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Short title: DNA fragmentation, chromatin compaction and hyaluronic acid binding affinity

Sedimentation properties in density gradients correspond with levels of sperm DNA fragmentation, chromatin compaction and binding affinity to hyaluronic acid

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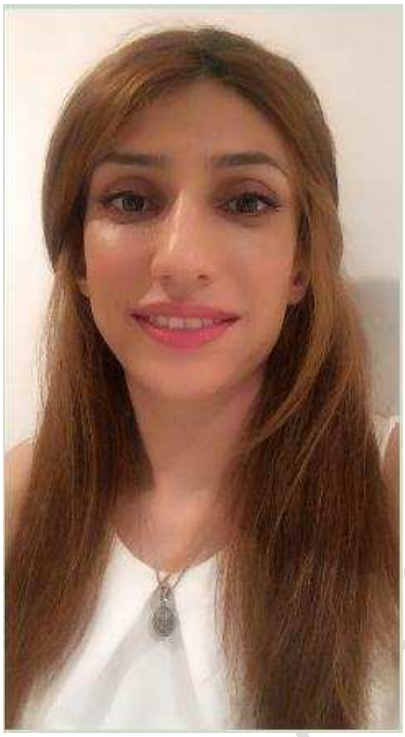

Author biography

Forough Torabi is a Marie-Curie Sklodowska sponsored PhD student (Reprotrain; http://cordis.europa.eu/result/rcn/149644_en.html) currently completing her studies on the dynamics of sperm maturation and capacitation in relation to hyaluronic acid binding. Before coming to the University of Leeds, Forough undertook a master's programme in Biochemistry at the Islamic Azad University, Science and Research Branch, Tehran, Iran in collaboration with the Avicenna Research

Institute, Tehran, Iran, graduating with distinction in 2011. She has presented aspects of her MSc project work at the Molecular Immunology and Immunogenetics Congress in April 2012, Antalya, Turkey and the seventh European Congress of Andrology (November 2012, Berlin, Germany).

Abstract

Mature spermatozoa bind hyaluronic acid in the extracellular matrix via hyaladherins. Immature spermatozoa may be unable to interact because they do not express the appropriate hyaladherins on their surface. Fresh human semen samples were fractionated using differential density gradient centrifugation (DDGC) and the ability of these fractions to bind hyaluronic acid was evaluated. The presence of sperm hyaladherins was also assessed. CD44 was located mainly on the acrosome and equatorial segment and became more restricted to the equatorial segment in capacitated spermatozoa. Hyaluronic acid-TRITC (hyaluronan acid conjugated with tetramethylrhodamine isothiocyanante), a generic hyaluronic-acid-binding reagent, labelled the membrane and the neck region, particularly after capacitation. Sperm populations obtained after DDGC or after interaction with hyaluronic acid were assessed for DNA fragmentation and chromatin maturity. Strong relationships between both measures and sperm sedimentation and hyaluronic-acid-binding profiles were revealed. Capacitation enhanced hyaluronic-acid-binding of both DDGC-pelleted sperm and sperm washed free of seminal fluid. In conclusion, hyaladherins were detected on human sperm and a higher capacity for sperm hyaluronic-acid-binding was shown to correspond with their DDGC sedimentation profiles and with lower levels of DNA fragmentation and better chromatin maturity. Capacitation induced changes in the distribution and presence of hyaladherins may enhance hyaluronic-acid-binding.

KEYWORDS: DNA integrity, chromatin maturity, hyaluronic acid, hyaladherins, capacitation, acrosome reaction

<A>Introduction

With the advent of intracytoplasmic sperm injection (ICSI) in the early 1990s, male reproductive dysfunction could be treated effectively for the first time (Palermo et al., 1992). Overall success rates for assisted conception procedures, however (including ICSI), have remained relatively static, with about 24% of cycles for all age groups delivering a term pregnancy (≥37 weeks) (HFEA, 2015). The choice of sperm for ICSI, particularly given the

procedure's increasing popularity and uptake (HFEA, 2008), however, has focused on various techniques aimed at enrichment of a viable, fertile sperm population.

Standard sperm washing and sorting technologies, such as swim up and differential density gradient centrifugation (DDGC), are routinely used to prepare sperm for assisted reproduction procedures (Akerlof et al., 1987). On the basis of classical parameters such as concentration, viability, motility and morphology under WHO guidelines, these techniques do enrich for more fertile spermatozoa (WHO, 2010). Functional properties of DDGC-enriched populations, such as DNA integrity and chromatin maturity that could explain the improved fertility of these populations of spermatozoa, are, however, usually not assessed. Available evidence suggests that, although spermatozoa prepared using standard routine sperm preparation methods such as DDGC or swim-up may have generally lower levels of DNA fragmentation or compromised chromatin compared with spermatozoa from unprocessed semen samples, and may therefore be suitable for use in assisted reproduction techniques, such preparations may not exclude sperm that are compromised in these regards (Zini et al., 1999; Brahem et al., 2011b; Henkel, 2012; Mortimer and Mortimer, 2013). This is a particular issue for ICSI in which sperm selection relies almost exclusively on the embryologist's judgement. Hence, alternative methods are being sought to prepare spermatozoa with optimum quality for ICSI procedures (Yetunde and Vasiliki, 2013; Zhao et al., 2014). In response to this demand, several novel procedures have been, or are being, developed, aiming at the enrichment or isolation of high-quality spermatozoa for motility, morphology, DNA integrity and maturity (Said and Land, 2011). One of these, hyaluronic acid binding, is being mooted as a viable, non-destructive sperm selection method based on its ability to discriminate mature sperm with low levels of chromosomal aneuploidies and DNA fragmentation (Jakab et al., 2005; Huszar et al., 2006).

