Asian Journal of Andrology (2020) 22, 447–453
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**ORIGINAL ARTICLE**

Sperm Biology

# Influence of *in vitro* capacitation time on structural and functional human sperm parameters

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**A cascade of dramatic physiological events is linked to the sperm acrosome reaction and binding to the oocyte's zona pellucida during human sperm capacitation. However, structural and functional sperm changes during capacitation currently remain poorly defined. Here, we performed a multibiomarker approach based on the utilization of sperm concentration, motility, viability, morphology, acrosome reaction, tyrosine phosphorylation, DNA fragmentation, and lectin-binding sites to analyze the impact caused by swim-up selection times (uncapacitated, 1 h capacitated, and 4 h capacitated) on sperm function and structure in normozoospermic samples. We found that a 4 h swim-up capacitation increased sperm quality, because a large number of cells with normal morphology and lower DNA fragmentation rates were recovered. Furthermore, the long-term capacitation induced a higher percentage of cells with tyrosine phosphorylation of the principal piece as well as a redistribution of lectin-binding sites. Overall, the multivariate biomarkers analyzed showed a less variable distribution on spermatozoa recovered after 4 h capacitation than that with the shorter capacitation time. These findings stress the importance of capacitation time as a relevant factor in sperm quality with potential biological reproductive implications both for basic research and in assisted reproduction techniques.**
*Asian Journal of Andrology* (2020) **22**, 447–453; doi: 10.4103/aja.aja_104_19; published online: 15 October 2019

**Keywords:** acrosome reaction; capacitation; DNA fragmentation; glycocalyx; lectin-binding sites; membrane integrity; protein phosphorylation; sperm selection

## INTRODUCTION

The success of mammalian fertilization depends largely on the spermatozoon's ability to acquire functionality through the female reproductive tract.[1] This process, known as capacitation,[2–4] refers to biochemical and physiological changes permitting the acrosome reaction and interaction with the oocyte.[5,6] Early reports showed that capacitation can also be achieved *in vitro* in several species,[7] including humans.[8]

Examples of the most outstanding changes in human sperm capacitation are the efflux of membrane cholesterol;[9] the redistribution of glycoconjugates;[10] the entry of calcium into the cytoplasm followed by the increase of tyrosine phosphorylation;[11] and the acquisition of hyperactivated motility.[12] This cascade of changes in biomarkers associated with sperm capacitation has recently been outlined.[13,14] Nevertheless, *in vitro* capacitation time in human spermatozoa covers an extensive range from 3 h to 24 h.[15] In this context, a study has reported that spermatozoa need to undergo capacitation *in vitro* for at least 4 h in order to recognize the oocyte's zona pellucida.[16] Wider capacitation ranges have been linked to the vast heterogeneity of semen samples, which result in sperm subpopulations with varying degrees of functionality and membrane cholesterol content.[17,18] Finally, other reports have shown that capacitation timing in human spermatozoa differs among men despite being reproducible within each man.[19]

Standard semen analysis results (*e.g.*, sperm concentration, motility, morphology, and viability) have limited predictive power for fertilization success.[20–22] Thus, new potential sperm biomarkers have been developed. These include the acrosome reaction, DNA damage, and oocyte's binding molecules.[23–25] The World Health Organization (WHO) stipulates times for conventional sperm selection protocols with a capacitating medium (for selection and capacitation), 1 h for swim-up and 15–30 min for discontinuous density gradients.[26] Therefore, studies on the influence of preparation time on sperm biomarkers only differ in longer incubation time after selection in the capacitation medium.[27–29] Optimal incubation times have been obtained for tyrosine phosphorylation,[27] DNA fragmentation,[28] and the acrosome reaction,[29] among others. However, studies that have considered sperm biomarkers after a longer combined sperm selection/capacitation time are particularly scarce.[30] This paper shows the first application of a novel multivariate approach to analyze the impact that different swim-up selection times with a capacitating medium (1 h and 4 h) have on quality and structural sperm biomarkers.

## MATERIALS AND METHODS

### *Experimental design*

Semen samples were obtained from 15 normozoospermic donors with their written informed consent. The research was approved by

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Received: 06 March 2019; Accepted: 04 July 2019

the Ethical Committee of the University of Alicante (Alicante, Spain) based on the Declaration of Helsinki principles. Each sperm sample was divided to obtain the three selected physiological conditions: uncapacitated (T0), 1 h selected under capacitating conditions (T1), and 4 h selected under capacitating conditions (T4). T1 was chosen in accordance with the WHO swim-up protocol[26] and T4 was based on previous protocols.[16] On the basis of these different physiological conditions, we examined a set of biomarkers, namely, basic sperm parameters, tyrosine phosphorylation, spontaneous and induced acrosome reactions, lectin-binding sites, and DNA fragmentation.

