

**SPERMATOZOA BOUND TO SOLID STATE HYALURONIC ACID SHOW
CHROMATIN STRUCTURE WITH HIGH DNA CHAIN INTEGRITY: AN ACRIDINE
ORANGE FLUORESCENCE STUDY.**

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Running Title: DNA in Hyaluronic Acid-selected sperm

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ABSTRACT

During human spermiogenesis, the elongated spermatids undergo a plasma membrane remodeling step which facilitates formation of the zona pellucida and hyaluronic acid (HA)-binding sites. Various biochemical sperm markers indicated that human sperm bound to HA exhibit attributes similar to that of zona pellucida-bound sperm, including minimal DNA fragmentation, normal shape, and low frequency of chromosomal aneuploidies. In this work, we tested the hypothesis that HA-bound sperm, would be enhanced in sperm of high DNA chain integrity and green acridin orange fluorescence (AOF) as compared to the original sperm in semen. Sperm DNA integrity in semen and in their respective HA-bound sperm fractions was studied in 50 men tested for fertility workup. In the semen samples the proportions of sperm with green AOF (high DNA integrity) and red AOF (DNA breaks) were $54.9\pm 2.0\%$ and $45.0\pm 1.9\%$, whereas in the HA-bound sperm fraction, the respective proportions were 99% and 1.0%. The data indeed demonstrated that HA show high degree of selectivity for sperm with high DNA integrity. These findings are important from the points of view of human sperm DNA integrity, sperm function, and the potential efficacy of hyaluronic acid mediated sperm selection for intracytoplasmic sperm injection.

Key words: acridin orange, DNA integrity, sperm maturity, sperm selection by hyaluronic acid,

INTRODUCTION

The structural integrity of sperm chromatin plays an important role in human fertilization and support of embryonic development. DNA chain breaks are known to adversely affect the paternal contribution of sperm to the zygote. The relationship between sperm paternal contribution and diminished DNA integrity was studied by various approaches, including acridine orange fluorescence (AOF, whether by flow cytometry or microscopy, Evenson et al. 1980; Tejada et al. 1984), the TUNEL assay (Borini et al, 2006), chromatin and protamine studies (Sati et al, 2004; Aoki et al, 2006), morphometry and fluorescence in situ hybridization, FISH (Celik-Ozenci et al, 2003, Jakab et al, 2005) and by various other methods (Aitken et al, 2003; Cayli et al, 2004;; Seli and Sakkas, 2005).

AOF distinguishes sperm with intact double stranded DNA (green AOF) or sperm with DNA strand breaks [red AOF, (Evenson et al, 1980)]. Subfertile men show increased levels of sperm with (Eggert-Kruse et al, 1996; Evenson et al. 1980; Hoshi et al, 1996; Liu and Baker, 1992b). Further, sperm DNA breaks affect the time to pregnancy, IVF fertilization and implantation rates, and increases in miscarriage rates (Lopes et al, 1998; Evenson et al, 1999; Larson et al, 2000; Aitken et al, 2003; Evenson and Wixon, 2006;).

In other lines of experiments directed to sperm development and fertility, an elevated sperm creatine phosphokinase content, reflecting surplus cytoplasm (Huszar et al, 1988a; Huszar et al, 1988b; Huszar and Vigue, 1993), as well as a low expression of the HspA2 chaperone was identified in spermatozoa of subfertile men, a consequence

of arrested sperm maturation, including cytoplasmic extrusion in spermiogenesis (Huszar et al, 2000; Cayli et al, 2003; Huszar et al, 2007; Sati et al, 2008).

Spermatozoa of arrested maturity with retained cytoplasm were deficient in zona-pellucida binding. This was demonstrated in sperm-hemizona (halved unfertilized human oocytes) complexes immunostained with CK, in which all bound spermatozoa were clear-headed without cytoplasmic retention (Huszar and Vigue, 1994; Huszar et al, 2000). This selectivity was attributed to a sperm plasma membrane remodeling step during spermiogenesis which facilitates the formation of the sperm zona pellucida binding site, as well as the binding sites for hyaluronic acid (Huszar and Vigue, 1990; Huszar and Vigue, 1994; Huszar et al, 1997).