Hyaluronic acid is a negatively charged, non-sulphated glycosaminoglycan that is a constituent of the extracellular glycocalyx environment in soft and connective tissues (Chen and Abatangelo, 1999) and is also found at high levels in the female genital tract, including the cervical mucus and the cumulus oophorus complex (Eppig, 1979; Toole, 2004). Hyaluronan synthases produce hyaluronic acid in vertebrates, which interact with hyaladherins including the sperm-egg interacting factor PH20 (SPAM1), CD44 and RHAMM (receptor for hyaluronic-acid-mediated motility). These proteins can also influence cell

motility, survival and proliferation (Forteza et al. 2001; Day and Prestwich, 2002; Toole, 2004; Plazinski and Knys-Dzieciuch, 2012).

Hyaluronic acid may play an important role in fertilization by 'capturing' spermatozoa-expressing hyaladherins that can either bind it directly, e.g. CD44 (Bains et al., 2002) or facilitate sperm penetration of the cumulus layers via a hyaluronidase activity followed by accessing and binding to the zona pellucida, e.g. SPAM1 (McLeskey et al., 1998). It has been shown that spermatozoa binding to hyaluronic acid in vitro have better indicators of nuclear maturation, cytoplasmic extrusion and plasma membrane remodelling than spermatozoa that do not bind hyaluronic acid (Huszar et al., 2003; Parmegiani et al., 2010a; Rengan et al., 2012).

On the basis of the likely functional aspects of hyaluronic acid and hyaladherins in the fertilization process, protocols have been developed in which spermatozoa are allowed to interact with, and bind to, a prepared surface coated with hyaluronic acid before collection and use for ICSI (Jakab et al., 2005; Nasr-Esfahani and Marziyeh, 2013). Several reports indicating some efficacy for the procedure have been published in relation to clinical pregnancy, pregnancy loss and, more recently, live birth rates of which the largest clinical trial to date made use of the commercially available physiological ICSI platform (Worrilow et al., 2013). See also (Nasr-Esfahani et al., 2008; Parmegiani et al., 2010a; Mokanszki et al., 2014; Beck-Fruchter et al., 2015).

Processing crude semen by DDGC works by favouring the sedimentation of mature sperm with higher density and motility into and through the denser layer(s) of a gradient (Sakkas et al., 2000; Sakkas, 2013). If sperm binding to hyaluronic acid can be shown to reflect the characteristics related to good cell maturity and quality, including lower levels of DNA fragmentation, further development of the method as a means of selecting higher quality sperm for ICSI and possibly IVF is justified.

The aims of the study were to examine sperm for evidence of hyaluronic acid (hyaluronan) binding proteins (hyaladherins), to monitor their expression in response to capacitation and the acrosome reaction and to compare levels of DNA fragmentation and chromatin compaction in sperm processed by standard DDGC or after their interaction with a hyaluronan-coated surface.

The study was considered and nationally approved by the relevant UK Integrated Research Application System (IRAS) Ethics Committee (NRES 12_NE_0192) on 13 January 2013 and locally approved by the University of Leeds' School of Medicine Research Ethics Committee (SoMREC/13/017) on 28 November 2013.

<A>Materials and methods

<B>Patient samples

All semen samples were ethically obtained from young male volunteers of unproven fertility by masturbation into sterile, tissue culture grade universal containers after 3 days of abstinence. Collected semen samples were liquefied for 30 min at 37°C. A basic semen assessment was undertaken to exclude grossly abnormal samples and ensure that all samples were within the ranges expected for normal semen. The included volunteers were aged between 19 and 36 years with a mean age (±SD) of 22.43 ± 4.25 years and only those with normal semen parameters as defined by WHO (2010) criteria were included in the study. A more complete description of the volunteer semen profiles is presented in **Table 1**.

<B>Differential density gradient centrifugation

Semen samples were processed using a two-layer density gradient (90–45%) of SupraSperm™, a silane-coated colloidal silica-based HEPES-buffered density gradient medium (Origio, Denmark) and spermatozoa harvested from the 90% pellets and 45–90% interface regions after swing-out centrifugation at 300 ×g for 20 min. These fractions are henceforth referred to as 90% and 45% fractions. Spermatozoa from both fractions were washed by resuspension and mixing with Quinn's sperm washing medium (Origio, Denmark) and centrifugation at 300 × g for 10 min (two repeat washes). Sample motility was then assessed using a Leitz Laborlux 12 light microscope (Mazurek Optical Services, UK) and spermatozoa counted (after killing by dilution with water) in a Neubauer haemocytometer (**Table 1**). 1×10^6 pelleted and interface spermatozoa were cytospun (Thermo-Shandon, UK) on to poly-L-Lysine coated slides (VWR, UK) for later fixation and staining.

