

Effects of hyaluronate and immediate motile sperm isolateion on the seminal fertility parameters*

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We aimed to clarify the effects of hyaluronic acid (HYA), and immediate isolateion of motile spermatozoa from nonfertile ones, on the male fertility indices. In 22 normozoospermic and 12 asthenooligozoospermic samples, sperm preparations with swim-up in HYA (SwUp in HYA) swim-up/centrifugation (SwUp/Cent) and conventional centrifugation/swim-up (Cent/SwUp) methods were compared. Motility parameters in normozoospermic samples treated by SwUp in HYA, SwUp/Cent and Cent/SwUp were as follows, respectively (meantSE): % motility 31.05±6.02, 57.95±6.3 and 61.77±6.83; %progressive motility 15.09±3.53, 43.91±5.46 and 42.32±5.82; sperm velocity 26.91±3.05, 42.77±2.74 and 39.68±2.52 pm/s. The same parameters in oligozoospermic samples treated by SwUp in HYA, SwUp/Cent and Cent/SwUp were as follows, respectively: % motility 6.75±2.71, 16.92±1.88 and 37.42±4.74; %progressivemotility 3.67±2.14, 15.25±1.95 and 21.08±4.50; sperm velocity 12.42±3.8, 40.83±2.64 and 35.33±1.67 um/s.

We conclude that 1) in preparation of normozoospermic samples for in vitro fertilization (IVF), SwUp/Cent procedure, which is significantly superior to the other two in terms of sperm velocity, can be preferred, 2) in preparing oligozoospermic samples for IVF, Cent/SwUp method, which is significantly superior to the other two in terms of % motility, can be preferred, and 3) hyaluronate fails to improve sperm fertility indices, and hence it might not be safe, from a toxicological point of view, to use HYA in sperm preparation. [Turk J Med Res 1994, 12(5): 200-205]

Key Words: Spermatozoa, Hyaluronic acid, Sperm motility

Reactive oxygen species (ROS), which are produced in certain nonfertile fractions of the sperm population, damage fertile spermatozoa by peroxidation of membrane lipids, and spermatozoa with poor motility respond to the mechanical stimulus of repeated centrifugation by the enhanced generation of ROS (1,2). Hence, immediate, isolation of the fertile from the nonfertile spermatozoa is recommended in many studies, and it's reported that Percoll gradient centrifugation or swim-up/centrifugation has to be preferred to the traditional centrifugation/swim-up method in preparing semen samples for in vitro fertilization (IVF) (1,3,4).

The primary product of the sperm NADPH-

oxidase system appears to be superoxide anion, a portion of which is converted to hydrogen peroxide (H_2O_2) by the action of superoxide dismutase (SOD), and H_2O_2 then combines with superoxide anion to generate the extremely reactive hydroxyl radical (1,5). In order to avoid harmful effects of ROS, it has also been reported that it would be logic to incorporate antioxidants into the culture medium during sperm preparation (1,5,6). Antioxidants from seminal plasma such as SOD and catalase, besides low molecular weight components, protect spermatozoa against ROS (7).

On the other hand, sperm preparation for IVF by swim-up in a solution of hyaluronic acid has been reported to improve retention of sperm motility and velocity (8-11). Hyaluronic acid, which improves sperm capacitation and acrosome reaction, is thought to be physiological because it is a component of cervical mucus, follicular fluid, seminal fluid and also extracellular matrix of the human oocyte-cumulus complex (12-15).

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The purpose of the present study was to compare three different sperm preparation techniques, the

traditional centrifugation/swim-up, swim-up/centrifugation, and the new swim-up in HYA, to assess their effects on sperm fertility indices.

MATERIALS AND METHODS

Sample selection

The study was based on seminal samples from men (n=34) undergoing physical examination at Çukurova University Hospital, Division of Urology. Semen was collected by masturbation into sterile plastic containers from 22 normozoospermic and 12 oligozoospermic (sperm concentration $<20 \times 10^6$ spermatozoa/ml) men, following 2 days of abstinence. Seminal samples were defined according to World Health Organization criteria (16). Oligozoospermic samples also had asthenozoospermia ($<50\%$ motile spermatozoa). 12 of 24 asthenozoospermic samples were normozoospermic, and there was no case of teratozoospermia ($>50\%$ morphologically abnormal spermatozoa). Nine of the samples exhibited agglutination, and eight possessed genital tract infection (polymorphonuclear leukocytes $>1.0 \times 10^6$ /ml).

Fresh ejaculates were allowed to liquefy at room temperature for 30 min, and motile spermatozoa were obtained from each sample by three different methods.

