Blackwell Publishing

Asian J Androl 2007; 9 (6): 731–737
DOI: 10.1111/j.1745-7262.2007.00311.x

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·*Review*·

Detecting subtle changes in sperm membranes in veterinary andrology

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Abstract

Thanks to the increasing use of flow cytometry in research in veterinary spermatology, many new membrane integrity assays have been developed over the past decade. These assays are important because of their superior ability to forecast fertility when compared with other tests, such as sperm motility. This major component of the sperm quality assessment has generated new investigations with the aim of developing tests that can detect membrane damage in a very early state. Using phospholipid transposition tests, early changes in membrane permeability and fluidity can be assessed in a large number of spermatozoa using fluorescent probes in combination with flow cytometry. *(Asian J Androl 2007 Nov; 9: 731–737)*

Keywords: sperm membranes; flow cytometry; early membrane changes; veterinary

1 Introduction

Detection of subtle changes in sperm membranes is an issue of utmost importance in human and veterinary andrology [1–12]. Inter-individual and inter-species variation in the ability of the ejaculates to withstand cryopreservation [13–20] and other biotechnologies make it necessary to continuously improve and adapt cryopreservation protocols, depending on the species or individual. This is even more important in those species in which the current cryopreservation protocols are still suboptimal. In this regard, evaluation of sperm membranes is a key step in the process of development of new cryopreservation protocols, because these sperm structures are extremely sensitive to the stress induced by freezing and thawing. In view of this fact, methods to disclose minimal and/or early pathological changes in sperm membranes are of critical importance.

2 Classical fluorescent methods to detect damaged membranes

Although some methods based on histological stains for sperm membranes are still used in clinical practice and even sometimes in research, the use of fluorescent staining together with flow cytometry is the usual practice in the world leading laboratories in the field of veterinary andrology [21–25]. Although numerous fluorescent dyes have been tested, such as carboxifluorescein acetate combined with propidium iodide, Hoescht 33258,

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Received 2006-12-08 Accepted 2007-05-16

or calcein acetate in combination with ethidium homodimer, the combination of SYBR-14 with either propidium iodide or ethidum homodimer is the combination of probes most frequently used [26]. Both are DNA-binding probes. However, both probes differ in their ability to penetrate sperm membranes. SYBR-14 is an acylated DNA binding probe [26]. Because of the acetyl moieties, this probe easily penetrates intact sperm membranes. In living cells, the probe is deacylated by intracellular esterases, leaving the probe entrapped in the cell. However, both ethidium homodimer and propidium iodide are non-permeable probes, thus staining only the sperm nucleus if there is a fracture in the sperm plasma-lemma. These probes are used in combination, because the range of excitation is similar (488 nm) while the range of emission differs. SYBR-14 emits in the green wavelength (515 nm) whereas both ethidium homodimer and propidium iodide emit in the red wavelength (610 nm).

When both probes are used in combination, as is the usual practice, two to three sperm subpopulations are easily identified. The first subpopulation comprises spermatozoa showing green fluorescence. These are cells with physically intact membranes. The spermatozoa showing red fluorescence are cells with complete membrane damage, whereas those cells showing green and red fluorescence are spermatozoa with damage in the sperm membrane, which allows ethidium homodimer or propidium iodide to enter, and displace or quench SYBR-14. Also, these doubly-stained spermatozoa observed in the scattergrams might result from two sperm, one green and one red, being simultaneously measured as they pass through the flow cell. However, if an adequate gate is selected based on forward and side scatter properties of the spermatozoa, together with a low flow rate, this artifact can be minimized [26].

This is a good technique for quality control of the semen samples at the artificial insemination station. However, the need for improvement of semen freezing protocols, or the possibility of developing "individual adapted" protocols requires new tests that can detect damages of sperm membranes in the very early stages, and even the detection of not only physical, but also biochemical injuries in the sperm membranes.