<B>Detection of sperm hyaladherins

Spermatozoa were centrifuged by DDCG as described above. Pelleted spermatozoa were resuspended in 1x phosphate buffered saline (PBS) (Gibco, UK) before placing on poly-L-Lysine coated slides (VWR, UK). After air drying, 100 µl of 3% bovine serum albumin

(Sigma-Aldrich, UK) in PBS was placed on the slides and incubated for 60 min at room temperature to block non-specific binding. A generic probe for hyaladherins was first applied by incubating slides with 100 µl (10 µg/ml) of hyaluronic acid-TRITC (hyaluronictetramethyl rhodamine isothiocyanate 1500kDa; Creative PEGWorks, USA) for 75 min at room temperature. Slides were then washed with PBS before incubation with 100 µl of a biotinylated monoclonal anti-CD44 antibody (Abcam, UK; 1:100 dilution of 0.5 mg/ml, in PBS for 90 min at room temperature. Slides were washed twice with PBS for 15 min each and then incubated with streptavidin conjugated with fluorescein isothiocyanate (FITC) (BD Pharmingen, UK; 1:1000 dilution of 0.5mg/ml in PBS) and DAPI (4, 6-diamidino-2-phenylindole) (Sigma-Aldrich, UK) for 60 min at room temperature in the dark and then rinsed with PBS. Coverslips were mounted with a drop of polyvinyl alcohol mounting medium (Sigma-Aldrich, UK) and samples were viewed with a Leica LEITZ DMRB fluorescence microscope (Mazurek Optical Services, UK). To act as controls for non-specific fluorescence, spermatozoa were incubated with unlabelled hyaluronic acid (Creative PEGWorks, USA) or with the biotinylated secondary antibody and streptavidin-FITC (BD Pharmingen, UK) layers alone (non-immune). Image background noise reduction and contrast enhancement were undertaken using open source Image J software (https://imagej.nih.gov/ij/).

<**B**>Evaluation of hyaladherins before and after capacitation and the acrosome reaction

Capacitation was accomplished according to the World Health Organization 2010 (WHO) guidelines with minor modifications (WHO, 2010). Briefly, spermatozoa were separated by DDCG as described earlier. To induce capacitation, pelleted spermatozoa (10^6) were incubated for up to 3 h in 1 ml of a hepes-buffered Ham´s F10 solution (Gibco, ThermoFisher Scientific, UK) supplemented with 3.5% (w/v) BSA, 0.2% (w/v) $NaHCO_3$ 5mM $CaCl_2$, 0.36% (w/v) sodium lactate and 0.003% (w/v) sodium pyruvate (all reagents sourced from Sigma-Aldrich, UK) at 37°C with constant rotation. After the incubation period, capacitated spermatozoa were recovered by centrifugation at 300 ×g for 10 min and washed twice with PBS. Pelleted spermatozoa resuspended in the buffer supporting capacitation, incubated for 0 h (non-capacitated), were used as a control.

To induce the acrosome reaction, 10 µl of Ca^{2+} ionophore A23187 (Sigma-Aldrich, UK, stock solution 1.0 mM) (was added to about 10^6 capacitated spermatozoa in 1 ml (final concentration 0.01 mM) and incubated at 37°C for 15 min. The reaction was stopped by adding 70% ethanol and spermatozoa were recovered as indicated above. An incubation with DMSO (Sigma-Aldrich, UK) only did not induce acrosome reaction. Spermatozoa were then placed on poly-L-Lysine coated slides and labelled with HA-TRITC and biotinylated anti-CD44 (Abcam, UK) as described above (section 2.04). As acrosome reaction is more efficiently accomplished by capacitated spermatozoa, in addition to monitoring hyperactive motility, capacitation was also assessed by counting spermatoza labelled as PSA-FITC (lectin from Pisum sativum [pea] FITC conjugate; Abcam, UK) after incubation in buffer-supporting capacitation for 0 h and 3 h and subsequent acrosome reaction (Bailey, 2010; De Jonge and Barratt, 2013).

<**B**>Spermatozoa binding to hyaluronic-acid-coated slides

Slides coated with hyaluronic acid (HBA® slides, Origio, Denmark) were used to check the ability of sperm to bind to hyaluronic acid according to the manufacturer's protocol. Briefly, after separation of human spermatozoa by density-gradient centrifugation (described earlier), about 1×10^6 (10 µL) spermatozoa (pelleted and interface) were placed onto the assay chamber and incubated for 15 min at room temperature. Spermatozoa with hyaluronic acid receptors are able to bind to the hyaluronic acid-coated slide with an actively beating tail, whereas spermatozoa lacking hyaluronic acid receptors are not able to bind to the slide and can move around freely. The percentage of hyaluronic-acid-bound spermatozoa (pelleted and interface) was calculated as follows: (% bound = bound motile/total motile × 100). In the absence of computer-assisted semen analysis, hyaluronab-binding assay slides were also used to manually assess hyperactive motility induced by capacitation.

<**B**>Isolation of spermatoza bound and unbound by hyaluronic-acid

Human semen samples were washed twice with Quinn's sperm washing medium at 300 × g for 10 min. The pellet was then re-suspended in Quinn's sperm washing medium. Spermatozoa were loaded on to a specially prepared dish coated with hyaluronic acid (Biocoat, USA) at a concentration of 50×10^6 /ml. After 15 min incubation at 37°C and 95% air: 5% CO_2, spermatozoa unbound by hyaluronic acid were removed by gently rinsing the dish with Quinn's sperm washing medium for 2 min with constant pipetting. Spermatoza

bound to the plates were recovered by more vigorous washing and both bound and unbound samples were centrifuged at 300 ×g for 15 min and washed twice with PBS.

<**B**>Acridine orange staining and quantitation

Spermatozoa resolved by DDGC, ability to bind to hyaluronic acid, or both, were loaded onto slides coated with poly-L-Lysine and fixed with a modified Carnoy's solution (9:1 ratio of methanol and glacial acetic acid) for 2 h at room temperature (Yagci et al., 2010). Slides were then stained for 5 min at room temperature with 12 µg/ml of acridine orange ready to use 2% solution, (Polysciences Inc, USA) and then rinsed with double-distilled water. After excitation with blue light, acridine orange emits green fluorescence when associated with a double stranded DNA (dsDNA; wavelength: 530 nm), whereas single-stranded DNA (ssDNA) emits red fluorescence (ssDNA; wavelength: 600nm). Three acridine orange categories of green (++), yellow (±) and red (--) fluorescence were used to evaluate the levels of DNA fragmentation in 90% and 45% fractions of spermatozoa corresponding to low, medium and high levels of DNA fragmentation, respectively. At least 150 spermatozoa per sample were categorized using a Zeiss LSM510-META upright confocal microscope (Zeiss, Germany) by two independent observers.

