Hyaluronic acid substantially increases the retention of motility in cryopreserved/thawed human spermatozoa

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We have demonstrated previously that hyaluronic acid (HA) improves the velocity and the retention of motility in freshly ejaculated human spermatozoa. In the present work, we examined the effect of HA on cryopreserved/thawed spermatozoa in four paradigms: (i) effect of HA on sperm motility and velocity in semen; (ii) stabilizing effect of HA after 4 h of incubation when the decline of sperm motility is already detectable; (iii) the duration of improved motility after the separation of spermatozoa from HA by Percoll gradient centrifugation; and (iv) motility of sperm cryopreserved in the presence of HA. HA improved the retention of sperm motility in thawed spermatozoa. Indeed, the motility values after 30 h were ~100% higher in the HA compared with the control samples. This effect of HA was also evident in the stabilization of spermatozoa with already declining motility. After removal of the HA from the incubation medium, significantly increased motility in the HA-exposed spermatozoa was still detectable for at least 4 h. Cryopreservation of spermatozoa in the presence of HA did not improve the recovery of motility. The data indicate that HA improves the retention of motility of cryopreserved/thawed spermatozoa, even after the removal of HA from the incubation medium. The utilization of HA will probably prove beneficial in assisted reproduction: in intrauterine insemination and in in-vitro fertilization (IVF), the extended sperm motility and velocity will enhance the fertilizing efficiency; in intracytoplasmic sperm injection (ICSI), the improved motility will facilitate the identification of viable spermatozoa. Because HA is a physiological component of the cumulus and of the female and male reproductive tracts, administration of HA should not cause ethical concerns.

Key words: assisted reproduction/cell locomotion/cumulus/fertility/hyaluronan

Introduction

Sperm cryopreservation has become an increasingly important addition to the methods of assisted reproduction in the past decade. It is utilized for sperm preservation by men prior to chemo-, or radiotherapy and vasectomy, as well as by couples in which the husband is away from home or unable to produce semen samples under time pressure for assisted reproduction procedures. Sperm cryopreservation is also of primary importance in facilitating the quarantine procedures for donor semen for the enhancement of safety in light of the human immuno-deficiency virus (HIV) threat. In cryopreserved/thawed semen samples, the recovery of sperm motility is between 25–50% of that of the prefreezing value, and there are substantial variations between individuals (Critser et al., 1987a; Keel and Webster, 1987; Kolon et al., 1992, 1995). In addition to the lower recovery of motility, there are differing opinions on whether spermatozoa in cryopreserved semen also show an abbreviated retention of motility and viability after thawing. For instance, in one study the investigators have found a diminished maintenance of sperm motility and viability (Keel and Webster, 1987), while in another laboratory, after the removal of the cryopreservation medium with Percoll centrifugation, the investigators did not find a difference in spermatozoa survival between fresh and cryopreserved/thawed samples (Le Lannou, 1991). In a third study, the authors report that the decline of sperm motility in the post-thaw sample was not different from that of fresh semen, but the ability of the spermatozoa to penetrate zona-free hamster oocytes diminished quickly, most likely due to loss of acrosomal integrity (Critser et al., 1987a,b).

The reasons underlying the decline are not well understood. The most likely factors are the (i) diminished integrity of the sperm membrane due to the freezing and thawing process and cleavage by the proteolytic enzymes of the seminal fluid, and (ii) cryodamage of membranes among the intracellular compartments of spermatozoa, which affects energy synthesis and utilization, and causes a premature exhaustion of the sperm ATP pool. As a consequence of the membrane damage and diminished motility, the efficacy of cryopreserved/thawed spermatozoa in causing pregnancy on a per cycle basis is thought to be lower compared to that of fresh semen (Smith et al., 1981; Richter et al., 1984; Hammond et al., 1986; Brown et al., 1988; reviewed in Le Lannou et al., 1995). However, there is no universal agreement on this issue either, because others reported no difference between the fecundability of spermatozoa in fresh versus cryopreserved/thawed samples (Bordson et al. 1986). In any case, in order to increase the efficacy of cryopreserved/thawed spermatozoa and maximize the chances for pregnancy, a direct placement of spermatozoa in the uterus and two inseminations around the time of ovulation are applied in clinical practice (Huszar and DeCherney, 1987; Byrd et al., 1990, Hurd et al., 1993).