3 Detecting subtle changes in sperm membranes

3.1 Phospholipids transposition assays

When the cell membrane is disturbed, the phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, probably related to either a decrease in the activity of a putative translocase, or an increase in the activity of scramblases, or both [27]. This is one of the earliest changes in the sperm membrane, and can be monitored by the calcium-dependent binding of Annexin-V (A). Recently, it has been demonstrated that freezing-thawing of human [28], bull [29, 30] and boar [10] spermatozoa induces membrane PS translocation. A binding appears to be more sensitive in detecting a deterioration of membrane functions compared to propidium iodide (PI) staining in frozen-thawed human semen samples [31] and is also more sensitive than the combination of SYBR-14/PI for bull semen [29]. Moreover, a considerable percentage of the live human spermatozoa appear to have dysfunctional plasma membranes besides those dead or moribund cells. Apoptosis plays a major role during spermatogenesis, and also a significant role during the final stages of sperm differentiation. In Drosophila, the remodelling of the surplus cytoplasm during spermatid differentiation seems to be a caspase-mediated apoptotic mechanism [32]. However, once mature spermatozoa are ejaculated, they are theoretically unable to undergo apoptosis, because they are terminal cells that do not retain the necessary caspase-mediated mechanism [33]. Despite this basic assumption, a number of spermatozoa might present aminophospholipid exposure after ejaculation [34], remainders of an earlier, abortive apoptotic change. Such spermatozoa would, supposedly, be non-functional, but they would, on a routine spermiogramme, be misjudged as normal spermatozoa. Some apoptotic-like phenomena have been claimed to occur in mature spermatozoa, such as the translocation of PS from the inner to the outer leaflet of the sperm membrane [35], which accompanies subtle changes in sperm membranes related to cryoinsult or capacitation [20, 36, 37]. However, not all the studies have detected transposition of PS in capacitated spermatozoa [38–41], or in spermatozoa subjected to biotechnological procedures such as sorting [42]. The reasons for these discrepancies might be related to differences in the experimental design and a lack of consensus in the techniques used to detect capacitation. In spite of this, new data indicate that an apoptosis-like phenomena is involved in cryodamage [43–46], and has been suggested that PS transposition is mainly related to early membrane degeneration as well as dead cells as occurs in somatic cells [41]. It is of interest to note that

PS is also present in the outer acrosomal membrane [38]. The fact that a recent human study shows that the annexin assay provides information for predicting the outcome of preservation [47] adds value to this technique.

The molecular mechanism involved in such putative apoptosis is still under debate. However, recent experiments demonstrate the involvement of caspases in mature spermatozoa, both in humans [44–46] and animals [48]. Apparently, the mitochondrial pathway of apoptosis is present in mature spermatozoa [39]. Also, a caspase mediated apoptotic mechanism has been demonstrated to be involved in sperm death induced by *Chlamidia trachomatits* [49]. We have detected active caspases in our laboratory in frozen thawed canine and pig spermatozoa using the caspase inhibitor VAD-FMK. Interestingly, in some human studies, ejaculates showing lower cryotolorerance, in infertile patients, showed a higher susceptibility to caspase activation [45, 50, 51].

In the last decade the use of aminophospholipid exposure assays to evaluate sperm membranes has received increased interest both in human [28, 31] and veterinary andrology [29, 30, 36]. The use of this technique to evaluate sperm membranes is assumed to be a more accurate and discriminative method than the combination of classical fluorescent probes, such as the well-validated combination of SYBR-14/PI [26]. The higher value of aminophospholipid exposure assays in the evaluation of sperm membranes relies on their ability to detect changes in the membrane at an earlier stage than other fluorescent probes. In fact, the population of live sperm, as evaluated using SYBR-14/PI, is a heterogeneous group of spermatozoa because we can detect living spermatozoa that has experienced the transposition of PS from the inner to the outer leaflet of the membrane, intermingled with living spermatozoa without this transposition (by definition, the latter are to be considered completely viable spermatozoa) [29, 36]. Live spermatozoa with PS translocation are probably not completely competent. In fact, the presence of these cells increases after cryopreservation in bull [29] and human semen [31]. Interestingly, those boar ejaculates having large numbers of spermatozoa presenting PS-transposition are more sensitive to the freezing-thawing procedures [36]. Although yet speculative, we hypothesized that the subpopulation of live spermatozoa with PS translocation might essentially represent the subpopulation of spermatozoa that would have a reduced life span *in vivo* [52, 53]. However, PS exposure after cryopreservation is pancellular, whereas bicarbonate induced PS exposure is specific for mature sperm cells, and only in the apical plasma membrane area of the sperm head of acrosome intact live cells [35, 37]. Under the stress of cryopreservation, enzymes involved in maintaining phospholipids asymmetry are silenced, which results in exposure of aminophospholipid, especially in the sperm mid-piece area [35, 37]. The identification and quantification of this subpopulation is, obviously, of extreme importance when designing freezing and thawing procedures.