<**B**>Aniline blue staining and quantitation

Spermatozoa resolved by DDGC and abilty to bind to hyaluronic acidy were assessed for excessive histone retention indicating defective chromatin compaction and hence maturity using aniline blue staining. Briefly, spermatozoa were loaded on to poly-L-Lysine coated slides and fixed with a 3:1 solution of methanol: acetic acid for 1 h at room temperature. Slides were then stained with aniline blue solution (2.5% in 2% acetic acid, Sigma-Aldrich, UK) for 5 min at room temperature (Huszar et al., 2003). At least 150 spermatozoa per sample were evaluated by brightfield light microscopy under oil immersion (100x objective). Spermatozoa with unstained (--), moderately stained (±) and extensively stained (++) nuclei were scored as sperm with fully, partially and weakly compacted chromatin, respectively, by two independent observers.

<**B**>Changes in abilty to bind to hyaluronic acid after capacitation

Capacitation of sperm obtained directly after centrifugation and washing of semen or from DDGC pellets was carried out and binding to hyaluronic acid was assessed as described earlier. The percentage of capacitated and non-capacitated spermatozoa binding to hyaluronic

acid was estimated after treatment of bound and unbound cells with Ca^{2+} ionophore and labelling with PSA-FITC (De Jonge and Barratt, 2013).

<B>Statistical analysis

Experimental data were analysed using GraphPad Prism (version 6, Graphpad Software, USA). Data were not normally distributed even after transformation and except for values obtained from binding capacity of hyaluronic acid in the 90% and 45% DDGC fractions, which were analysed using the Mann–Whitney U test; all other group-based comparisons were analysed using Kruskall–Wallis analysis of variance with Dunn's post-hoc multiple comparisons test applied to compare groups. The P-values for median rank differences are indicated in all figures as a (0.0001) > b (0.001) > c (0.01) > d (0.05).

<A>Results

<B>General semen assessment of volunteers participating in this study

A cohort of healthy young male volunteers of unproven fertility (n = 16) from the student population of the University of Leeds was used in this study (**Table 1**). The average age of the cohort was 22.4 (range 19–36 years). The average sperm concentration, total sperm count, semen volume and per cent sperm motility (range) were, respectively, 136.6 (36–251); 361 (93.6–1205); 2.9 (0.6–4.8) and 77.5 (56–92).

<B>Microscopic evaluation of hyaluronic-acid-binding in human sperm

To investigate the general presence, capacity and conditions for sperm to recognize and bind hyaluronic acid, spermatozoa were recovered from 90% pellets and incubated with hyaluronic acid labelled with (TRITC) and an antibody to the common hyaladherin, CD44 and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (**Figure 1** and **Supplementary Figure 1**). The images shown were processed for background noise reduction and contrast enhancement to help improve clarity. The original images are presented in **Supplementary Figure 1** for comparison. Hyaluronic-acid TRITC labelled the acrosome and the tail and more strongly, the neck region (A, E) whereas an antibody to CD44 (green) labelled the equatorial segment and the acrosome (C, E). We next looked for changes in hyaluronic-acid-binding in response to sperm capacitation and the acrosome reaction. On capacitation, CD44 acrosomal labelling was slightly reduced and more restricted to the equatorial segment (D, F) and was reduced further after the acrosome reaction (I, K).

Labelling with TRITC-tagged hyaluronic-acid, however, intensified after capacitation (compare A, E with B, F), particularly on the neck region. On repeating the experiment with samples pre-incubated with excess unlabelled soluble hyaluronic acid, signals for both CD44 (J, L) and hyaluronic acid-TRITC (H, L) were strongly reduced but not abolished altogether. Incubation with secondary antibody alone did not elicit a fluorescent signal (not shown). Acrosomal staining with PSA-FITC after Ca^{2+} ionophore triggering of the acrosome reaction was also much reduced confirming the efficiency of capacitation (**Figure 2**).

<**B**>DNA fragmentation and chromatin compaction in spermatozoa recovered from DDGC fractions and from populations bound and unbound by hyaluronic acid

Significantly more spermatozoa recovered from hyaluronic acid bound by 90% fractions compared with sperm recovered from 45% fractions ($P < 0.0001$) (**Figure 3**). To assess the relevance of this relationship to spermatozoal viability, levels of DNA fragmentation and chromatin compaction in 90% and 45% DDGC spermatozoal fractions and in hyaluronic-acid-binding and non-binding populations of washed spermatozoa were assessed by acridine orange and aniline blue staining. Examples of each are shown in **Figure 4** in relation to acridine orange for DDGC (A, B) and samples bound by hyaluronic acid (C, D), respectively and in **Figure 5** in relation to AB for DDGC (A, F) and samples bound by hyaluronic acid (C, D), respectively. Keys for the subjective measurement of staining are shown alongside. Acridine orange staining generated predominantly green (++) fluorescent nuclei in both 90% fractions and samples bound by hyaluronic acid indicating sperm with relatively low levels of DNA fragmentation compared with more mixed colours (++, ±, --) in 45% fractions and samples not bound by hyaluronic acid. Similarly, AB only weakly (±) or did not (--) stain sperm from 90% fractions and samples bound by hyaluronic acid compared with sperm from 45% fractions and samples that did not bind to hyaluronic acid that were more strongly (++) stained. Hence, hyaluronic acid and aniline blue staining essentially mirrored each other in relation to DNA fragmentation and chromatin compaction with each indicating better quality sperm in 90% DDGC fractions and sperm bound to hyaluronic acid from washed samples.