Chemicals and media

Sodium hyaluronate was dissolved in liquid Ham's F-10 medium to a final concentration of 1 mg/ml and equilibrated in an incubator set at 37°C and 5% CO₂ in air. Hyaluronate (HYA) was obtained from Sigma (H5388, St Louis, USA) and Ham's F-10 from Biochrom Seromed (F0713, Berlin, Germany). The high-molecular weight sodium hyaluronate used in this study, like Sperm Select from Pharmacia, was prepared from rooster combs. The medium was supplemented with 0.3 mg/ml streptomycin (Sigma, S6501), 0.3 mg/ml penicillin G (Sigma, P3032) and 5 µg/ml L glutamin (Biochrom Seromed, K0281). The osmolarity of the medium was adjusted to 280-290 mosm/l. Filtration was achieved by using Sterivex™-GS 0.22µm filters (Millipore Inc., Bedford, MA, USA). For sperm preparation, inactivated human serum was added to a final concentration of 10%. Human serum, which was prepared from O Rh (-) young individuals taking no drugs, was supplied from Blood Bank of Çukurova University Hospital.

Sperm preparation

1. Swim-up in hyaluronate (SwUp in HYA): In order to isolate motile spermatozoa from nonferile fractions immediately, and to check the effect of hyaluronate, we modified the procedure previously described by Wikland et al (17). In a tube of ~ 5 mm diameter. 0.50 ml semen was layered under 0.50 ml HYA solution. The tube was incubated in a vertical position at 37°C and 5% CO₂ in air. After 60 min, 0.25 ml of the upper

phase, containing motile spermatozoa, was removed and the fertility parameters determined.

2. Swim-up/centrifugation methods (SwUp/Cent): Immediate isolation of spermatozoa was achieved by modifying the technique previously described (18). 1 ml of semen was layered under 1.5 ml Ham's F-10 in a tube of 15 mm diameter and the spermatozoa were allowed to swim up under the same conditions as those used with HYA. Two swim-up tubes were used for each ejaculate. After swim-up, 1 ml of the upper phase was aspirated, the aliquots from the two tubes pooled and centrifuged for 5 min at 200 g. The pellet was resuspended in 3 ml Ham's F-10, whereafter the sperm parameters were determined.

3. Traditional centrifugation/swim-up methods (Cent/SwUp): 2 ml of Ham's F-10 was added on 1 ml semen sample in a tube, the mixture was harvested, and then centrifuged for 10 min at 260 g. Supernatant was immediately aspirated, the pellet was resuspended in 3 ml Ham's F-10, and then centrifuged for 10 min at 260 g. After this second centrifugation, 0.5 ml portion of the supernatant was left, and the spermatozoa were allowed to swim up under the same conditions as those used with the above two methods. After 60 min, 0.25 ml of the upper phase, containing motile spermatozoa, was removed and the sperm parameters determined.

In order to evaluate the survival of spermatozoa at different preparation techniques, sperm suspensions prepared by the three methods were kept at room temperature in capped tubes, and after 24 h, samples were taken for evaluation of sperm motility.

Determination of sperm quality measures

Semen analysis and preparation commenced within 1 h of ejaculation. The sperm parameters were determined using a computerized sperm analysis system (Hamilton Thorn Research, Inc. HTM-C 2030 Motility Analyzer. Version 7.2, Danvers, USA) Using a 10 µm deep ARH counting chamber (Arnold D Horwell Ltd., London) and an Olympus microscope (Olympus CH-2) equipped with a phase contrast objective, a minimum of three fields from 5 µl of each sample were studied. Spermatozoa with average velocity >0 µm/s were considered as motile.

We have monitored sperm concentration, %motility (fraction of total cells for which velocity >0 µm/s), progressive motility (fraction of all cells moving with velocity >25 µm/s, and straightness >80), and velocity.

Statistics

In the statistical evaluation of the results, the non-parametric Wilcoxon test and correlation-regression analysis were used (CSS: Complete Statistical System). All data were expressed as mean ± SEM, and sperm concentrations also as median.

Table 1. Semen quality in the groups taken for sperm preparation (mean±SE)

Variable	Groups	
	Normozoospermia (n=22)	Oligozoospermia (n=12)
Sperm concentration (x10 ⁷ /ml)	58.07±7.05 (52.6)* (22.3-131.5)**	8.47±1.19(8.45) (3.8-18.4)
% Motility	43.55±4.68	18.83±2.87
% Progressive motility	16.23±2.62	4.75±0.96
Velocity (µm/s)	38.41±1.91	28.92±1.42
% Abnormal spermatozoa	33.86±1.79	34.58±1.89
Median		
Minimum-maximum values		

RESULTS

Semen quality of the normozoospermic and Oligozoospermic groups are shown in Table 1.