Sperm of arrested maturation also exhibit increased rates of lipid peroxidation and consequential DNA fragmentation (Aitken et al, 1994; Huszar and Vigue, 1994; Aitken et al, 2003; Alvarez 2003). Increase in DNA chain fragmentation may also be related to inadequate delivery of DNA repair enzymes and to arrest in the histone-transition protein-protamine replacement sequence, a likely consequence of the decreased HspA2 chaperone activity (Allen et al, 1996; Eddy, 1999; Huszar et al, 2000; Carrell et al, 2007;). Indeed, a close relationship was established recently between HspA2 function and transition protein transport (Govin et al, 2006). However, mature sperm that are able to bind to HA and to zona pellucida are viable, show high DNA integrity and high proportion of sperm with normal Tygerberg morphology (Cayli et al, 2004; Huszar et al, 2003, 2009).

In earlier sperm DNA integrity studies with AO, chromatin structure of zona pellucida-bound spermatozoa was examined in sperm removed from zona pellucida of unfertilized

oocytes (Liu and Baker, 2007). The authors reported > 85% frequency of sperm with green AOF reflecting high DNA integrity. In the present study, we examined the hypothesis that HA-bound sperm will be enriched in sperm with high DNA integrity green AOF compared to their respective semen fractions.

METHODS AND MATERIALS

Experimental Design

The subjects for this HA-sperm selection/acridine orange study were husbands of infertile couples who were referred for semen analysis in the Sperm Physiology Laboratory, Department of Obstetrics, Gynecology and Reproductive Sciences, Yale University School of Medicine. Semen samples were obtained by masturbation after 2-4 days abstinence. All studies were approved by the Yale Human Investigation Committee.

Sperm preparation and HA sperm selection

We have studied semen of 50 men [sperm concentrations: $84.3 \pm 9.3 \times 10^6$ sperm/ml (min-max 11.2-284), sperm motility $46.1 \pm 3.4\%$ (4-86%)]. The mean proportion of sperm with green and red AOF was $54.9 \pm 2.0\%$ and $45.1 \pm 1.9\%$ in the 50 samples.

After liquefaction of the semen a routine semen analysis was carried out by CASA and, the sperm were washed by diluting the semen to 1:4 with HTF (Human Tubal Fluid, Irwin Scientific, CA), and centrifugation at 600xg for 10 minutes at room temperature. Further, the washed sperm were re-suspended in HTF media to a density of approximately 30 million sperm/mL HTF for the AO experiments.

HA-selection of mature sperm

For HA-selection of mature sperm, we utilized 5 cm. diameter IVF Falcon Petri dishes equipped with four 100 micron diameter solid state hyaluronic acid spots. (PICSI Sperm Selection Device, MidAtlantic Scientific, Mount Laurel, NJ, USA). An aliquot of the sperm suspension (10-20 μ l) was layered over the HA spot of the Petri dish. The sperm cells freely moved, until the mature sperm bound to the HA head-first (similar to sperm-zona pellucida binding (Huszar et al, 2003). The HA binding was observed by the lack of progressive motility and by the characteristic increase in tail cross-beat frequency of the bound sperm. Sperm lacking HA-binding capacity or HA-receptors, likely due to impaired/arrested spermiogenesis, have failed to “perceive” the HA and did not bind (Huszar et al, 1997 and 2003). Non-motile sperm and sperm without the characteristic higher tail beat frequency activity are not considered in the HA-bound sperm fraction. After a 15 minutes incubation/sperm binding period at room temperature, the unbound sperm were removed by slightly tilting the PICSI dish and applying HTF with a gentle drop-by-drop rinsing. As control for the sperm selection process, following the rinsing step another aliquot of the same sperm suspension (5-10 μ l) was smeared on the uncoated plastic areas between the HA-spots on same Petri dishes, and the HA-selected and unselected sperm were subjected to fixation and AO staining.

Technical aspects of sperm fixation for AO staining.