Relationships between sperm sedimentation and DNA fragmentation are shown in **Figure 6A**. These results were not unexpected considering the known ability of DDGC to differentiate between good and poor-quality sperm based on their relative density and motility. Hence, higher levels of sperm with low DNA fragmentation (++) were recovered

from the 90% fractions compared with the 45% fractions (as indicated by the mixed acridine orange colours in **Figure 4B**). Pelleted (90%) sperm also had lower proportions of sperm with medium (±) and high levels of DNA fragmentation (--) DNA. There were also higher numbers of sperm with medium levels of DNA fragmentation in 45% (interface) fractions compared with pellets and almost no sperm with high levels of fragmented DNA were recovered from sperm pellets. DNA fragmentation was also assessed in relation to capacity to bind to hyaluronic acid where sperm with little or no fragmentation dominated populations bound by hyaluronic acid (**Figure 6B**) and an almost complete clearance of sperm from populations bound by hyaluronic acid with medium levels of DNA fragmentation were observed. These findings were emphasized by the virtual absence of sperm with higher levels of DNA fragmentation in populations bound by hyaluronic acid (**Figure 4C**). Significant differences between categories are shown above the box whiskers for this and the charts shown in **Figures 7** and **8** (a; $P < 0.0001$, b; $P < 0.001$, c; $P < 0.01$, d; $P < 0.05$).

Aniline blue staining was used to assess incomplete protamination and so levels of chromatin compaction or maturity in DDGC fractionated and sperm populations bound and unbound by hyaluronic acid. Very low proportions of sperm staining strongly with aniline blue (++) were recovered from 90% fractions that were instead dominated by sperm that did not stain with this dye (--; **Figure 7A**). Although higher levels of strongly stained sperm were recovered from the 45% fractions, differences were not statistically significant compared with other groups and weakly stained (±) or unstained sperm indicating good chromatin compaction were well represented in 45% fractions. Proportions of weakly stained sperm essentially mirrored those of strongly stained sperm in both DDGC fractions. Similarly, sperm with ++ and ± AB staining characteristics were more highly prevalent in samples unbound by hyaluronic acid with unstained sperm dominating the bound fractions (**Figure 7B**). In these experiments, the virtual clearance of sperm with any stained nuclei was observed in samples bound by hyaluronic acid suggesting that hyaluronic-acid-binding was a better discriminator than DDGC for removing sperm with relatively immature (±) chromatin.

<B>Changes in sperm hyaluronic-acid-binding and hyperactive motility after capacitation

The effect of capacitation on the ability of spermatozoa to bind to hyaluronic acid was further assessed using slides coated with hyaluronic acid (**Figure 8**). Higher numbers of sperm recovered and capacitated from washed, sperm samples could bind to hyaluronic acid

compared with non-capacitated controls, although the difference was not statistically significant. In the 90% fractions, DDGCpromoted hyaluronic-acid-binding, regardless of whether the conditions for capacitation were favourable. This, in itself, suggests that the 90% fraction contains more sperm competent to bind hyaluronic acid and that capacitation of sperm from this fraction makes little difference to the ability to bind to hyaluronic acid. On the other hand, the data suggests that conditions favourable to capacitation may improve hyaluronic-acid-binding in samples of simply buffer washed sperm. A large increase in hyperactive motility (more vigorous, asymmetrical tail beating and occasional rotation of sperm immobilised on the HBA slides) was observed after 3 h but not after 0 h incubation in capacitation supportive buffer for both Quinn's washed and DDGC (90%) processed sperm (**Table 2**).

<A>Discussion

At present, assisted reproductive techniques (such as ICSI and IVF) account for about 5% of births in the Western world (Lewis and Kumar, 2015) and, with ICSI, some checkpoints of natural fertilization are bypassed. As a result, low-quality spermatozoa with features such as abnormal morphology, low motility, damaged DNA, aneuploidy and poor zona binding potential that are not normally able to participate in natural fertilization, may be selected for ICSI procedure inadvertently (Cummins and Jequier, 1995; Tournaye, 2003; Alukal and Lamb, 2008). As spermatozoa have no mechanism to repair DNA damage, DNA strand breaks can be transferred to the oocyte during ICSI, and will rely on the oocyte to repair them (Lewis and Kumar, 2015). Therefore, sperm with higher levels of DNA fragmentation may contribute to embryonic mortality (Bonduelle et al., 2002; Jakab et al., 2005; Heytens et al., 2009; Marchesi et al., 2010). Hence, new methods to select sperm of optimum quality for use in assisted reproduction techniques are still being developed, including morphological (The International Mobile Subscriber Identity), electrical (flow cell, Zeta potential) and cell sorting methods (fluorescent-activated cell sorting and magnetic-activated cell sorting) (Chan et al., 2006; Nasr-Esfahani et al., 2012; Delaroche et al., 2013; Sakkas, 2013; Teixeira et al. 2013)