3.2 Changes in sperm membrane permeability

Detection of increased membrane permeability is used in different cell types to distinguish different status of membrane organization [54–57]. In determined physiological or pathological situations, live cells are unable to exclude YO-PRO-1, but are still not permeable to other dead-cell discriminatory dyes, like propidium iodide or ethidium homodimer. YO-PRO-1 is an impermeable membrane probe and can leak in, only after destabilization of the membrane, under conditions where ethidium homodimer does not. Because several ATP-dependent channels have been detected in spermatozoa [58], it seems plausible that this is a result of the silencing of a multidrug transporter. This multidrug transporter is involved in transporting amphipathic small molecules like YO-PRO-1, which in intact cells is actively pumped out but not after destabilization of the plasma membrane, maybe because sub-viable cells lack appropriate amounts of ATP to transport YO-PRO-1 back out of the cell [55]. Therefore, the use of a fluorescence probes, such as YO-PRO-1, which penetrates cells as they undergo changes related to cryoinjury, where membranes become slightly permeable, makes YO-PRO-1 a useful tool for detecting early membrane changes [59, 60].

We have developed a triple staining combining SNARF-1, YO-PRO-1 and ethidium homodimer [10, 11] to disclose early changes in sperm membranes in a similar way as the annexin assay, but using a simpler, lower cost, and more suitable assay for microscopy than the use of annexin.

The triple staining technique offers some advantages over the A/PI assay. Whereas in the A/PI assay there is always an unstained subpopulation, the triple stain labels all the spermatozoa in the sample, an obvious advantage when using manual counting in fluorescence microscopy. If a flow cytometer is available, because only sperm cells are stained with the triple staining technique, spermato-

zoa and debris can be easily separated based not only on scatter properties of the particles but also on their fluorescent properties. This fact is important because in bull semen, it has been demonstrated that egg-yolk particles can be easily misjudged as spermatozoa based only on their scatter properties [21], requiring preliminary washing and centrifugation to cleanse the cells. Centrifugation might cause sperm damage and, therefore, mask other effects caused by the cryopreservation.

The three probes are easily distinguished both in flow cytometry and in fluorescence microscopy. The absorption and emission maxima for YO-PRO-1 are 491 nm and 509 nm, respectively, and 528 nm and 617 nm, respectively, for ethidium homodimer to be detected in the Flow Cytometer with the FL1 and FL3 photomul-tipliers. The absorption and emission maxima of SNARF-1 is 488 nm and 575 nm, respectively, and is detected on FL2. Because of the emission and excitation of the probes used in combination, the assay can be easily monitored using conventional fluorescence microscopy, without the need to change filters, making this combination of fluorophores especially useful for routine conditions, without using expensive flow cytometers.

This triple staining distinguishes, as in the A assay, four sperm subpopulations. The first is the subpopulation of spermatozoa which are stained with the SNARF-1, considered alive and without any membrane alteration. It is noteworthy that SNARF-1 stains the whole cell, making their discrimination easy from those membrane-damaged, ethidium homodimer-positive cells (that stain red, but at the sperm head), a difference easily readable with epi-fluorescence. Another subpopulation is the YO-PRO-1 positive cells. In early stages of apoptosisin somatic cells, there is a modification of membrane permeability that selectively allows entry of some semi-permeant DNA-binding molecules [54, 60]. This subpopulation is the spermatozoa might also show early damage or a shift to another physiological state, because membranes become slightly permeable during the first steps of cryoinjury, enabling YO-PRO-1 but not ethidium homodimer to penetrate the plasma membrane in somatic cells. None of these probes enters intact cells Finally, two subpopulations of cryoinjury-induced necrotic spermatozoa are easily detected: early necrotic spermatozoa stained both with YO-PRO-1 and ethidium homodimer, and late necrotic spermatozoa, cells stained only with ethidium homodimer. Although both YO-PRO-1 and ethidium are DNA-binding probes, and YO-PRO-1 penetrates both early and late damaged membranes, producing green fluorescence in the sperm nucleus, ethidium homodimer permeates only through damaged membranes, producing red fluorescence. When the plasma membrane looses integrity, ethidium homodimer penetrates to the nucleus and quenches or displaces YO-PRO-1 with the consequent change in colour [56]. The subpopulation of live cells using the new triple staining concurs with the subpopulation of live cells using the well validated A/PI assay. In addition, the staining protocol is much easier than the A/PI because the staining is made from stock solutions and is not necessary to use a binding buffer. As the staining of the probes is not dependent on Ca^{2+}, as is the case binding FITC-A, the preparation and using of a Ca^{2+} enriched buffer is not necessary.