### *Preparation of semen samples*

Donor semen samples were obtained by masturbation after 3–4 days of abstinence and subsequently analyzed in the laboratory of the Department of Biotechnology at the University of Alicante. The samples were allowed to liquefy for 15 min at room temperature, and basic seminogram analysis was performed by following the WHO guidelines.[26] Sperm concentration and motility assessment was undertaken using a Makler® (BioCare Europe, Rome, Italy) counting chamber; Papanicolaou staining (Panreac Química S.L.U., Barcelona, Spain) served for the evaluation of morphology, and vitality was studied by eosin–nigrosin staining (Projectes i Serveis R+D S.L., Paterna, Spain).

### *Sperm capacitation*

Semen aliquots were selected/capacitated by swim-up in human tubal fluid medium (HTF, Origio®, Måløv, Denmark) supplemented with 5 mg $ml^{-1}$ bovine serum albumin (BSA, Sigma-Aldrich®, Saint Louis, MO, USA) at 37°C and 5% (*v*/*v*) $CO_2$ for 1 h (T1) and 4 h (T4). The supernatant fraction was subsequently collected and washed three times for 5 min in phosphate-buffered saline without calcium or magnesium, pH 7.4 (PBS, Biowest, Nuaillé, France) by centrifugation (250*g*, 10 min). Concentration, motility, morphology, and viability for recovery of each mobile spermatozoon (REM) were estimated. Cells from the different times considered (T0, T1, and T4) were fixed in 2% (*w*/*v*) paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 45 min at 4°C, after which paraformaldehyde was replaced with PBS and the samples were stored at 4°C.

### *Assessment of spontaneous and inducted acrosomal reaction*

An aliquot of capacitated cells was induced with 0.01 mmol $l^{-1}$ of calcium ionophore A23187 (Sigma-Aldrich) and 2 mmol $l^{-1}$ calcium chloride (Panreac Química S.L.U., Barcelona, Spain) for 1 h at 37°C and 5% (*v*/*v*) $CO_2$ following previous protocols.[31] Only calcium chloride was added to the controls for the purpose of detecting spontaneous acrosomal reaction. We also evaluated the percentage of spontaneously reacted cells in the T0 condition.

A total of 5 µl sperm suspension from each physiological condition was placed on coverslips and fixed in methanol for 30 min for the assessment of acrosomal status. After drying the smear, the cells were washed three times in PBS and unspecific binding was blocked by 2% (*w*/*v*) BSA-PBS for 30 min. The smears were then incubated in the dark with *Pisum sativum* lectin conjugated to fluorescein-5-isothiocyanate (PSA-FITC, Sigma-Aldrich) 50 µg $ml^{-1}$ for 30 min. After three washes in PBS, the samples were mounted with Vectashield® and 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI, Vector Laboratories, Burlingame, CA, USA). DAPI helped with identification of the cell nucleus (**Figure 1a**). The whole process took place at room temperature.

### *Immunolocalization of tyrosine phosphorylation*

A total of 5 µl of sperm suspension from each paraformaldehyde-fixed condition was deposited on a coverslip. When dry, the cells were washed three times in PBS and permeabilized by incubation in 0.1% (*v*/*v*) Triton X-100 (Sigma-Aldrich) for 10 min. In an attempt to prevent unspecific binding, spermatozoa were blocked with 2% (*w*/*v*) BSA-PBS for 30 min. Tyrosine phosphorylation was detected using an anti-phosphotyrosine primary antibody produced in mice (PY20, Sigma-Aldrich) at a 1:500 dilution for 1 h and a secondary anti-mouse IgG (H + L) antibody conjugated to Cyanine ™3 (Jackson ImmunoResearch, Ely, UK) at a 1:300 dilution for 1 h in the dark, taking previous protocols as reference.[11] Slides were rinsed with PBS between both incubations. Finally, coverslips were washed again three times with PBS and subsequently mounted with Vectashield and DAPI.