Previous studies have suggested that sperm able to bind the endocervical mucus have better morphology and that selecting for sperm with good morphology, inter alia should also select for sperm with other good parameters, including progressive motility and low DNA fragmentation (Sati et al., 2008; Prinosilova et al., 2009; Yagci et al., 2010). A potential link between hyaluronic-acid-binding (which can be considered a surrogate for sperm binding to

the endocervical mucus and the hyaluronan-rich cumulus oopherous complex) and spermatozoal viability, was established in the 1990s (Vandevoort et al. 1997; Cayli et al. 2003). This, and related work, suggest that spermatozoa capable of binding to hyaluronic acid have completed their maturation processes, most specifically plasma membrane remodelling and full chromatin condensation, and subsequently have an increased fertilizing capacity (Cayli et al., 2003; Huszar et al., 2003; Mokanszki et al., 2014). Different studies have suggested that hyaluronic-acid-binding improves embryo quality and development after ICSI, although all these studies were relatively small (Nasr-Esfahani et al., 2008; Parmegiani et al., 2010a; Parmegiani et al. 2010b; Mokanszki et al., 2014; Beck-Fruchter et al., 2015).

Considering that intermittent sperm binding to hyaluronic acid during their journey across the female genital tract may be an important aspect of natural fertilization (Henkel, 2012), the current study was set up to investigate the dynamics of sperm binding to hyaluronic acid in vitro and to investigate the relationship between hyaluronic-acid-binding and standard methods of sperm preparation for IVF (in this case, differential density gradient centrifugation) and measures of sperm quality that included DNA fragmentation and chromatin condensation. Initially, microscopy was used to detect hyaladherins, including CD44 on human sperm, which was detected on the acrosome as has been reported elsewhere (Bains et al., 2002) and equatorial regions of washed spermatozoa. Compared with CD44, however, the signals obtained with a fluorescently tagged probe for hyaluronic-acid-binding (HA-TRITC), capable of generically recognizing hyaladherins (including CD44) seemed to be more widely distributed over the sperm surface with some localized areas of high intensity, particularly in the neck region. In this regard, signals for CD44 and hyaladherins in general were both substantially blocked by incubating sperm with excess hyaluronic acid beforehand. These results confirm previous reports that hyaladherins (CD44 among them) are present on human sperm (Kornovski et al., 1994; Cherr et al,. 2001; Bains et al., 2002; Kim et al., 2008; Martin-Deleon, 2011). The failure to completely abolish labelling of hyaladherins after cold incubation with hyaluronic acid probably reflects the transient interaction of hyaluronic acid with its receptors compared with the stronger and more stable interaction between antibodies and their ligands (Plazinski and Knys-Dzieciuch, 2012). Other background signals may be caused by non-specific interaction of small amounts of free TRITC with ‘sticky’ sperm surfaces.

During transit through the female genital tract, sperm undergo capacitation, which involves protein tyrosine phosphorylation, hyperactive motility and the dramatic re-distribution of membrane proteins and lipids including the efflux of membrane cholesterol (Zaneveld et al. 1991; Liu et al., 2007; Gadella et al., 2008; Leahy and Gadella, 2011). Other changes may also affect the distribution of hyaladherins in preparation for encountering the descending egg mass (Baldi et al., 2000). In this regard, striking changes in the labelling of hyaladherin were observed after incubation under conditions favouring capacitation and after the acrosome reaction. General fluorescence, particularly with hyaluronic acid-TRITC increased after capacitation and the acrosome reaction, suggesting greater accessibility of this detection reagent to its ligands in keeping with the likely redistribution of membrane components (Leahy and Gadella, 2011). Capacitation, as reported originally by Yanagimachi (1969) using hamster spermatozoa is now a widely recognised phenomenon that is linked with the acquisition of hyperactive motility (Fraser, 1998; Suarez, 2008; Bailey, 2010), the observed redistribution of hyaladherins accords with reports showing that cytoplasmically mature and actively motile spermatozoa have completed their plasma membrane remodelling, which probably includes the redistribution of hyaladherins (Cayli et al. 2003; Huszar et al. 2003; Prinosilova et al. 2009; Parmegiani et al. 2010a; Yagci et al. 2010). Hyperactive motility is not an absolute requirement for sperm binding to hyaluronic acid, but it may be a requirement for sperm penetration of the hyaluronic acid-rich, cumulus complex surrounding the oocyte in vivo (Hong et al., 2009). It is likely that the higher hyaluronic-acid-binding scores in capacitated sperm reflect this process in vitro.

The sperm-preparation procedure, DDGC, widely used in assisted reproduction techniques (WHO, 2010), is thought to enrich for higher quality spermatozoa with a higher density and motility caused by prior cytoplasmic extrusion and greater chromatin condensation. These denser and more motile, mature cells can more readily (and rapidly) sediment through high-density silica-based media (Bolton and Braude, 1984; Mortimer and Mortimer, 2013). In this regard, sperm populations from 90% fractions had generally higher abilities to bind to hyaluronic acid and lower levels of DNA fragmentation than 45% fractions, suggesting that either hyaladherins are more abundant or have a higher affinity for hyaluronic acid in pelleted sperm (also with low levels of DNA fragmentation) and agreeing with the likelihood that these sperm are more cytoplasmically mature. We also showed that hyaluronic-acid-binding is highly effective at excluding spermatozoa with higher levels of DNA fragmentation and particularly so for excluding sperm with lower levels of chromatin maturity. The post-hoc

Dunn's test reports the most significant ranking differences between group medians after K-W analysis of variance. Although the differences in DNA fragmentation between unpaired 90% and 45% fractions or between hyaluronic-acid-binding and non-binding samples were occasionally not statistically significant using this test, the trends were clear nonetheless. These findings are supported by other studies showing that spermatozoa from the 90% fractions of DDGC processed samples have a high percentage of normal morphology and chromatin maturity and generally lower levels of DNA fragmentation (Le Lannou and Blanchard, 1988; Sakkas et al. 2000; Tomlinson et al., 2001; Brahem et al. 2011a).