The agreement between both techniques (A and YO-PRO-1/Eth/SNARF-1)was good, although the percentage of live spermatozoa was slightly higher in the triple staining method [36]. Also, the percentage of early damaged spermatozoa was higher with the A/PI assay. This might reflect an increase in membrane permeability, preceeding the transposition of PS in the evolution of the cryodamage, or in a yet to be determined physiological change probably being a very early step of both processes related to changes in cell volume regulation and movement of ions, occurring during the initiation of apoptosis [61] or cryoinjury [62]. In addition, an earlier inactivation of enzymes involved in maintaining membrane asymmetry than those involved in transporting amphipatic small molecules like YO-PRO-1 might explain this fact.

Recently we used this triple staining to predict the ability of different ejaculates to sustain cryopreservation [11]. The interpretation of the results of this technique should take into account all the patterns of staining. First, not only the percentage of YO-PRO-1 spermatozoa is correlated to sperm survival, but the percentage of SNARF-1 positive spermatozoa also correlates to cryosurvival. In addition, the percentage of ethidium positive spermatozoa before freezing correlates negatively with cryosurvival ($r = -0.727$, $P < 0.01$). In view of these facts, boar ejaculates showing high percentages of SNARF-1, YO-PRO-1 positive spermatozoa and low percentages of ethidium positive cells should be elected for freezing. Also, the subpopulation YO-PRO-1 positive cells might represent cells showing higher fluidity of the membrane, and an increase on the fluidity of the mem-

brane has been related to a better ability to face the stress of cryopreservation [63]. Experiments in progress in our laboratory clearly demonstrate the importance of detecting subtle changes in sperm membranes. We compared two extenders for chilled canine semen. Motility and the percentage of propidium idodide negative cells were equivalent for all incubation times for both extenders. Only the percentage of YO-PRO-1 positive spermatozoa changed as a result of the extender used. It is clear that if we had used a classical combination of probes, this fact would have been missed.

4 Conclusion

The increasing use over the past decade of flow cytometry in the leading laboratories in human and veterinary andrology has dramatically increased our knowledge of sperm function under physiological and biotechnological conditions. Among many other recent advances, such as the identification of sperm subpopulations [64–67], detecting subtle changes in sperm membranes is an issue of major practical importance. Sperm membranes are an accurate biosensor of the status of the whole cell. Cryopreservation procedures will benefit from new flow cytometry protocols, which will enable the early detection of membrane damage.

Acknowledgment

The investigations of the author have been supported by grants from the Ministry of Education and Science of Spain AGL 2004 –01722 (GAN) and Fundación ONCE del Perro Guía Spain.