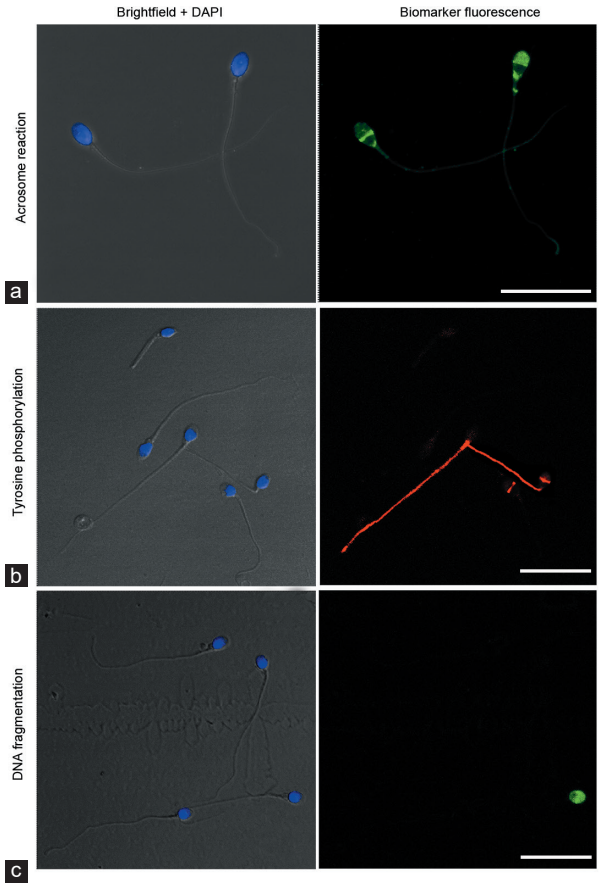


**Figure 1:** Sperm biomarker fluorescence. (**a**) PSA-label. Spermatozoa that fluoresced in the acrosomal region were considered not reacted (right) and those with labeling of the equatorial band were deemed as reacted (left). (**b**) PY20 fluorescent patterns. The equatorial segment, the principal piece, and the combined principal piece plus equatorial segment. (**c**) TUNEL assay. Green staining indicated DNA fragmentation. The sperm nucleus was detected by means of DAPI (blue). Scale bars = 20 µm. PSA: *Pisum sativum* lectin; TUNEL: terminal-deoxynucleotidyl transferase-mediated nick end labeling; DAPI: 4',6-diamidino-2-phenylindole.

### *Assessment of DNA fragmentation*

A terminal-deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay was performed using the *In Situ* Cell Death Detection Kit: Fluorescein according to the manufacturer's guidelines (Roche Diagnostics GmbH, Mannheim, Germany). A total of 5 µl fixed cell suspension from each experimental group (T0, T1, and T4) was first deposited on the slides and then rinsed in PBS and permeabilized with 0.2% (*v*/*v*) Triton X-100 for 5 min. The TdT-labeled nucleotide mixture was added and incubated at 37°C for 1 h in a dark, humid

chamber, after which the slides were washed three times in PBS and mounted with Vectashield with DAPI.

***Lectin cytochemistry***

As lectins bind to the carbohydrate group of glycoconjugates, we used several lectins to characterize the spermatozoa surface carbohydrate distribution by means of cytofluorescence. More specifically, four lectins were conjugated with FITC (Vector Laboratories): *Aleuria aurantia agglutinin* (AAA), *Concanavalin A* (ConA), *Peanut agglutinin* (PNA), and *Wheat germ agglutinin* (WGA). Each lectin presents a high specificity for a different carbohydrate. AAA recognizes fucose residues;[32] ConA has a high affinity for mannose;[33] PNA for galactose;[34] and WGA for sialic acid.[35] A total of 5 µl sperm suspension from each paraformaldehyde-fixed condition was deposited on a coverslip. Once dry, the smear was rehydrated three times with PBS and incubated in a 2% (*w/v)* BSA-PBS block solution for 1 h. The cells were subsequently incubated with each FITC-conjugated lectin at a final concentration of 20 µg $ml^{-1}$ for 1 h, at room temperature, and in a dark, humid chamber as in previous studies.[36] Finally, spermatozoa were rinsed three times in PBS and mounted with Vectashield with DAPI.

The data related to the acrosome reaction, tyrosine phosphorylation, the TUNEL assay, and lectin-binding sites were examined using Leica TCS-SP2 confocal microscopy (Leica Microsystems GmBH, Wetzlar, Germany), and Leica Confocal Software helped obtain (1024 × 1024 pixel) images. At least 200 cells were evaluated for each biomarker and physiological condition (T0, T1, and T4). Added to this, the appropriate negative controls served to corroborate the specificity of each biomarker.

***Statistical analyses***

The Shapiro–Wilk test performed with the aim of testing the distribution and equal variance in the biomarkers under study showed that 81.5% of the biomarkers did not have a normal distribution (W = 0.540–0.946; $P < 0.05$). The nonparametric Kruskal–Wallis test was used to assess differences on the effect of uncapacitated (T0) and 1–4 h (T1–T4) capacitated sperm samples within each biomarker. A principal component analysis (PCA) on the variance–covariance matrix allowed calculation of individual specific biomarker patterns that accounted for most of the variability observed among uncapacitated and capacitated conditions. Descriptive and statistical results were obtained by means of IBM SPSS Statistics 19.0 (IBM, Armonk, NY, USA) and SYSTAT® 11 (Systat Software, Inc., Point Richmond, CA, USA). Two-sided $P < 0.05$ was deemed statistically significant.