Although the data collected in this study were derived from men of unproven fertility, our results are relevant when considering assisted reproduction techniques for infertile men, where higher levels of sperm DNA fragmentation may be associated with increased chances of early pregnancy failure after IVF and ICSI (Zini et al., 2008; Robinson et al., 2012; Zhao et al., 2014; Osman et al., 2015). Earlier studies have shown that, compared with spermatozoa recovered after a general washing step, sperm from the same semen sample binding to hyaluronic acid were more mature in relation to cytoplasmic and nuclear condensation (Huszar et al., 2003; Yagci et al., 2010) and that hyaluronic-acid-selected sperm from crude semen samples had low levels of DNA fragmentation (Yagci et al., 2010). These data suggest that hyaluronic-acid-binding may be a useful augmentation or alternative to DDGC for preparing crude semen samples for ICSI and potentially other IVF procedures, particularly where sperm numbers are too low for DDGC. Clinical support for these findings, however, is equivocal. One highly relevant prospective study (Nijs et al., 2009), whereas establishing clear connections between conventional sperm assessment methods and more advanced techniques, including assays of DNA fragmentation and chromatin maturity, could not link these connections through to the sample's ability to bind to hyaluronic acid. A later, smaller study appeared to uphold this disconnect (Nijs et al., 2010). Recent meta-analyses of numerous studies that included use of a selection step for hyaluronic acid step before ICSI have so far also failed to support efficacy with the caveat that the studies concerned were relatively small (Zini et al., 2008). The largest study to date reported a significant drop in pregnancy loss but unfortunately failed to report birth outcomes (Worrilow et al., 2013). A more recent report showed a significant improvement in live births although the lack of available raw data led to the study's exclusion from a later meta-analysis that recommended the need for better clinical trials with full data reporting (Mokanszki et al., 2014; Beck-Fruchter et al., 2015).

In conclusion, to the best of our knowledge, this is the first study to bring together compelling evidence for the relationship linking a standard sperm-processing technique (DDGC) with affinity of sperm for hyaluronic acid. Sperm DNA fragmentation and chromatin compaction status, therefore, reflects this relationship and supports claims for a positive sperm quality benefit for hyaluronic-acid-based sperm selection. Whether this benefit translates into significantly improved live birth outcomes for ICSI remains to be determined. In this regard, the use of more specific, anti-hyaladherin-based procedures for sperm selection (initially targeting CD44, RHAMM and SPAM1) should also be considered. We are currently undertaking the largest clinical trial to date (Witt et al., 2016; ISRCTN99214271) of an sperm-selection procedure based on hyaluroni acid for ICSI that is sufficiently sensitive to detect a beneficial effect Our findings will be reported in the autumn of 2017.

In the absence of computer-assisted seman analysis, hyperactive motility (more vigorous and irregular tail beating often accompanied by whole cell rotation) was observed on four samples (D3, D5, D11 and D14) of sperm bound to hyaluronic acid using hyaluronan binding assay slides. To assess the effect of capacitation, normal sperm motility before capacitation was first assessed in sperm from the crude semen samples immediately after washing (column 2) and after processing by DDGC (90% fractions; column 3). The same samples were then re-assessed for hyperactive motility after 0 h (columns 4 and 6) and 3 h (columns 5 and 7) capacitation.

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Declaration

The authors report no financial or commercial conflicts of interest.

Figure 1: Immunofluorescence images of sperm probed with reagents targeting hyaladherins. Sperm samples from 90% fractions are shown after Image J background noise reduction and contrast enhancement after labelling with hyaluronan acid conjugated with tetramethylrhodamine isothiocyanante (TRITC) directly (A, B, G, H), or indirectly with an antibody to CD44 (C, D, I, J). Panels E, F, K, L shows the corresponding merged images with an additional DAPI layer included to aid definition of sperm nuclei. Panels A, C and E show sperm samples labelled before capacitation. Panels B, D, F show sperm samples labelled after capacitation. Panels G, I, K show sperm that were acrosome reacted. Panels H, J, L (controls) show sperm pre-incubated with excess unlabelled hyaluronic acid prior to incubation with hyaluronic-acid-TRITC or antibodies to CD44. Equatorial regions of some sperm are indicated by arrow and the scale bar is 5 µm.

Figure 2: Demonstration of the efficiency of capacitation by inducing the acrosome reaction. To determine the efficiency of capacitation, sperm samples from 90% fractions were incubated in capacitation supportive buffer for 0 h (A) and 3 h (B) and then acrosome reacted before labelling with fluorescein-conjugated Pisum sativum agglutinin (green) and counterstaining with 4',6-diamidino-2-phenylindole (DAPI) (blue) in each case. Acrosome reacted sperm are indicated by a star '*'. The scale bar is 5 µm.

Figure 3: Assessment of hyaluronic-acid-binding in sperm recovered from DDGC fractions. Binding of sperm recovered from the 90% and 45% fractions to hyaluronan was assessed using HBA® slides (n = 15). Box-whisker plots are drawn showing quartiles, minimum and maximum values with the significance indicated as 'a' ($P \leq 0.0001$; Mann–Whitney U test).