References

1 Alvarez JG, Storey BT. Evidence that membrane stress contributes more that lipid peroxidation to sublethal cryodamage in cryopreserved human sperm: glycerol and other polyols as sole cryoprotectant. J Androl 1993; 14: 199–209.
2 Amann RP, Hammerstedt RH. *In vitro* evaluation of sperm quality: an opinion. J Androl 1993; 14: 397–406
3 Eriksson BM, Rodríguez–Martínez H. Effect of freezing and thawing rates on the post-thaw viability of boar spermatozoa frozen in FlatPacks and Maxi-straws. Anim Reprod Sci 2000; 63: 205–20.
4 Eriksson BM, Vázquez JM, Martínez EA, Roca J, Lucas X, Rodríguez-Martínez H. Effects of holding time during cooling and type of package on plasma membrane integrity, motility and in vitro oocyte penetration ability of frozen thawed boar spermatozoa. Theriogenology 2001; 55: 1593–605.
5 Gillan L, Maxwell WM. Flow cytometric evaluation of sperm parameters in relation to fertility potential. Theriogenology 2005 63: 445–57.
6 Graham LK. Assessment of sperm quality: a flow cytometric approach. Anim Reprod Sci 2001; 68: 239–47.
7 Harrison RAP. Sperm plasma membrane characteristics and boar semen fertility. J Reprod Fertil 1997; 52 (Suppl): 195–211.
8 Medrano A, Watson PF, Holt WV. Importance of cooling rate and animal variability for boar sperm cryopreservation: insights from the cryomicroscope. Reproduction 2002; 123: 315–22.
9 Parks JE, Graham JK. Effects of cryopreservation procedures on sperm membranes. Theriogenology 1992; 38. 209–22.
10 Peña FJ, Saravia F, Johannisson A, Walgren M, Rodriguez-Martinez H. A new and simple method to evaluate early membrane changes in frozen-thawed boar spermatozoa. Int J Androl 2005; 28: 107–14.
11 Peña FJ, Saravia F, Johannisson A, Walgren M, Rodríguez-Martínez H. Detection of early changes in sperm membranes integrity pre-freezing can estimate post-thaw quality of boar spermatozoa. Anim Reprod Sci 2007; 97: 74–83.
12 Petrunkina AM, Gropper B, Günzel-Apel AR, Töpfer-Petersen E. Functional significance of the cell volume for detecting sperm membrane changes and predicting freezability in dog semen. Reproduction 2004;128: 829–42.
13 Fraser L, Gorszczaruk K, Strzezek J. Relationship between motility an membrane integrity of boar spermatozoa in media varying in osmolarity. Reprod Domest Anim 2001; 36; 325–9.
14 Grunewald S, Paasch U, Wuendrich K, Glander HJ. Sperm caspases become more activated in infertility patients than in healthy donors during cryopreservation. Arch Androl 2005; 51: 449–60.
15 Janett F, Thun R, Niederer K, Burger D, Hässig M. Seasonal changes in semen quality and freezability in the Warmblood stallion. Theriogenology 2003; 60: 453–61.
16 Núñez Martínez I, Moran JM, Peña FJ. A three-step statistical procedure to identify sperm kinematic subpopulations in canine ejaculates: changes after cryopreservation. Reprod Domest Anim 2006; 41: 408–15.
17 Núñez-Martínez I, Morán JM. Peña FJ. Two-step cluster procedure after principal component analysis identifies sperm subpopulations in canine ejaculates and its relation to cryoresistance. J Androl 2006; 27: 596–603.
18 Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Antioxidant supplementation of boar spermatozoa from different fractions of the ejaculate improves cryopreservation: changes in sperm membrane lipid architecture. Zygote 2004; 12: 117–24.
19 Peña FJ, Saravia F, Núñez-Martínez I, García Herreros M, Johannisson A, Wallgren M, *et al.* Identification of sperm morpholometric subpopulations in two different portions of the boar ejaculate and its relation to postthaw quality. J Androl 2005; 26: 716–23.
20 Peña FJ, Saravia F, Núñez-Martínez I, Johannisson A, Wallgren