There is major interest in extending the motile lifetime of spermatozoa. Several motility enhancing agents have been tried
thus far, including the phosphodiesterase inhibitors caffeine, pentoxifylline, theophylline, various bioactive peptides and 2-deoxyadenosine (Aitken et al., 1986; Hammitt et al., 1989; Yovich, 1993; Lanzafame et al., 1994; McKinney et al., 1994; Kolon et al., 1995). In different approaches, we added to fresh semen the energy source creatine phosphate in combination with calcium, as well as proteoglycans isolated from human follicular fluid. Both creatine kinase–calcium (as measured by verapamil sensitivity), and a large proteoglycan improved the retention of sperm motility and velocity (Fakih et al., 1986; Eriksen et al., 1994). However, until now none of the enhancing agents has been reported to cause a sustained improvement of motility in cryopreserved/thawed spermatozoa after their removal from the incubation medium.

Another line of research directed towards an increased retention of sperm motility and velocity was initiated in our laboratory with hyaluronic acid (HA; Huszar et al., 1990). HA, or as it is now called, hyaluronan, is a linear polysaccharide which is a mediator of cell locomotion (Torvard, 1987; Turley, 1992). HA also occurs physiologically in the reproductive system in cervical mucus, the cumulus and the follicular and seminal fluids (Eppig, 1979; Grimek et al., 1984; Lee et al., 1985). In previous studies we tested the effects of HA on fresh semen. Within 3 h HA caused a 26% increase in sperm velocity, and an improved retention of sperm motility and velocity in long-term incubations of normozoospermic and oligozoospermic semen samples. We suggested that the motility enhancement in spermatozoa was initiated by a direct action of HA on sperm membrane receptors which modulate either the regulation of sperm motility or the energy synthesis and the ATP pool (Huszar et al., 1990). Indeed, the presence of HA receptors in spermatozoa has been recently confirmed by two laboratories (Kornovsky et al., 1994; Ranganathan et al., 1994). The effects of HA on human spermatozoa were also explored in other clinical and research applications (Mortimer et al., 1990; Karlstrom et al., 1991; Neuwinger et al., 1991; Slotte et al., 1993). In a very recent report an HA mediated increase in acrosomal activation was demonstrated in monkey spermatozoa (Vandevoort et al., 1997).

Following up on our finding that HA causes an increased retention of sperm motility and velocity in fresh semen, in the present study we investigated whether HA would extend the motile lifetime of cryopreserved/thawed spermatozoa. The study design is based on four experimental paradigms: (i) retention of sperm motility in the presence of HA; (ii) the stabilizing effect of HA in thawed semen in which the addition of HA occurred after a 4 h incubation, during which sperm motility was declining; (iii) the duration of the improved sperm motility after the removal of HA by Percoll centrifugation; and (iv) retention of motility in semen samples cryopreserved in the presence of HA or with previously frozen HA.

Materials and methods

Sperm preparation

The sperm samples used in the studies were cryopreserved for a period of 6-24 months. After rapid thawing at 37°C, the spermatozoa were washed twice in human tubal fluid (HTF; Irvine Scientific, Co., Irvine, CA, USA) containing 0.3% bovine serum albumin, 0.5 mg/ml streptomycin, 1 mg/ml erythromycin lactobionate and 15 mM sodium bicarbonate (pH 7.2), at 500 g for 15 min. The pellets were resuspended in 1.0 ml HTF. Each sample was divided into four aliquots: two controls and two study samples of 0.25 ml each. To all study samples 50 µl sodium hyaluronate solution (Pharmacia AB, Uppsala, Sweden; Select Medical Systems, Inc., Williston, VT, USA) was added to a final concentration of 0.25 mg/ml HA. The control semen samples were respectively adjusted to the same volume with 50 µl HTF. The incubations were carried out during a 2-day long experimental period.