The usual fixative for sperm AO staining is the Carnoy solution which is composed of 3:1 ratio of methanol and glacial acetic acid. However, this solution is too corrosive for the HA spot used for selection of mature sperm. We have tried variations of the Carnoy solution which would fix the sperm, and also would allow the preservation of the integrity of the HA selection spots. The fixative that was satisfactory on both accounts is a 9:1

ratio of methanol and glacial acetic acid, that was used in the further experiments. In order to validate the fixation/AO staining results, we performed, in conjunction with each of the 50 experiments, a control study of unselected sperm suspension fixed on glass slides with both the Carnoy and 9:1 ratio methanol and glacial acetic acid solutions. The AO staining patterns following the two fixation methods showed excellent agreement ($r= 0.93$, Figure 1).

AO staining of the sperm fractions

The fixed Petri dish regions, including the HA spots with the selected sperm, and the areas between the spots with the unselected sperm suspension, as well as the glass slides of the fixation validation aliquots were stained with Polysciences Inc. acridine orange solution for 10 minutes. Subsequently, the Petri dishes and glass slides were gently rinsed, mounted with distilled water and cover slips were applied. The AOF patterns with the Carnoy or 9:1 methanol and glacial acetic acid solutions were similar (the proportion of sperm with green AOF; $r=0.97$, with red AOF $r=0.89$, respectively). We also compared 2 acridine orange solutions from Polysciences (Warrington, PA, USA). The first AO “lab reconstituted” staining solution (**#04539**) was prepared daily as follows: 1 ml of 1% AO stock solution was added to a mixture of 4 ml of 0.1 M citric acid and 0.25 ml of 0.3 M Na_2HPO_4 , pH 2.5. The 1% AO stock solution was stored in the dark for up to 4 weeks. The second AO solution (**#24603**) was “ready to use,” a much simpler and less work-intensive process, was utilized according to manufacturer, Polysciences Inc. directions.

Scoring of AO green and red sperm

The percentage of sperm with normal DNA was determined on each slide or Petri dish regions as follows: We selected 10 or more individual fields and evaluated a total of 200 sperm (40,000 sperm in 50 samples) under an Olympus BX51 fluorescence microscope with x400 magnification and excitation of 450-490nm. The observation of the fields did not exceed 40 seconds, in order to prevent fading. The spermatozoa were recorded as “green” or “red.” Orange or yellow heads, as well as those displaying green and red color simultaneously, were also considered “red” or sperm with fragmented DNA (Tejada et al, 1984). Each stained slide was evaluated freshly, immediately after staining.

Statistical analysis

A paired t-test was used to compare the percentages of green sperm after treatment with the lab-made vs. ready-to-use Polysciences fixatives. Unpaired t-tests were used to compare sperm concentrations, and green and red sperm percentages, between the lowest 10 and highest 10 sperm concentration samples. Correlation analysis was performed by the Pearson test. Analyses were conducted with the Sigma Stat program (Jandel Scientific, San Rafael, CA). A value of $p < 0.05$ was considered statistically significant. All data are expressed as mean \pm SEM.

RESULTS

Experimental Design

We have carried out three lines of experiments on each of the 50 samples. The first two studies addressed the validation of the methods regarding the fixative solution used and the type of the AO reagent, which is a very important element. Once we validated the methods, in the third study we focused upon the actual AO staining of unselected spermatozoa and HA-selected spermatozoa.

In **Experiment 1**, directed to the fixation method, sperm aliquots were fixed to glass slides with both Carnoy fixation solution (3:1 methanol:glacial acetic acid) and 9:1 methanol and glacial acetic acid solution, and the proportion of sperm with green or red AOF (acridin orange fluorescence) were assessed, in order to assure that the two fixatives give comparable results. In **Experiment 2** the two Polysciences Inc. AO reagents were procured (lab-reconstituted AO and “ready to use” AO) and compared for further validation of the methods. In **Experiment 3**, Falcon Petri dishes with HA-spots were used for sperm selection. Further, the proportions of sperm with green and red AOF, that were fixed and stained in an identical manner, were compared within the HA-selection spots and outside “control” sperm fractions.