## RESULTS

***Effects of capacitation time on sperm parameters***

A significant decrease in sperm concentration ($P < 0.001$) along with an increase in motility and vitality ($P < 0.001$) became visible after both capacitation times (T1 and T4), compared with uncapacitated cells (**Table 1**). The results showed an increase up to 26.6% in normal forms ($P < 0.001$) after 4 h capacitation compared with T0. No statistical significance corresponded to normal sperm morphology differences between T0 (9.7%) and T1 (19.3%).

***Effects of capacitation time on spontaneous and induced acrosomal reactions***

The spermatozoa that fluoresced in the acrosomal region were regarded as not having reacted. Those with a label in the equatorial segment were considered reacted (**Figure 1a**). We did not find any differences after the induction of acrosome reaction using the calcium ionophore either at T1 or at T4 ($P > 0.05$). Furthermore, a high percentage of spontaneously acrosome-reacted cells was identified at T4 compared with T0 ($P = 0.028$) together with a lack of significant differences between T1 and T4 (**Table 1**).

***Effects of capacitation time on sperm tyrosine phosphorylation***

We defined three fluorescent tyrosine phosphorylation patterns, located at the equatorial segment (ES), the principal piece (PP), and the combined equatorial segment plus principal piece (ES + PP) (**Figure 1b**). We generally found a significant growing percentage ($P < 0.05$) of cells with tyrosine phosphorylation at every sperm location (ES, PP, and ES + PP) when a comparison was made between capacitation times (T1 and T4) and T0. The most prominent increase between T1 and T4 corresponded to phosphorylation of the principal piece, which showed a significant increase from 17.2% to 25.3% ($P = 0.004$) (**Table 1**).

***Effects of capacitation time on DNA sperm fragmentation***

Apoptotic cells were recognized by means of positive green fluorescence (**Figure 1c**). Fourteen percent of the analyzed cells showed DNA fragmentation at T0. The comparison of T0 with capacitating conditions ($P < 0.05$) revealed that the percentage of cells with fragmented DNA decreased to 3.3% ($P = 0.077$) after 1 h of capacitation and up to 2.0% after 4 h of capacitation ($P = 0.047$; **Table 1**).

***Effects of capacitation time on plasma membrane sugar-binding sites***

The findings revealed a high degree of carbohydrate surface heterogeneity, because sperm subpopulations with different location patterns appeared in all three physiological conditions (T0, T1, and T4). This allowed us to characterize five clearly defined patterns (**Figure 2**): Pattern 1 (P1), characterized by a highly stained acrosomal region; Pattern 2 (P2), defined by a highly stained acrosomal and postacrosomal region with no labeling in the equatorial segment; Pattern 3 (P3), with labeling in the equatorial segment; Pattern 4 (P4), which showed a dotted label in the whole head; and Pattern 5 (P5), with a faint labeling in the whole head. Patterns 1 to 5 (P1–5) were quantified for both AAA and ConA lectins. However, only three patterns (P1, P3, and P5) emerged for the PNA lectin and four for WGA (P1, P3, P4, and P5).

Significant differences in the redistribution of glycoconjugates from T0 to T1 only became visible in the AAA and WGA-labeling patterns (**Table 1**). We detected a significant increase ($P < 0.001$) in AAA lectin-binding sites at both the acrosomal and the postacrosomal region (P2: from 6.0% to 32.4%), with decrease ($P < 0.001$) in the whole head dotted staining (P4: from 45.2% to 4.8%). Likewise, the percentage of cells with WGA-binding sites in the cap (P1) decreased from 59.7% to 41.3% ($P = 0.005$), while P4 increased to 47.4% from 23.1% ($P < 0.001$). We detected no differences after 1 h capacitation in the percentage of ConA and PNA-binding site patterns compared with T0.

Along with the differences in the redistribution of the sugar residues described for the T1 condition, changes were recorded after 4 h of capacitation in comparison with T0. The most noticeable was the increase from 26.0% to 45.0% ($P = 0.02$) of cells with ConA-labeling in the acrosomal as well as the postacrosomal region (P2). We additionally observed an increase in the number of cells that relocated the residues recognized by the AAA lectin to the acrosomal region (P1: from 28.7% to 59.3%; $P < 0.001$). No significant differences were found in the head spatial distribution of PNA-binding residues in any of the sperm capacitation times under study (**Table 1**).