Experimental design

Experiment 1

In the experiments focusing upon the effects of HA on sperm motility and velocity (15 samples from 14 donors), HA or HTF (control) was added to aliquots of cryopreserved/thawed/washed spermatozoa. The sperm samples were incubated at 37°C and were evaluated at seven time points, time 0, 30 min, 1, 4, 10, 24 and 30 h, by computer-assisted semen analysis (CASA).

Experiment 2

In the second set of experiments, a possible ‘stabilizing’ effect of HA was examined (10 samples from 10 donors). In these studies, 50 µl of HA (study) or 50 µl of HTF (control) were added to samples in which the sperm motility was already declining after incubation for 4 h in HTF. CASA analyses were carried out at seven time points, time 0, 30 min, 1 and 4 h (when the HA was added). Following the addition of HA, the samples were further analysed at 6, 20 and 26 h (total observation times 10 h, 24 h and 30 h).

Experiment 3

In the third experiment HA was added immediately to the thawed washed samples (eight samples from eight donors). Samples were then incubated for 4 h (analysed at 0, 1 and 4 h). After 4 h, the HA was removed by separating the spermatozoa from the medium containing the HA by centrifugation through 40/80% discontinuous Percoll gradients (Huszar and Vigue, 1993), and by further HTF washing of the sperm pellet at 500 g for 5 min. The control samples were treated similarly. The experimental and control sperm samples were then incubated further, and CASA analyses were carried out at time 0, 1, 4, 10 and 24 h following the recovery of spermatozoa from the Percoll pellets (eight time points in all).

Experiment 4

Semen samples combined with cryopreservation medium were frozen in the presence of 0.25 mg/ml HA or were exposed to previously frozen HA (six samples from six donors). Sperm motility was evaluated at time 0, 1, 4 and 8 h.

CASA analysis

At each of the time points, five to 10 fields from each of two drops of the two HA and control semen aliquots (50–60 fields in the eight drops) were videotaped and analysed using a 10 µm deep Makler chamber (Zygote Systems, Inc., Springfield, MA, USA) and an Olympus microscope (Olympus, Lake Success, NY, USA) equipped with a phase contrast objective. The CASA system parameters (CASA, HTM-IVOS version 10.5, Hamilton-Thorne Research, Beverly, MA, USA) were as follows: 15 frames at 30 frames/s, threshold velocity 8 µm/s and maximum velocity 150 µm/s. We monitored sperm concentration, motility (%), curvilinearity velocity, linearity, mean amplitude of lateral head displacement and beat/cross frequency. All references to sperm velocity refer to curvilinear velocity.

Statistical evaluation

Data analysis was carried out with Statview, using the one-way repeated measures analysis of variance (ANOVA) or Friedman
HA improves motility of cryopreserved spermatozoa

Figure 1. Retention of motility in the hyaluronic acid (HA) (0.25 mg/ml) and control samples during the 30 h incubation period (mean ± SEM). Asterisks show significant differences: (*) P < 0.05, (**) P < 0.01, (***) P < 0.001.

Figure 3. Stabilizing effect of hyaluronic acid (HA). Thawed samples were preincubated for 4 h. After the preincubation, the samples were divided and 0.25 mg/ml HA was added to the HA samples and human tubal fluid (HTF) to the control aliquots. The HA and control aliquots were further incubated for another 26 h as shown (mean ± SEM). Asterisks show significant differences: (**) P < 0.01, (***) P < 0.001.

Figure 2. Retention of velocity during the 30 h incubation period. Values are mean ± SEM.