Another important consideration was related to the study samples. The primary goal was to have sufficient sperm within the un-selected and HA-selected fractions to provide populations for a convincing evaluation of the green and red AOF pattern. Thus, considering the likely attrition due to manipulation at the steps of sperm binding incubation, the rinsing off the un-bound sperm, the fixation and AO treatments and the washing steps, as indicated in Methods and Materials, the starting sperm suspensions

were re-suspended to an approximately 30 million/per ml. sperm concentrations. However, the studies are relevant for all constituent sperm, as the membrane remodeling and HA-binding properties of individual spermatozoa are independent from the other motile sperm within the samples (Huszar et al, 2007).

Development of the AO fixative for the HA-studies (Experiment 1)

The first series of experiments were aimed at the development of a sperm fixative solution that is comparable to the Carnoy solution (3:1 methanol and glacial acetic acid), but does not affect the integrity of the solid state HA coating on the Petri dish. The use of Carnoy-like solution with 9:1 methanol and glacial acetic acid satisfied these requirements. In order to validate the substitution, from each of the 50 study samples two sperm aliquots were applied to glass slides, and following fixation with both the Carnoy and 9:1 solutions, the sperm were stained with the AO reagents, and the fluorescence staining patterns were compared. The results were very similar (Carnoy vs. 9:1 methanol:glacial acetic acid: green sperm: $45.7 \pm 2.1\%$ vs. $45.0 \pm 2.0\%$, $r = 0.93$, $p < 0.01$ Fig.1; red sperm: 46.1 ± 2.3 vs. $41.6 \pm 2.1\%$; $r = 0.85$, respectively, $p < 0.01$, $N = 50$ pairs).

Reproducibility of the AO stains (Experiment 2)

In the second set of experiments, the reproducibility of AOF patterns by the lab-reconstituted and ready to use AO solutions from Polysciences Inc. was tested. The two stains provided very similar results in the proportions of sperm with green and red AOF whether using either the Carnoy or the 9:1 methanol:glacial acetic acid solutions. The data also indicated close similarity between the ready to use and lab-made Polyscience Inc. reagents in the proportions of AO stained sperm [green sperm with Carnoy:

54.7±2.2% vs. 54.7±2.1% (lab-reconstituted and ready to use); green sperm with 9:1 solution: 57.6±2.1% and 54.9±2.0% (lab-reconstituted and ready to use). Thus, we used the ready made Polysciences Inc. reagent in the experiments which facilitated well the execution of studies yet was less work-intensive.

Proportion of sperm with green and red AOF in the HA-selected and semen sperm fractions (Experiment 3)

The comparison of sperm with green and red AOF patterns indicated that in the unselected semen sperm fractions placed on the “outside” (uncoated region) of the Petri dishes were comparable to the AOF patterns in the original semen aliquots. However, in the regions of the HA spot where the “HA-selected sperm” was bound (and the unbound sperm were gently rinsed off), the proportion of sperm with green AOF was in the > 99% range (Table 1). This indicated that (deleted) HA-binding promotes the selection of sperm with high DNA chain integrity (Figure 2).

Considering the overall semen parameters, there was no correlation between proportion of sperm with green AOF and sperm concentrations. However, there were moderate correlations between green AOF sperm and sperm motility or total motile sperm ($r = 0.45$, $p < 0.01$ and $r = 0.36$, $p < 0.01$, respectively).

Due to the known association in semen samples between oligozoospermia and sperm with arrested maturity, we expected that in the low sperm concentration samples there will be a higher representation of red AOF sperm. We addressed this question by comparing the proportion of green and red AOF sperm in samples with the 10 highest and 10 lowest sperm concentrations of the 50 men (Table 2). The sperm concentrations in the two groups showed an approximately 8-fold difference. The proportion of the

green fluorescing sperm was higher in the group with higher sperm concentration. However, the differences in green red AOF proportions were nearly not as dramatic. Further, it is of note that the proportion of sperm was 99% with green AOF in the HA-bound fraction of both concentration groups.