**Table 1: Descriptive and statistical results of sperm biomarkers in uncapacitated and swim-up capacitated conditions**

| | *Uncapacitated, mean±s.d.* | *1 h capacitated, mean±s.d.* | *4 h capacitated, mean±s.d.* |
|---|---|---|---|
| Concentration ($10^6$ ml$^{-1}$) | 54.7±26.2 | 8.6±5.9[a] | 8.9±3.5[b] |
| Total motility (%) | 70.9±14.8 | 97.5±1.9[a] | 96.3±1.5[b] |
| Viability (%) | 82.5±15.1 | 96.7±2.3[a] | 95.9±1.6[b] |
| Normal morphology (%) | 9.7±6.2 | 19.3±9.2 | 26.6±13.8[b] |
| Acrosomal reaction (%) | | | |
| Spontaneous | 12.0±6.7 | 16.5±9.5 | 23.8±8.2[b] |
| Calcium ionophore | – | 80.9±6.8 | 81.2±6.9 |
| Tyrosine phosphorylation (%) | | | |
| ES | 1.5±3.2 | 16.5±6.0[a] | 18.1±7.2[b] |
| PP | 3.2±1.9 | 17.2±5.6[a] | 25.3±4.5[b,c] |
| ES + PP | 0.0±0.0 | 5.5±4.4[a] | 9.7±7.2[b] |
| DNA fragmentation (%) | 14.0±12.7 | 3.3±4.0 | 2.0±2.3[b] |
| Sugar-binding patterns, AAA lectin (%) | | | |
| P1 | 28.7±16.2 | 43.3±20.1 | 59.3±15.4[b] |
| P2 | 6.0±7.3 | 32.4±17.6[a] | 21.1±10.6[b] |
| P3 | 4.0±6.6 | 2.3±6.2 | 2.2±5.7 |
| P4 | 45.2±15.1 | 4.8±5.8[a] | 3.6±4.8[b] |
| P5 | 16.0±11.8 | 17.0±18.3 | 13.6±11.1 |
| Sugar-binding patterns, ConA lectin (%) | | | |
| P1 | 55.5±16.9 | 49.0±24.0 | 38.9±20.2 |
| P2 | 26.0±13.9 | 26.4±14.9 | 45.0±17.0[b,c] |
| P3 | 3.4±4.5 | 1.3±4.0 | 2.7±4.8 |
| P4 | 4.6±10.7 | 3.2±5.8 | 7.9±4.7 |
| P5 | 10.2±8.5 | 19.8±26.5 | 5.3±6.6 |
| Sugar-binding patterns, PNA lectin (%) | | | |
| P1 | 45.8±29.9 | 67.8±21.8 | 68.0±21.6 |
| P3 | 19.9±15.9 | 9.8±10.0 | 17.9±13.3 |
| P5 | 34.2±30.9 | 22.3±22.6 | 14.0±14.0 |
| Sugar-binding patterns, WGA lectin (%) | | | |
| P1 | 59.7±12.9 | 41.3±11.2[a] | 35.1±7.3[b] |
| P3 | 7.4±9.0 | 4.5±6.3 | 13.4±10.9 |
| P4 | 23.1±10.3 | 47.4±12.0[a] | 42.1±17.0[b] |
| P5 | 9.9±10.7 | 6.7±9.1 | 9.2±10.2 |

Letters a, b, and c indicate significant differences at $P<0.05$ level according to Kruskal–Wallis test between capacitation times: [a]T0 versus T1, [b]T0 versus T4, and [c]T1 versus T4. ES: equatorial segment; PP: principal piece; AAA: *Aleuria aurantia agglutinin*; ConA: *Concanavalin A*; PNA: *Peanut agglutinin*; WGA: *Wheat germ agglutinin*; s.d.: standard deviation

### *Difference in sperm biomarkers*

The PCA results provide strong evidence that the sperm biomarkers used can distinguish semen samples (**Table 2** and **Figure 3**). Eight principal components were obtained with eigenvalues >1 accounting for 89.3% of the total variance. Nonetheless, little variation appeared after the first two components (≤10% of variance), which is why we considered only the first two principal components (PC1 and PC2), which accounted for 57.1% of the total variance. Similarly, significant differences (Kruskal–Wallis; $P < 0.05$) were only observed for the first (PC1: $\chi^2 = 27.289$; $P < 0.001$) and second (PC2: $\chi^2 = 7.023$; $P = 0.008$) components when comparing uncapacitated and capacitated samples.

PC1 (38.4% of the variance) clearly separated uncapacitated (PC1 negative values) and capacitated samples mainly clustered together (**Figure 3a**). Therefore, the sperm biomarkers with the strongest correlations reported significant changes during the capacitation process (**Table 2** and **Figure 3b**). PC1 was marked by uniformly high and positive values ($r > 0.4$; $P < 0.01$) associated with changes in motility, viability, tyrosine phosphorylation, and spontaneous acrosomal reaction at both capacitation times. Other biomarkers, including sperm concentration and DNA fragmentation, showed significant negative correlations ($r > -0.6$; $P < 0.01$) because lower values were recorded during capacitation for both T1 and T4. It became obvious too that several lectin patterns (P4WGA, P1-P2AAA, P1PNA, and P2ConA) were represented mostly on the PC1 axis during capacitation time ($r = 0.3–0.7$; $P < 0.05$). Instead, others such as P1WGA, P4AAA, P5AAA, and P5PNA underwent a significant reduction ($r = -0.3–-0.8$; $P < 0.05$) (**Table 2**).