Results

Experiment 1: retention of motility in the presence of HA

As previously with the fresh semen (Huszar et al., 1990), in the cryopreserved/thawed semen samples the retention of sperm motility was increased in the presence of HA and significant differences were detectable after 30 min of incubation (Figure 1). The retention of motility followed an almost straight line in the HA samples throughout the 30 h observation period, whereas there was a continuous decrease of sperm motility in the control samples. Sperm velocity, which showed an immediate increase in response to HA in fresh semen, did not exhibit such a pattern in the HA compared with the control samples of the cryopreserved/thawed spermatozoa, as shown by the lack of significant differences (Figure 2). However, the velocity index (%motility×velocity), which reflects the efficiency of potential sperm-oocyte encounters, was 263% higher in the HA compared with the control samples after the 30 h incubation period (12.9 versus 4.9, P < 0.001). There were no differences in linearity, lateral head displacement and beat/cross frequency in any of the experiments (data not shown). In experiment 1, as well as in experiments 2 and 3 there was a significant effect of treatment over time (repeated measures ANOVA, P < 0.001)

Experiment 2: the stabilizing effect of HA

In this experiment, HA or HTF was added to the study and control sperm samples, respectively after 4 h incubation period, when the decline in sperm motility was already perceptible. In this group the initial drop of motility was not as sharp as expected because two samples remained steady and another showed a slight increase in motility at the 1 h time point. However, during hours 4–30 of the experiments, sperm motility in the HA samples was maintained at an almost even level, whereas in the control samples sperm motility continued to decrease. The patterns of motility decline in the HA and control samples clearly indicated that the effects of HA on the retention of sperm motility were similar to those in freshly thawed spermatozoa (Figure 3). Thus, HA stabilized sperm motility in the thawed samples even after the initiation of motility decline immediately post-thaw. As in the first series of experiments (Figure 2), there were no differences in sperm velocity (data not shown). For instance, the velocities in the HA and control samples after 1, 10 and 24 h incubations were: 36 ± 4 and 39 ± 4, 33 ± 3 and 32 ± 3 and 35 ± 3 and 30 ± 3 µm/s respectively.

Experiment 3: sperm motility after the separation of sperm and HA

In these experiments, the duration of the improved sperm motility after the removal of HA from the incubation medium
was investigated. In order to study this, (i) semen samples were incubated in the presence of HA for 4 h as in the first experiment; (ii) after observing the increased retention of sperm motility (P < 0.001, Figure 4) HA exposed and control samples were centrifuged through a 40/80% discontinuous Percoll gradient; (iii) the sperm fractions that originated in the 80% Percoll pellets of the HA-treated and control semen were resuspended in HTF and observed for another 24 h. In the spermatozoa recovered from the pellet of the HA samples, the motility was ~30% higher after 1 h and 10% higher after 4 h (P < 0.001 and 0.05 respectively) than that of the corresponding control sperm fractions. Subsequently, the decline of motility followed a similar rate in the two sperm fractions (Figure 4; motility was not tested between 4 and 10 h time points). This indicated that after the removal of HA from the incubation medium, significantly higher sperm motility was maintained for at least 4 h. There was no HA effect on sperm velocity (data not shown). The velocity values in the HA and control samples after the initial 4 h, post-Percoll 4 h and 24 h incubations were: 40 ± 2 and 37 ± 3, 44 ± 2 and 41 ± 3 and 42 ± 2 and 42 ± 3 μm/s respectively.