Figure 4: Examples of acridine orange stained sperm from experimental samples. Sperm were recovered from the 90% (A) and 45% (B) differential density gradient centrifugation fractions (n = 15) or after brief incubation on surfaces coated with hyaluronic acid (n = 10) and which remained bound to the substrate after gentle washing (C) or were recovered from the unbound washes (D). After washing, the samples were cytospun on to coated slides and stained with acridine Orange. Note the more uniform staining with acridine orange (green) in 90% and hyaluronic-acid-binding fractions compared with 45% fractions and hyaluronic acid-unbound populations (mixed colours). The panels to the right of the main figures shows the subjective scoring system used to assign staining categories (++, ±, --) to the samples. The scale bar is 20 µm (A–D).

Figure 5: Examples of acridine-orange-stained sperm from experimental samples. Sperm were recovered from the 90% (A) and 45% (B) differential density gradient centrifugation

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fractions or after brief incubation on surfaces coated with hyaluronic acid and which remained bound to the substrate after gentle washing (C) or were recovered from the unbound washes (D). After washing, the samples were cytospun on to coated slides and stained with Aniline Blue. Note the weaker staining (--) in 90% and hyaluronic-acid-binding fractions compared with 45% and hyaluronic-acid-unbound fractions (a mix of strongly and marginally stained sperm). The panels to the right of the main figures shows the subjective scoring system used to assign staining categories (++, ±, --) to the samples. The scale bar is 10 µm (A–D).

Figure 6: Box-whisker plots of acridine orange category data from the study. Semi-quantitative data for acridine orange staining in relation to differential density gradient centrifugation sedimentation into 90% and 45 % fractions (A; n = 15) and into hyaluronic-acid-binding and unbound populations (B; n = 10) are plotted with quartiles, minimum and maximum values shown. Acridine orange categories indicate low (++), medium (±) and high (--) DNA fragmentation. Note the generally inverse relationships between staining categories and sedimentation or hyaluronic-acid-binding profiles. The K-W, post-hoc Dunn's test assigns the significance alpha between group medians hierarchically with a (<0.0001) > b (<0.001) > c (<0.01) > d (<0.05). HA, hyaluronic acid.

Comment [S7]: Typesetter: X axis in A: remove percentages and add as lavel (%)

Figure 7: Box-whisker plots of AB category data from the study. Semi-quantitative data for AB staining in relation to differential density gradient centrifugation sedimentation into 90% and 45 % fractions (A; n = 15) and into hyaluronic-acid-binding and unbound populations (B; n = 10) are plotted with quartiles, minimum and maximum values shown. AB categories indicate fully (--), partially (±) and weakly (++) compacted chromatin. Note the generally inverse relationships between staining categories and sedimentation or hyaluronic-acid - binding profiles. The K-W, post-hoc Dunn's test assigns the significance alpha between group medians hierarchically with a (<0.0001) > b (<0.001) > c (<0.01) > d (<0.05). HA, hyaluronic acid.

Comment [S8]: Typestter: as above.

Figure 8: Box-whisker plots of hyaluronic-acid-binding in relation to capacitation. The effect on hyaluronic-acid-binding of capacitation in washed sperm (n = 10) or sperm recovered from 90% fractions (n = 10) is shown with quartiles, minimum and maximum values shown and the most significant difference being between washed, non-capacitated sperm and 90% capacitated sperm indicated (a). Although not statistically significant due to outlying values, note the clear increase in hyaluronic-acid-binding following capacitation of washed sperm.

Comment [S9]: Typesetter: change Y axis to Hyaluronic-acid-binding spermatoza.

The K-W, post-hoc Dunn's test assigns the significance alpha between group medians hierarchically with a (<0.0001) > b (<0.001) > c (<0.01) > d (<0.05).

Supplementary Figure 1. As for Figure 1 but showing the original unenhanced images.

Table 1: General semen assessment of young male volunteers (unproven fertility) participating in the study.

Sample	Volunteer age (years)	Sperm concentration (million/ml)	Total sperm count ($\times 10^6$)	Semen volume (ml)	Sperm motility (%)
D1	20	159	238.5	1.5	56
D2	21	69	345	5	94
D3	22	122	244	2	70
D4	36	200	300	1.5	90
D5	21	56	224	4	67
D6	21	120	360	3	86
D7	20	156	93.6	0.6	85
D8	19	120	240	2	70
D9	28	233	349.5	1.5	92
D10	24	185	370	2	75
D11	21	90	270	3	65
D12	24	36	108	3	85
D13	19	251	1204.8	4.8	87
D14	20	179	716	4	61
D15	22	154	385	2.5	83
D16	21	55	330	6	75
Mean ± SD	22.4 ± 4.2	136.6 ± 64.5	361 ± 264.53	2.9 ± 1.5	77.5 ± 11.7

Table 2. Hyperactive motility after capacitation.[a]

Sample	Sperm motility, *Quinn's* washed semen (%)	Sperm motility after DDGC, 90% fraction (%)	Sperm hyperactive *motility, Quinn's* washed semen at 0 h cap (%)	Sperm hyperactive *motility, Quinn's* washed semen at 3 h cap (%)	Sperm hyperactive motility, 90% fraction at 0 h cap (%)	Sperm hyperactive motility, 90% fraction at 3 h cap (%)	Hyperactive m incubation in c conditions (%)
D3	70	88	0.53	44.5	0.6	47.5	47.5
D5	67	81.5	0.2	41.0	0.4	43.4	43.4
D11	65	84.3	0.4	39.8	0.4	44.0	44
D14	61	79	0.27	42.8	0.3	39.6	39.6
Mean ± SD	65.8 ±3.8	83.2 ±3.9	0.35 ±0.2	42 ± 2.01	0.43 ± 0.13	43.6 ±3.23	**43.63 ± 3.23**

[a]See also **Figure 8**.