Moreover, sample distribution along PC2 (18.7% of the variance) was mainly affected by interindividual lectin changes after 1 h of capacitation. Interestingly, the significant changes in lectins that occurred during capacitation showed a clearly less variable distribution after 4 h of capacitation (**Figure 3a** and **3b**). In turn, P1 and P5 patterns were conversely distributed, affecting the four different lectins and capacitated sample variability. On the whole, we found that PC2 drew a distinction where P4-5WGA, P2 and P5AAA, P5PNA, and P5ConA patterns received positive loadings ($r \geq 0.4$; $P < 0.05$), as opposed to P1WGA, P1AAA, P1PNA, and P1ConA, which carried negative loadings ($r \geq -0.3$; $P < 0.05$) (**Figure 3b** and **3c**).

## DISCUSSION

Although the physiological phenomenon of sperm capacitation was described more than half a century ago,[1] contradictory information

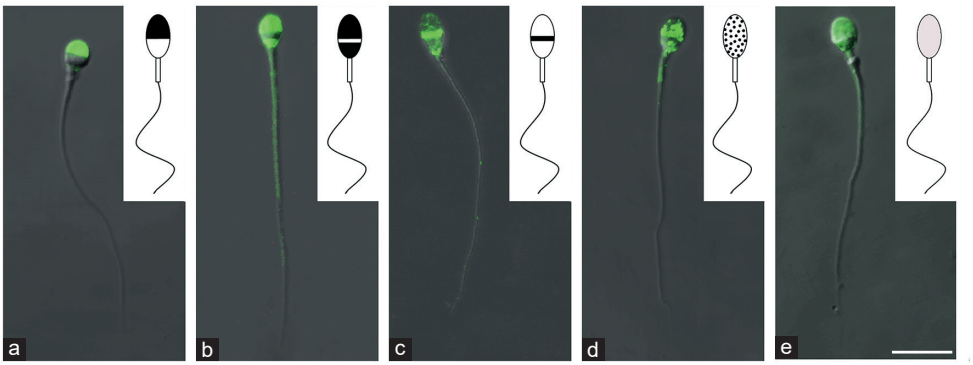


**Figure 2:** Superposition of bright field and lectin-binding fluorescent patterns. (**a**) Pattern 1, highly stained acrosomal region. (**b**) Pattern 2, highly stained acrosomal and postacrosomal region. (**c**) Pattern 3, highly stained equatorial segment. (**d**) Pattern 4, dotted staining over the head. (**e**) Pattern 5, faint staining over the head. Scale bar = 10 µm.

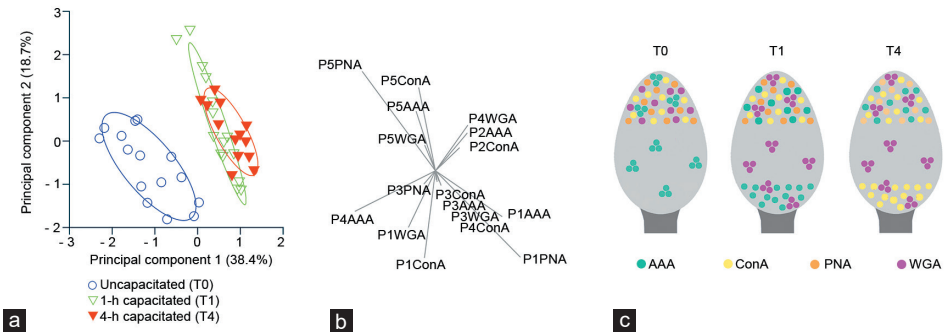


**Figure 3:** Swim-up capacitation time changes on sperm biomarkers. (**a**) Binary plot of the first two principal components (PC1 and PC2) accounting for 57.1% of total variance. The ellipses include 95% confidence regions of within-subpopulation sample variability. (**b**) The labeled rays show the loadings for lectin patterns onto PC1 and PC2 axes (see **Table 2** for additional biomarker correlation details). (**c**) Glycocalyx sketch based on strong lectin correlations in different physiological conditions according to **b** data. T0 is composed of P4AAA, P1ConA, P1PNA, and P1WGA; T1 is composed of P2AAA, P1ConA, P1PNA, and P4WGA; T4 is composed of P1AAA, P2ConA, P1PNA, and P4WGA. AAA: *Aleuria aurantia agglutinin*; ConA: *Concanavalin A*; PNA: *Peanut agglutinin*; WGA: *Wheat germ agglutinin*.