**Experiment 4: sperm cryopreservation in the presence of HA**

Spermatozoa cryopreserved in the presence of HA, or addition of previously frozen HA to thawed semen, showed no increased retention of sperm motility or velocity (data not shown). This was consistent with the finding that exposure to sub-freezing temperatures causes deterioration of the HA structure (Torvard, 1987)

**Discussion**

It is becoming increasingly accepted that cryopreserved and thawed spermatozoa have diminished motility and viability compared to that of freshly ejaculated spermatozoa. For this reason, intrauterine insemination (IUI) treatment of women with cryopreserved versus fresh semen is less effective in terms of per cycle pregnancy rates (Smith et al., 1981; Richter et al., 1984; Hammond et al., 1986; Critser et al., 1987a,b; Keel and Webster, 1987; Brown et al., 1988; Byrd et al., 1990; Le Lannou et al., 1995). In several studies, various pharmacological agents and bioactive peptides have been employed to improve and extend the motility of cryopreserved/thawed spermatozoa, but most papers indicate only an inconsistent or short-term motility improvement or maintenance (Hammit et al., 1989; Lanzafame et al., 1994).

Previously, in fresh semen, we found that HA, which is present in the cumulus and in the female and male reproductive tracts, caused an initial increase in sperm velocity and a higher retention of sperm motility and velocity in long-term incubation (Huszar and Vigue, 1990). In the present study, we examined whether HA also enhances the motility of cryopreserved/thawed spermatozoa. We anticipated that HA would affect sperm motility rather than viability, because using the combined methods of supravital staining and hypo-osmotic swelling in probing membrane integrity in the head and tail of fresh spermatozoa, we found that the increased retention of motility and velocity, in response to HA, was not associated with or caused by a membrane protective effect of HA (Huszar et al., 1990).

When we added HA to cryopreserved spermatozoa immediately after thawing, there was a significant increase in the retention of sperm motility within 30 min. Indeed, after 30 h the motility in the thawed samples with HA was nearly double that of the control samples. The velocity index, which reflects the fertilizing efficiency of the sample and is a good measure of sperm functional integrity (Keel et al., 1987), was also more than twice as high. The benefits of HA in the clinical setting are further supported by a retrospective study of 1147 IUI cycles with cryopreserved/thawed donor spermatozoa, in which the most significant predictors of pregnancies were sperm velocity and the number of motile spermatozoa inseminated (Marshburn et al., 1992). Along with other investigators (Keel et al., 1987; Kolon et al., 1992), we have also noticed a substantial variation in the pattern of post-thaw motility in the maintenance of post-thaw motility. An example of this is the short-term motility differences between the samples of experiments 1 and 2 (Figure 1 versus 3), which in the subsequent 30 h showed a consistent pattern of decline.

Contrary to our experience in fresh semen, in the cryopreserved/thawed samples, sperm velocity showed no improvement in response to HA in any of the experimental paradigms. Thus, the pathway promoting the HA-mediated velocity response is not operational after cryopreservation. In order to determine whether the motility enhancement by HA would be present only immediately after thawing, or if it is possible to stabilize spermatozoa which are already declining in motility, we added HA to spermatozoa that were incubated for 4 h following the thaw. Upon the addition of HA, sperm motility stabilized and remained at significantly higher levels for the following 26 h, compared to the declining motility in the control samples (Figure 3).
To examine further whether the continuous presence of HA is required to extend the maintenance of sperm motility values, after 4 h of incubation we separated the HA from the spermatozoon by centrifugation through a 40/80% discontinuous Percoll gradient. The previously HA exposed spermatozoa retained the significantly enhanced motility for at least another 4 h, but even after 10 h there was a detectable difference (Figure 4). The fact that the increased motility was retained after the removal of HA for an extended period is consistent with the idea that HA acts via a membrane HA receptor, and that the effect is likely to be linked to the lifetime of the receptor–HA complex (Huszar et al., 1990). Indeed, blocking of the HA-receptor with an antibody diminishes sperm motility, velocity and cross-beat frequency (Kornovsky et al., 1994).