DISCUSSION

Sperm DNA integrity is an important factor in the paternal contribution of human sperm to the fertilization process and the maintenance of pregnancy (Aitken et al, 2009). Regarding DNA integrity, zona pellucida-bound sperm contain a high proportion of sperm with green AOF (Liu and Baker, 2007). In the present study, we have examined the hypothesis that, (deleted) the HA-bound sperm fraction will be enriched in sperm with high DNA integrity and green AOF. Indeed, in the 50 experiments there was an almost exclusive presence (> 99%) of sperm with green AOF and high DNA integrity in HA-selected bound sperm. (Figure 2)

In preparing for the studies, we had to address the vulnerability of the HA coating to the corrosive effects of Carnoy fixative. An alternative fixative (the 9:1 methanol and glacial acetic acid solution) has satisfied the requirements of sperm fixation and preservation of the HA spot. Further, the 9:1 methanol and glacial acetic acid solution was validated with an >90% correlation by the proportion of sperm with green and red AOF, using both fixatives on glass slides (Figure 1). The staining methods were also validated by the comparable proportions of sperm with green and red AOF using either of the two AO stains. In these comparative studies that were aimed at HA-selected and unselected

spermatozoa of common origin, treated and examined identically, the individual microscopic observation facilitated the comparative studies appropriately and efficiently.

With respect to semen parameters and AOF staining, there are three points of interest:

(a) In semen samples with lower sperm concentrations there was a higher proportion of sperm with DNA breaks, most likely due to the known relationship between reduced sperm production, sperm development and increased DNA fragmentation. (b)

Accordingly, there was a higher proportion of green AOF sperm in samples with the 10 highest vs. the 10 lowest sperm concentrations (Table 2). However, in the HA-bound sperm fractions both showed >99% frequency of green AOF sperm. Thus, selection power of HA is related to the proportion of individual mature spermatozoa in the individual semen. In previous experiments, we have already demonstrated that HA-binding properties of individual spermatozoa are independent from other constituent sperm in the samples (Huszar et al, 2007). For instance, the HA-bound sperm fractions of normozoospermic and individually selected sperm from ICSI-range oligozoospermic samples showed similar frequencies of chromosomal aneuploidy, regardless the initial sperm concentrations or aneuploidy frequencies (Jakab et al, 2005).

(c) There was a moderate correlation ($r=0.45$, $p<0.01$) between the proportion of sperm with green AOF and sperm motility. In earlier sperm chromatin structure assay studies, a similar relationship was found ($r=-0.53$, Giwercman et al, 2003).

The similarity in sperm selection power by the zona pellucida and HA may be explained by the related formation of their receptors via the plasma membrane remodeling in spermiogenesis (Huszar et al, 2007; Huszar et al, 1998; Huszar and Vigue, 1994).

Further confirmation of this relationship was supported by the similar degree of

enrichment in sperm with Tygerberg normal morphology in the zona pellucida and HA-bound sperm fractions vs. their respective semen sperm fraction [approximately 4.0x and 3.8-times (Menkveld et al, 1996; Prinosilova et al, 2009)]. The focus of the present work was the DNA integrity of the HA-bound sperm fractions. Sperm morphology was not a goal because of the above very recent 2009 study. Another consideration was the harsh fixation method and the red/green fluorescence of AO staining that would have made sperm shape and morphology unreliable.

The present work independently confirms the notion that the sperm selection attributes of HA and of zona pellucida (as reported by Liu and Baker, 2007) are similar in selecting sperm with high DNA integrity. This is important information, as lack of ZP-binding is a major cause of IVF fertilization failure (Angelopoulos et al, 1998; Claassens et al, 1992; Liu and Baker, 1992a; Liu and Baker, 2007; Menkveld et al, 1996).