1. Normozoospermic samples

Sperm parameters of the treatment groups in normozoospermia are shown in Table 2.

In normozoospermic samples, sperm preparation by swim-up/centrifugation produces sperm suspensions that are of higher quality except per cent motile spermatozoa, which is improved better by the reverse procedure. The superiority of swim-up/centrifugation to both of the other two methods was significant only in terms of sperm velocity. Hyaluronate failed to improve sperm parameters, when compared to the other two procedures, and these action of HYA was very significant in regard to sperm motility indices ($p < 0.001$). Centrifugation/swim-up in HYA (Table 2).

2. Oligozoospermic samples

Sperm parameters of the treatment groups in oligozoospermia are shown in Table 3.

In Oligozoospermic samples, treatment by

centrifugation/swim-up produces sperm suspensions that are of higher quality in terms of % motility and % progressive motility, whereas sperm recovery and velocity are improved the best by the reverse procedure. Except sperm recovery, all the other parameters were influenced negatively by swim-up in HYA, when compared to the other two methods. Improved sperm recovery in swim-up/centrifugation is significant when compared to centrifugation/swim-up, which is significantly superior to both of the other two methods in terms of per cent motile spermatozoa, an important male fertility index. Centrifugation/swim-up is also significantly superior to the reverse procedure when sperm concentration is considered. Hyaluronate significantly failed to improve sperm motility parameters, when compared to the other two methods (Table 3).

3. Motility after 24 h

Motility after 24 h in the treatment groups are shown in Table 4.

When all samples were considered together, per cent motility was found to be superior with the centrifugation/swim-up treatment at 0 h, whereas with reverse procedure at 24 h (Table 4). Superiorities of the methods to their reverse procedures in both cases were determined to be significant ($p < 0.05$).

Hyaluronate significantly failed to improve % motility at both times, when compared to the other two methods.

4. Dependence of the final sperm concentration, on the seminal fertility parameters

For the purpose of evaluating the efficacy of the three methods for recovering spermatozoa from semen, the dependence of the concentration of motile spermatozoa after preparation on the sperm concentration, per cent motility, per cent progressive motility and per cent abnormal spermatozoa in semen were determined by correlation-regression analysis. The results of this analysis are presented in Table 5.

Table 2. Sperm parameters in normozoospermic samples (n=22) after preparation with 3 different methods (mean±SE)

Variable	Method		
	HYA ¹	SwUP/Cent	Cent/SwUP
Sperm concentration (7.85) (x10 ⁷ /ml)	9.81 ± 3.13 ^a (5.5)* (0.9-6.3)**	19.89±6.11 (6.6) (0.6-108.4)	17.05±5.22 (0.8-102.8)
Recovery ² (%)	15.07±3.20	25.81 ± 5.78	25.11 ± 5.98
% Motility	31.05±6.02 ^a	57.95±6.3	61.77±6.83 ^a
% Progressive motility	15.09±3.53 ^a	43.91 ± 5.46	42.32±5.82 ^a
Velocity (µm/s)	26.91 ± 3.05 ^a	42.77±2.74 ^a	39.68±2.52 ^a

(1) Swim-up in HYA

(2) Recovery=100xconcentration of spermatozoa in the final suspension/concentration of spermatozoa in semen

* Median

** Minimum-maximum values

(a) Differences are significant in terms of HYA versus SwUp/Cent

(b) Differences are significant in terms of SwUp/Cent versus Cent/SwUp

(c) Differences are significant in terms of HYA versus Cent/SwUp

Table 3. Sperm parameters in Oligozoospermie samples (n=12) after preparation with 3 different methods (meantSE)

Variable	Method		
	HYA ^a	SwUP/Cent	Cent/SwUP
Sperm concentration (x10 ⁶ /ml)	1.78±0.32 (1.6) [*] (0.4-3.7) ^{**}	1.8±0.37b(1.55) (0.4-5.4)	1.9*0.1 (1.1) (0.5-1.7)
Recovery (%)	23.11±4.34	24.67±5.14 ^b	15.17±2.22
%Motility	6.75±2.71 ^o	16.92±1.88 ^b	37.42±4.74 ^o
% Progressive motility	3.67±2.14 ^a	15.25±1.95	21.08±4.50 ^o
Velocity (pm/s)	12.42±3.80 ^a	40.83±2.64	35.33±1.67 ^o

(1) Swim-up in HYA

(2) Recovery-100xconcentration of spermatozoa in the final suspension/concentration of spermatozoa in semen

^{*} Median^{**} Minimum-maximum values

(a) Differences are significant in terms of HYA versus SwUp/Cent

(b) Differences are significant in terms of SwUp/Cent versus Cent/SwUp

(c) Differences are significant in terms of HYA versus Cent/SwUp

For swim-up/centrifugation, the sperm concentration in the final suspension is the most dependent on the fertility parameters in semen, except per cent progressive motility; for swim-up in HYA, this dependence was determined to be the least (Table 5).

