women age $\geq$ 37 years old).

However, some other studies failed to show any effect of selecting vacuole-free sperm on ART outcome [82, 85, 86]. One possible explanation therefore is the patient selection. Probably IMSI indications are not precise enough, and doing IMSI in an unselected or a bad-selected population will not be advantageous. Another point is that IMSI seems to promote blastocyst development when selecting vacuole-free spermatozoa (see precious point on vacuoles and embryo development). So in addition to implantation and pregnancy rates, we have to take in account pregnancies obtained with frozen-thawed supernumerary embryos, and to calculate cumulative pregnancy rate (fresh + frozen/thawed embryo transfers) per follicle puncture. Knez et al. [65] showed that there was no significant difference in the pregnancy rates between IMSI and ICSI procedures after blastocyst transfer. However, after ICSI more pregnancies terminated by spontaneous abortion, whereas after IMSI there was no spontaneous abortion. One explanation could be that IMSI procedure permits to select spermatozoa without defect and as consequence provide more "healthy" blastocysts, possibly, in spite of very comparable development and morphology in ICSI-derived blastocysts.

Vacuole-Like Structures and Postnatal Data

Still concerns remain about the long-term safety of injecting spermatozoa carrying vacuoles. We have to be cautious, especially in the light of Aitken's work [33] on the putative negative effects of sperm DNA fragmentation for the next generation. Depending on the level of sperm nuclear DNA fragmentation, oocytes may partially

repair fragmented DNA, producing blastocysts able to implant and develop up to live offspring. However the incomplete repair may lead to long-term pathologies. The work of Fernandez-Gonzalez et al. [87] on the mouse model indicates that the use of DNA-fragmented spermatozoa in ICSI can generate effects that only emerge in later life, such as, aberrant growth, premature aging, abnormal behavior and tumors derived from the mesenchymal lineage. Moreover the association of vacuoles with defects in chromatin packaging, which has an important role in epigenetic control of paternal genome as discussed earlier, is an argument in favor of the selection of vacuole-free sperm for oocyte injection.

Up to now, there are in sufficient numbers published studies concerning the health of children born after ICSI to draw any firm conclusions about the long-term safety of this procedure. However, it is important to emphasize that animal data are absolutely unequivocal on this point and clearly indicate that DNA damage in the male germ line is potentially hazardous for the embryo and therefore for the resulting offspring. According to two recently published papers, paper, sperm nucleus morphological normalcy, assessed at high magnification, could decrease the prevalence of de novo major fetal malformations in ICSI children [88, 89].

Conclusions

The introduction of MSOME and IMSI points to embryologists that more attention has to be paid during sperm selection, even when it is done with a conventional optic.

It is now confirmed in the literature that the occurrence of vacuole-like structures on the sperm head is related with sperm chromatin immaturity. However, the background and the relation between the two are still unclear. Do we face a chicken-and-egg problem? Do both, vacuoles and abnormal chromatin condensation occur at the same time or is one the consequence of the other? At this point the most probable explanation is that the vacuoles, which are in fact concavities in the sperm head membrane, first appear during the spermiogenesis rendering the nucleosome and DNA and connected molecules more vulnerable to intrinsic or extrinsic attacks by ROS. According to the level of ROS, DNA fragmentation may appear. More research on this area will bring light in these processes.

So the application of IMSI leads to more blastocysts of higher quality, increasing the chance to transfer an embryo with a high implantation potential and to obtain the birth of a healthy baby.

Seeing that this simple, noninvasive technique still arises debates and scepticism exists about its efficiency, mainly due to a low number of controlled randomized studies published yet, one fundamental question is whether we should—with the knowledge that sperm vacuoles are related with abnormal chromatin packaging and possibly with DNA fragmentation—select spermatozoa with these defects for injection if we have only to change the optics? As far as we know, there is no reason for not selecting the morphologically best spermatozoa.

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