At present, the mechanism of motility increase in response to HA is unclear. We assume that it is related to increased ATP level and improved flagellar function. This is supported by our previous studies, in which (i) the energy source creatine phosphate caused an increased retention of sperm motility, (ii) sperm velocity was directly related to the ATP/ADP ratio in spermatozoa (Fakh et al., 1986; Vigue et al., 1992), and (iii) sperm velocity was unrelated to sperm maturity, which in immature sperm fractions was detected by the increased sperm creatine kinase activity, which reflects the incomplete cytoplasmic shedding during spermogenesis. The retained cytoplasm is apparently metabolically inert, because in spite of the increased creatine kinase content in the immature sperm fractions the ATP/ADP ratios were not elevated (Huszar and Vigue, 1990, 1993; Vigue et al., 1992). The ATP pool concept may also explain why the protracted increase in long-term HA-mediated motility is common in both fresh and cryopreserved spermatozoa, whereas the quickly occurring velocity enhancement found in fresh spermatozoa (Huszar et al., 1990) is absent in cryopreserved spermatozoa. It may be relevant that the area with the highest density of HA receptors is the mid-piece, where the mitochondrial energy generating system is located (Kornovsky et al., 1994).

The improved retention of motility with HA favourably compares with the effects of the nine best studied motility enhancers in cryopreserved/thawed human spermatozoa (Hammit et al., 1989). Within a 60 min observation period, there was a consistent improvement of motility only with caffeine, pentoxifylline and 2-deoxyadenosine. Pentoxifylline appears to be the most efficient in fresh spermatozoa, since in most studies it was reported to improve and extend sperm motility and velocity (Mbizvo et al., 1993; Lanzafame et al., 1994). However, following the washing of the spermatozoa and removal of these three agents, none of the agents caused a continued motility stimulatory effect (Hammit et al., 1989). In the experiments by Hammit et al. (1989), the pentoxifylline-treated sperm fractions, in spite of the lack of extended motility stimulation, showed improved zona-free hamster oocyte penetration (SPA) rates after 24 h. This suggests that the increased SPA and in-vitro fertilization (IVF) rates reported with pentoxifylline (Yovich, 1993; Lanzafame et al., 1994) may not be exclusively related to motility improvement, but also to the increased cAMP levels that occur in response to phosphodies-}

**References**


Eppig, J.J. (1979) FSH stimulates hyaluronic acid synthesis by oocyte–terase inhibition and facilitate acrosomal activation (Tesarik et al., 1992; Gearon et al., 1994).

In comparing the efficiency of HA and other motility enhancers in cryopreserved sperm, an extended stimulation was reported in two studies (McKinney et al., 1994; Kolon, 1995), but in other studies, after the removal of pentoxifylline, unlike in the present HA studies with residual increased sperm motility, there was no further enhancement (Lanzafame et al., 1994). With respect to safety, there are concerns that, in spite of sperm washing, in cryopreserved/thawed spermatozoa the plasma membrane defects developing due to the freezing and thawing may provide binding sites for the transport of pentoxifylline into the oocyte. Such concern is not applicable to HA, which is present during sperm–oocyte interaction under physiological conditions.

In summary, we found that (i) in the presence of 0.25 mg/ml HA, sperm motility was maintained almost unchanged during the 30 h observation time. Thus, it appears to have a substantial advantage over other sperm motility enhancers, which have a temporary effect; (ii) HA caused a stabilizing effect and motility enhancement in sperm fractions that had already showed declining sperm motility; (iii) unlike the other motility enhancers, some of which cause short-term maintenance of improvement after removal of the agent, HA provided a significant motility increase for at least 4 h after the Percoll purification of spermatozoa. HA preserves motility in spermatozoa and will be useful in assisted reproduction in utilizing thawed semen samples: in intratruterine insemination and IVF, the extended motility will increase fertilizing efficiency, while in intracytoplasmic sperm injection (ICSI) the improved sperm motility will enhance the selection of viable spermatozoa. Because HA is a physiological component of both the male and female reproductive tracts, the use of HA in sperm preparation for assisted reproduction should not represent an ethical dilemma.

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