still exists about the molecular basis for orchestration of human sperm capacitation. A wide variety of conditions have been employed for capacitation time or medium composition, which, in turn, trigger a great diversity in the elucidation of molecular events.[37] Added to this, the high heterogeneity of human sperm populations, together with the subjectivity of conventional semen analytical techniques, leads to incorrect predictions of sperm functional status.[20,38] Hence, there is a lack of studies that recognize selection/capacitation time as an influential factor in the fertilization process. Here, we have been the first to undertake a multivariable study of several biomarkers and aspects of sperm structure and function before capacitation and after 1 h and 4 h of swim-up selection under capacitating conditions.

In agreement with previous studies,[39] our findings, based on the use of standard biomarkers, showed that 1 h of capacitation sufficed to recover a sperm subpopulation with high levels of motility and viability. The swim-up recovery of motile sperm directly implies reduction in the total number of recovered spermatozoa (<20%).[26] Our results match this, because the sperm recovery after swim-up was approximately 15% after 1 h and 4 h. Under our experimental conditions, 4 h of capacitation is required to recover a subpopulation with a significant increase of normal sperm forms. In contrast, a previous study recorded a significant increase in normal sperm forms just after 1 h of capacitation.[40] Such a discrepancy is probably related to staining methods.[41] Interestingly, previous observations have highlighted the importance of normal sperm morphology in the binding to the zona pellucida;[42] our results stressed that longer sperm selection times under capacitating conditions enrich the normal forms of morphology.

The ability to undergo the acrosome reaction after induction constitutes a parameter related to sperm quality. Moreover, only the spermatozoa that release their acrosomal content will be able to cross the zona pellucida and fuse with the oocyte.[43] In this context, our results showed that approximately 80% of spermatozoa were incubated to undergo the acrosome reaction by calcium ionophore, regardless of their selection/capacitation time (1 h or 4 h). Therefore, the number of acrosome-reacted spermatozoa depends on the induction time[29] rather than on the sperm selection time in capacitating media. For the spontaneous acrosome reaction, the percentage of reacted spermatozoa clearly increased with the swim-up selection/capacitation time. Likewise, reports have also proved the time-dependent characteristic of the spontaneous acrosome reaction, after 1 h, 3 h, and 5 h of sperm selection/capacitation.[30]

The key sperm capacitation marker is protein phosphorylation on tyrosine residues, because associations exist with hyperactivation, cumulus oophorus penetration, and zona pellucida binding.[11] After 4 h of capacitation, tyrosine phosphorylation was found to increase significantly in different sperm regions. In particular, the principal piece (25.3%) registered a higher percentage of phosphorylation than uncapacitated (3.2%) and 1 h capacitated (17.2%) cells. Likewise, a previous report linked sperm hyaluronic acid binding with time-related

**Table 2: Factor loadings corresponding to the first two PCs based on sperm biomarkers within uncapacitated and swim-up capacitated samples**

| | *r* | |
|---|---|---|
| *Biomarker* | *PC1* | *PC2* |
| Concentration | –0.833* | –0.341* |
| Total motility | 0.685* | 0.427* |
| Viability | 0.611* | 0.326* |
| Normal morphology | 0.606* | 0.147 |
| Acrosome reaction | | |
| Spontaneous | 0.405* | –0.001 |
| Calcium ionophore | 0.055 | –0.055 |
| Tyrosine phosphorylation | | |
| ES | 0.713* | 0.493* |
| PP | 0.763* | 0.268 |
| ES + PP | 0.561* | 0.407* |
| DNA fragmentation | –0.670* | –0.146 |
| Sugar-binding patterns, AAA lectin | | |
| P1 | 0.748* | –0.319* |
| P2 | 0.451* | 0.331* |
| P3 | 0.051 | –0.258 |
| P4 | –0.867* | –0.271 |
| P5 | –0.326* | 0.622* |
| Sugar-binding patterns, ConA lectin | | |
| P1 | –0.122 | –0.601* |
| P2 | 0.331* | 0.196 |
| P3 | 0.023 | –0.295 |
| P4 | 0.097 | –0.196 |
| P5 | –0.226 | 0.697* |
| Sugar-binding patterns, PNA lectin | | |
| P1 | 0.759* | –0.479* |
| P3 | –0.197 | –0.141 |
| P5 | –0.702* | 0.590* |
| Sugar-binding patterns, WGA lectin | | |
| P1 | –0.430* | –0.558* |
| P3 | 0.136 | –0.228 |
| P4 | 0.457* | 0.398* |
| P5 | –0.257 | 0.380* |