M, Rodríguez-Martínez H. Do different portions of the boar ejaculate vary in their ability to sustain cryopreservation? Anim Reprod Sci 2006; 93:101–13.
21 Nagy S, Jansen J, Topper EK, Gadella BM. A triple stain flow cytometric method to assess plasma and acrosome membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol Reprod 2003; 68: 1828–35.
22 Núñez Martínez I, Moran JM, Peña FJ. Do computer assisted derived morphometric sperm characteristics reflect DNA status in canine spermatozoa? Reprod Domest Anim 2005; 40: 537–43.
23 Peña AI, Johannisson A, Linde-Forsberg C. Validation of flow cytometry for assessment of viability and acrosomal integrity of dog spermatozoa and for evaluation of different methods of cryopreservation. J Reprod Fertil Suppl 2001: 57: 371–6.
24 Peña FJ, Johannisson A, Wallgren M, Rodríguez-Martínez H. Assessment of fresh and frozen thawed boar semen using an annexin assay: a new method of evaluating sperm membrane integrity. Theriogenology 2003; 60: 677–89.
25 Rodríguez-Martínez H. Laboratory semen assessment and prediction of fertility: still utopia? Reprod Domest Anim 2003; 38: 312–8.
26 Garner DL, Johnson LA. Viability assessment of mammalian sperm using SYBR-14 and propidium iodine. Biol Reprod 1995; 53: 276–84.
27 Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. Trends Cell Biol 2000; 10: 369–77.
28 Duru NK, Morshedi MS, Schuffer A, Oehninger S. Cryopreservation-thawing of fractionated human spermatozoa and plasma membrane translocation of phosphatidylserine. Fertil Steril 2001; 75: 263–8.
29 Anzar M, He L, Buhr MM, Kroetsch TG, Pauls KP. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. Biol Reprod 2002; 66: 354–60.
30 Januskauskas A, Johannisson A, Rodríguez Martínez, H. Subtle membrane changes in cryopreserved bull semen in relation with sperm viability, chromatin structure and field fertility. Theriogenology 2003; 60: 743–58.
31 Glander HJ, Schaller J. Binding of annexin V to plasma membranes of human spermatozoa: a rapid assay for detection of membrane changes after cryostorage. Mol Hum Reprod 1999; 5: 109–15.
32 Arama E, Agapite J, Steller H. Caspase activity and a specific cytochrome c are required for sperm differentiation in drosophila. Dev Cell 2003; 4: 687–97.
33 Weil M, Jacobson MD, Raff MC. Are caspases involved in the death of cells with a transcriptionally inactive nucleus? Sperm and chicken erythrocytes. J Cell Sci 1998; 111. 2707–15.
34 Sakkas D, Mariethoz E, Manicardi G, Bizzaro D, Bianchi PG, Bianchi U. Origin of DNA damage in ejaculated human spermatozoa. Rev Reprod 1999; 4: 31–7.
35 de Vries KJ, Wiedmer T, Sims PJ, Gadella BM. Caspase independent exposure of aminophospholipids and tyrosine phosphorylation in bicarbonate responsive human cells. Biol Reprod 2003; 68: 2122–34.
36 Peña FJ, Núñez-Martínez I, Morán J. Semen technologies in dog breeding: an update. Reprod Domest Anim 2006; 41(S2): 21–9.
37 Gadella BM, Harrison RA. Capacitation induces cyclic adenosine 3',5'-monophosphate-dependent, but apoptosis unrelated exposure of aminophospholipids at the apical head plasma membrane of boar sperm cells. Biol Reprod 2002; 67. 340–50.
38 Kurz A, Viertel D, Herrmann A, Müller K. Localization of phosphatidylserine in boar sperm cell membranes during capacitation and acrosome reaction. Reproduction 2005; 130: 615–26.
39 Baumber J, Meyers SA. Changes in membrane lipid order with capacitation in rhesus macaque (*Macaca mulatta*) spermatozoa. J Androl 2006; 27: 578–87.
40 Varum S, Bento C, Sousa AP, Gomes-Santos CS, Henriques P, Alameida-Santos T, *et al*. Characterization of human sperm populations using conventional parameters surface ubiquitination and apoptotic markers. Fertil Steril 2007; 87: 572–83.
41 Muratori M, Porazzi I, Luconi M, Marchiani S, Forti G, Baldi E. Annexin V binding and merocyanine staining fail to detect human sperm capacitation. J Androl 2007; 25: 797–810.
42 Spinaci M, De Ambrogi M, Volpe S, Galeati G, Tamanini C, Seren E. Effect of staining and sorting on boar sperm membrane integrity, mitochondrial activity and in vitro blastocyst development. Theriogenology 2005; 64: 191–201.
43 Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, Thomas AJ, *et al*. Cryopreservation and thawing is associated with varying extent of apoptotic machinery in subsets of ejaculated human spermatozoa. Biol Reprod 2004; 71: 1828–37.
44 Weng SL, Taylor SL, Morshedi M, Schuffner A, Duran EH, Beebe S, *et al*. Caspase activity and apoptotic markers in ejaculated human sperm. Mol Hum Reprod 2002; 8: 984–91.
45 Grunewald S, Paasch U, Said TM, Sharma RK, Glander HJ, Agarwal A. Caspase activation in human spermatozoa in response to physiological and pathological stimuli. Fertil Steril 2005; 83 (Suppl 1): 1106–12.
46 Martin G, Sabido O, Durand P, Levy R. Phosphatidylserine externalization in human sperm induced by calcium ionophore A23187: relationship with apoptosis, membrane scrambling and the acrosome reaction. Hum Reprod 2005; 20: 3459–68.
47 Sion B, Janny L, Boucher D, Grizard G. Annexin V binding to plasma membrane predicts the quality pf human cryopreserved spermatozoa. Int J Androl 2005; 27: 108–14.
48 Martin G, Sabido O, Durand P, Levy R. Cryopreservation induces an apoptosis-like mechanism in bull sperm. Biol Reprod 2004; 71: 28–37.
49 Eley A. Hosseinzadeh S, Hakimi H, Geary I, Pacey AA. Apoptosis of ejaculated human sperm is induced by co-incubation with *Chlamydia trachomanis* lipopoysaccharide. Hum Reprod 2005; 20: 2601–7.
50 Cayli S, Sakkas D, Vigue L, Demir R, Huszar G. Cellular maturity and apoptosis in human sperm: creatinine kinase, caspase-3 and Bcl-XL levels in mature and diminished maturity sperm. Mol Hum Reprod 2004; 10: 365–72.