DISCUSSION

It has been shown that there is a strong inverse relationship between the fertility of a semen sample and its rate of production of reactive oxygen species (ROS). ROS, which are produced in certain nonfertile fractions of the sperm population, damage spermatozoa by peroxidation of membrane lipids, resulting in an increase in the permeability of the plasma membrane. Spermatozoa characterized by poor motility and an impaired capacity for fertilization, appear to respond to the mechanical stimulus of repeated centrifugation by the enhanced generation of ROS (1,2). Hence, it is of great importance to isolate the fertile from the nonfertile spermatozoa as soon as possible. If all spermatozoa, fertile and nonfertile, are pelleted together, the former will be exposed to the reactive oxygen produced by the latter, and the proportion of fertile spermatozoa would decrease (1,3,4).

The primary product of the sperm NADPH-oxidase system appears to be superoxide anion, a portion of which becomes converted to hydrogen peroxide through the action of superoxide dismutase. If catalytic amounts of iron are available, the combination of superoxide anion and hydrogen peroxide generates the extremely reactive hydroxyl radical, which is a powerful initiator of lipid peroxidation (1,5). In order to avoid these harmful effects of ROS, it's recommended to separate fertile and nonfertile spermatozoa by Percoll gradient centrifugation or by swim-up in standard medium (1,3,4). On the other hand, sperm preparation for IVF by swim-up in a solution of hyaluronate has been reported to improve retention of sperm motility and velocity (8,9,10,11).

Table 4. Per cent motile spermatozoa in all samples (n=34) at various times after preparation with 3 different methods (meantSE)

Method	Hours after preparation	
	0	24
HYA1	22.47±4.46	6.88±2.87
SwUp/Cent	43.47±5.33	25.29±3.92
Cent/SwUp	53.18±5.09	16.38±3.76

(1) Swim-up in HYA

Table 5. Correlation analysis of the dependence of the sperm concentration in the final suspension on sperm concentration, per cent motility, per cent progressive motility and per cent abnormal spermatozoa in semen (n=34)

Variable	Treatment	r [*]	p
Sperm concentration in semen	HYA1	0.57	<0.001
	SwUp/Cent	0.75	<0.001
	Cent/SwUp	0.63	<0.001
% Motility	HYA	0.66	0.001
	SwUp/Cent	0.78	<0.001
	Cent/SwUp	0.70	<0.001
% Progressive motility	HYA	0.39	<0.05
	SwUp/Cent	0.49	<0.01
	Cent/SwUp	0.51	0.01
%Abnormal spermatozoa	HYA	-0.40	<0.05
	SwUp/Cent	-0.63	<0.001
	Cent/SwUp	-0.48	<0.01

(1) Swim-up in HYA

^{*} Coefficient of correlation

The present investigation shows that in normozoospermic samples, sperm preparation by swim-up/centrifugation method produces sperm suspensions that are significantly of higher quality in respect to sperm velocity (Table 2). On the other hand for swim-up/centrifugation, the sperm concentration in the final

suspension is the most dependent on the sperm concentration, percentage motile spermatozoa and percentage motile spermatozoa and percentage abnormal spermatozoa in semen (Table 5). Due to these two facts, swim-up/centrifugation method may be preferred to the other two methods, in preparing normozoospermic samples for IVF. Strong dependence of the final sperm concentration on seminal fertility indices makes swim-up/centrifugation a poor candidate for preparing oligozoospermic samples with poor sperm quality.

In oligozoospermic samples, sperm preparation by swim-up/centrifugation was shown to improve sperm recovery significantly, and sperm velocity non-significantly when compared to the conventional centrifugation/swim-up method. On the other hand, percentage motile spermatozoa, which is an important index for the fertility of a semen sample, was determined to improve very significantly by the centrifugation/swim-up, when compared to the reverse procedure (Table 3). This finding makes the centrifugation/swim-up method a preferable one in preparing oligozoospermic samples for IVF.

When the survival of spermatozoa prepared by 3 different methods was evaluated after 24 h, swim-up followed by centrifugation produced a sperm suspension that was of better quality in terms of per cent motile spermatozoa, and the difference was significant when compared to the other two procedures (Table 4). This investigation shows that immediate isolation of fertile spermatozoa from the nonfertile ones which produce ROS, improve their survival tested at 24 hours.