*Correlation (Pearson's *r*) at $P<0.05$. ES: equatorial segment; PP: principal piece; AAA: *Aleuria aurantia agglutinin*; ConA: *Concanavalin A*; PNA: *Peanut agglutinin*; WGA: *Wheat germ agglutinin*; PCs: Principal component 1 (PC1) and 2 (PC2)

increases of tyrosine phosphorylation in the sperm neck and principal piece.[27] Our values suggest that the sperm selection/capacitation process was adequate, because tyrosine phosphorylation increases in a time-related manner.[44]

One of the more popular indicators of sperm function is the detection of fragmented sperm DNA,[23] which impairs function and is a useful predictor of reproductive outcome.[45] The evaluation of DNA damage has also become a complementary test in recent clinical techniques such as magnet-activated cell sorting (MACS), developed additionally to eliminate apoptotic cells.[46] According to one study, DNA fragmentation rates increase significantly with incubation time, from 3.6% (0 h) to 6.2% (4 h), after swim-up sperm selection/capacitation.[47] However, our results showed that extending the selection/capacitation time up to 4 h made it possible to recover a sperm subpopulation with significantly lower DNA damage, from 14.0% in uncapacitated spermatozoa to 2.0% in 4 h capacitated cells. Our findings, thus, provide new insights into the potential of sperm selection time in a capacitating medium with DNA integrity.

The redistribution of glycoconjugates stands out as a major feature of sperm capacitation[10] and plays a prominent role in sperm–zona pellucida binding.[48] Nevertheless, to our knowledge, no information exists on the distribution of lectin-binding patterns during longer combined sperm selection/capacitation times. Our results revealed different sugar redistribution levels depending on the lectin used, as well as a high degree of heterogeneity in the patterns defined (P1–P5). In relation to the sugars recognized by the AAA lectin, a strong relocation in the acrosomal region was observed after 4 h of capacitation. This pattern was shown by approximately 60% of spermatozoa. Similarly, the sugars linked to the ConA lectin underwent a dramatic relocation in both the acrosomal and the postacrosomal region (45%) after a 4 h selection with a capacitating medium. ConA-binding sugar relocation acquires physiological importance, because the presence of those sugars in the postacrosomal region has been linked to proper sperm functionality.[49] For the PNA label pattern, the acrosomal region clearly appears as the principal residue position, regardless of the capacitation time, reaching almost 70% after 4 h. Regarding WGA lectin, the selection/capacitation process also influenced the positioning of its recognized sugar residues. In turn, we found redistribution from the acrosomal region, the most common pattern in the uncapacitated condition, to clusters after just 1 h of capacitation. The lack of differences in WGA pattern percentages at both capacitation times could be related to the WGA-specific sugar recognition, such as sialic acid, and removal during earlier capacitation stages, thus unmasking molecules involved in recognition.[50]

AAA, ConA, PNA, and WGA lectins recognize sugars involved in capacitation and zona pellucida recognition.[48] Therefore, differences in the location of glycocalyx residues during selection/capacitation could explain the lower capacity of spermatozoa to recognize and bind to the zona pellucida when incubated for a relatively short capacitation time (1 h).[16,51] We additionally identified strong lectin pattern correlations during 4 h capacitation, *e.g.*, P1 in AAA, P2 for ConA, P1 with PNA, and P4 for WGA. The role played by these sugar relocation patterns could be related to proper sperm function and adequate oocyte recognition. Functional tests such as the zona pellucida test would be necessary to confirm these findings.

## CONCLUSION

We found a functional sperm improvement after 4 h swim-up capacitation characterized by an increase in morphologically normal forms; a decrease in DNA fragmentation; a principal piece tyrosine phosphorylation increase; and a wide redistribution of glycocalyx sugars. Even though a shorter sperm preparation time may be more desirable in clinical routines, short-term sperm selection/capacitation may lead to fertilization failures in clinics owing to the lack of time-dependent events that orchestrate capacitation. In short, our data point at 4 h swim-up capacitation as an alternative to obtain a more homogeneous sperm subpopulation with a higher level of structural and functional differentiation. Four hours sperm swim-up capacitation has important potential implications for semen assessment in the future, both for basic research and assisted reproduction techniques in clinical practice.

## AUTHOR CONTRIBUTIONS

PSE, NHR, LRG, and MJGT performed the experiments and collected the data. PSE, AR, IV, and MJGT performed data analyses. PSE, MA, JA, AR, and MJGT conceived the experimental design. All authors contributed to drafting the manuscript. All authors read and approved the final manuscript.

## COMPETING INTERESTS

All authors declared no competing interests.

## ACKNOWLEDGMENTS

This research was funded by the Human Fertility Chair, the Department of Biotechnology of the University of Alicante (VIGROB-186), and the project of the Spanish Ministry of Economy and Competitiveness (AGL2015-70159-P).

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