51 Almeida C, Cardoso MF, Sousa M, Viana P, Gonçalves A, Silva J, *et al.* Quantitative study of caspase-3 activity in semen and after swim-up preparation in relation to sperm quality. Hum Reprod 2005; 20: 1307–13.
52 Watson PF. Recent developments and concepts in the cryopreservation of spermatozoa and the assessment of their post-thawing function. Reprod Fertil Dev 1995; 7: 871–91.
53 Watson PF. The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci 2000; 60–61: 481–92.
54 Cohen JJ. Apoptosis. Immunol Today 1993; 14: 126–30.
55 Ormerod MG, Sun XM, Snowden RT, Davies R, Fearnhead H, Cohen GM. Increased membrane permeability of apoptotic thymocytes: a flow cytometric study. Cytometry 1993; 14: 595–602.
56 Castañeda F, Kinne RK. Short exposure to millimolar concentrations of ethanol induces apoptotic cell death in multicellular HepG2 spheroids. J Cancer Res Clin Oncol 2000; 126: 305–10.
57 Reber F, Kasper M, Siegner A, Kniep E, Siegel G, Funk RH. Alteration of the intracellular pH and apoptosis induction in a retinal cell line by the AGE-inducing agent glyoxal. Graefes Arch Clin Exp Ophthalmol 2002; 240:1022–32.
58 Acevedo JJ, Mendoza-Lujambio I, de la Vega-Beltrán JL, Treviño CL, Felix R, Darszon A. KATP channels in mouse spermatogenic cells and sperm and their role in capacitation. Dev Biol 2006; 298: 395–405.
59 Idziorek T, Estaquier J, De Bels F, Ameisen JC. YOPRO-1 permits cytofluorometric analysis of programmed cell death (apoptosis) without interfering with cell viability. J Immunol Methods 1995; 185: 249–58.
60 Wronski R, Golob N, Grygar E, Windisch M. Two-color, fluorescence-based microplate assay for apoptosis detection. Biotechniques 2002; 32: 666–8.
61 Bortner CD, Cidlowski JA. A necessary role for cell shrinkage in apoptosis. Biochem Pharmacol 1998; 56: 1549–59.
62 Paasch U, Grunewald S, Wuendrich K, Jope T, Glander HJ. Inmunomagnetic removal of cryo-damaged human spermatozoa. Asian J Androl 2005; 7: 61–9.
63 Aboagla EM, Terada T. Trehalose-enhanced fluidity of the goat sperm membrane and its protection during freezing. Biol Reprod 2003; 69: 1245–50.
64 Abaigar T, Holt WV, Harrison RA, del Barrio G. Sperm subpopulations in boar (*Sus scrofa*) and gazelle (*Gazella dama mhorr*) semen as revealed by pattern analysis of computer-assisted motility assessments. Biol Reprod 1999; 60: 32–41.
65 Chantler E, Abraham Peskir J, Roberts C. Consistent presence of two normally distributed sperm subpopulations within normozoospermic human semen: a kinematic study. Int J Androl 2004; 27: 350–9.
66 Holt WV. Can we predict fertility rates? Making sense of sperm motility. Reprod Domest Anim 1996; 31: 17–24.
67 Holt WV, Van Look KJ. Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory tests of semen quality. Reproduction 2004; 127: 527–35.