We have established earlier that HA-selected spermatozoa do not exhibit attributes of arrested development, such as cytoplasmic retention, persistent histones, high levels of the apoptotic caspase 3 or DNA fragmentation (Huszar et al, 2003; Cayli et al, 2004). Moreover, individual sperm probed with multiple biochemical markers indicated an approximately 70% agreement between the various cytoplasmic and nuclear markers of normal and arrested development (Sati et al, 2008; Huszar et al, 2003) The present study, which is based on direct examination of single spermatozoa bound to HA, further extends the relationship between sperm cellular maturity, DNA integrity, and the selection role of HA for spermatozoa with attributes promoting fertilization potential and paternal contributions of sperm.

In addition to demonstration of an enhancement in DNA integrity in the HA-selected and zona pellucida-bound sperm (Liu and Baker, 2007), this study indicates that semen testing with the sperm-HA binding assay does reflect the proportion of sperm with high DNA integrity. Also, the almost exclusive presence of sperm with green AO fluorescence in the HA-bound sperm fraction further supports the potential advantages of HA-mediated sperm selection for ICSI treatment with sperm that would have been also selected by the zona pellucida during natural or conventional IVF fertilization (Gergely et al, 1999; Celik-Ozenci et al, 2004; Huszar et al, 2007; Huszar and Vigue, 1994; Nasr-Esfahani et al, 2008; Parmegiani et al, 2009).

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Figure Legends

Figure 1: Correlation between the proportions of sperm with green AOF using the Carnoy and 9:1 methanol and glacial acetic acid solutions as fixatives.

Figure 2: Acridine orange stained sperm in the area of the HA-selection spot, and in the outside control area of the Petri dish. Please note the very high proportion of sperm with green AOF within the HA-selected sperm fraction. The higher density of sperm around the perimeter is due to an HA-ridge, which is related to the application procedure tool used for the HA-coating.

Summary sentence

Binding to solid state hyaluronic acid selects spermatozoa with high DNA chain integrity, as detected by acridine orange fluorescence.

Table 1: Summary of the 50 HA-sperm selection experiments

Variables	n	mean±SD
Green sperm in semen (%)	50	54.9 ± 2.0
Green sperm (% , Petri outside control)	50	56.1 ± 1.9
Green sperm (% , Petri HA spot)	50	99.1 ± 0.2
Red sperm in semen (%)	50	45.0 ± 1.9
Red sperm (% , Petri outside control)	50	43.9 ± 1.9
Red sperm (% , Petri HA spot)	50	0.9 ± 1.9

Table 2: Comparison of the 10 samples with highest and lowest sperm concentrations

	Lowest 10 samples	Highest 10 samples	p
Concentration (sperm x 10 ⁶)	22.8±3.4	187.1±17.1	P<0.001
Green sperm %	52.1±2.9	65.4±3.6	P<0.05
Red sperm %	47.9±2.9	34.6±3.6	P<0.05

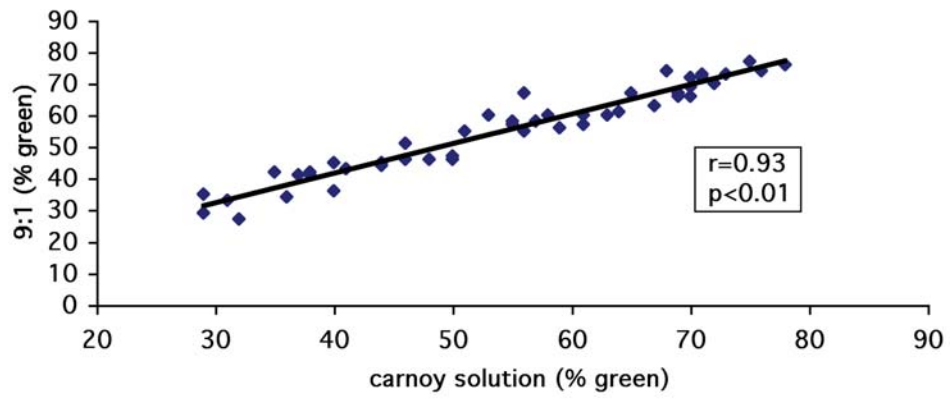


Figure 1. Correlation of sperm AOF patterns using the two fixatives.