In this present study, all sperm parameters, including the per cent motile spermatozoa at 24 hours, were influenced negatively when sperm preparation was carried out by using swim-up in HYA; in sight of these results, it's possible to conclude that hyaluronate exerted a toxic effect on the male fertility indices. Similar to our results, Sjoblom and Wikland (4) also couldn't confirm the previously reported superiority of sperm preparation with HYA over conventional methods. In contrast to our findings, hyaluronic acid was reported to improve retention of sperm motility, and sperm preparation with sodium hyaluronate was recommended as an alternative to traditional method of centrifugation/swim-up (8-11). In most of these previous studies, Sperm Select was used as sodium hyaluronate solution, but HYA used in this present investigation was obtained from Sigma, and this fact may contribute to the controversy of findings. Different media and different concentrations of HYA used in the studies may also add to these controversial results.

The recovery of spermatozoa after swim-up is highly dependent on how much of the swim-up layer is utilized; the height of the swim-up column is of great importance and directly affects the results. For this reason the figures for recovery should be interpreted cautiously and comparisons with results from other

laboratories are difficult (4).

The interface area/volume ratio is larger in the HYA system than in the swim-up/centrifugation system. This should allow a more rapid migration of spermatozoa from semen to medium in the HYA system. However, the final distribution of spermatozoa between semen and medium is also dependent on the time allowed for swim-up as well as on the rheological properties of the medium (4). No investigation of these factors was done, since the aim of this study was to evaluate the performance of the procedures.

Although sperm preparation with HYA has been frequently reported to improve fertility indices, the concentration of hyaluronan in seminal fluid was shown to be negatively correlated with sperm count and ejaculate volume (13). In this regard, future studies are needed to clarify the potential role of HYA in the male fertility parameters, and also to determine if view, to use HYA in sperm preparation

The overall evaluation of our results leads to the conclusion that swim-up /centrifugation seems more suitable for normozoospermic samples, whereas centrifugation/swim-up for poor semen specimens, and that HYA used in this study possibly exerts a toxic effect on the male fertility parameters.

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Hyaluronotın ve motil spermelerin hemen izolasyonunun, seminal fertilité parametreleri üzerine etkisi

Bu çalışmada hyaluronik asitin (HYA) ve motil spermelerin nonfertil olanlardan hemen ayrılmasının, erkek fertilité parametreleri üzerindeki etkileri araştırıldı. 34 erkekten alınan semen örnekleri (22 normozoospermi, 12 astenooligozoospermi) üçer kısma ayrılarak HYA'lı ortamda yüzeye çıkma (HYA yöntemi), yüzeye çıkma/santrifügasyon ve klasik santrifügasyon/yüzeye çıkma yöntemleriyle muamele edildi. Normozoospermik örneklerde sırasıyla HYA, yüzeye çıkma/santrifügasyon ve klasik yöntemlerle elde edilen ortalama \pm SE değerler: % motilité 31.05 \pm 6.02, 57.95 \pm 6.3 ve 61.77 \pm 6.83; % ilerleyici motilité 31.05 \pm 6.02, 57.95 \pm 6.3 ve 61.77 \pm 6.83; % ilerleyici motilité 15.09 \pm 3.53, 43.91 \pm 5.46 ve 42.32 \pm 5.82; sperm hızı 26.91 \pm 3.05, 42.77 \pm 2.74 ve 39.68 \pm 2.52 μ m/s. Oligozoospermik örneklerde sırasıyla aynı yöntemlerle elde edilen değerler: % motilité 6.75 \pm 2.71, 16.92 \pm 1.88 ve 37.42 \pm 4.74; % ilerleyici motilité 3.67 \pm 2.14, 15.25 \pm 1.95 ve 21.08 \pm 4.50; sperm hızı 12.42 \pm 3.8, 40.83 \pm 2.64 ve 35.33 \pm 1.67 μ m/s.

Bu bulgularla, 1) normozoospermik örneklerin in vitro fertilizasyon (IVF) için hazırlanmasında,

sperm hızı açısından anlamlı üstünlüğü olan yüzeyleme/santrifügasyon yönteminin tercih edilebileceği, 2) oligozoospermik örneklerin IVF için hazırlanmasında, % motilite açısından anlamlı üstünlüğü olan klasik yöntemin tercih edilebileceği ve 3) hyaluronatın sperm fertilité parametrelerini iyileştirmediği ve sperm hazırlamada HYA kullanımının toksikolojik açıdan sakıncalı olabileceği sonuçlarına varıldı. [Turk J Med Res 1994, 12(5): 200-205]

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