Improving outcome of *in vitro* sperm activation using non–liquefied versus liquefied semen of oligoasthenozoospermic patients

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Received November 2009; Revised and accepted February 2010

Abstract

Objective: To evaluate the efficacy of *in vitro* sperm activation (ISA) using non–liquefied versus liquefied asthenozoospermic semen samples for improvement of sperm parameters.

Materials and Methods: Fifty six oligoasthenozoospermic (OA) patients (age range: 22–44 years; mean: 32.089 years) were enrolled in this study. OA patients were classified according to type of infertility. Also, duration of infertility was recorded. Fifty six semen samples were collected, and seminal fluid analyses were done involving macroscopic and microscopic examinations were performed according to WHO methodology. Direct swim–up technique was used to separate the motile spermatozoa from seminal plasma. Minimum Essential Medium Eagle (MEME) enriched with 5% Human serum albumin (HSA) was used. One mL of either non–liquefied or liquefied semen was layered beneath 1 mL of MEME enriched with 5% HSA, and placed for incubation in an air incubator at 37 °C for 30 minutes. Then, one drop (10 μ L) from upper layer of culture medium was taken using automatic pipette to be examined under high power field (40 X) for assessment of sperm parameters.

Results: According to type of infertility, infertile patients were classified into patients with either primary infertility (no. 46; 82.15 %) or secondary infertility (no. 10; 17.85 %). In contrast to other parameters, significant (P<0.05) reductions were noticed in the percentages of sperm motility and progressive sperm motility for OA patients with primary infertility as compared to OA patients with secondary infertility. All sperm parameters were significantly (P<0.001) enhanced after *in vitro* activation of liquefied and non–liquefied semen samples when compared to pre–activation. In the present study, best results were achieved for non–liquefied semen samples as compared to liquefied semen samples. **Conclusion:** It was concluded that the outcome of ISA was enhanced in regard to sperm parameters when using non–liquefied semen of OA patients. Furthermore, some components of seminal plasma of OA patients may be have harmful effects on certain sperm functions when *in vitro* incubated for longer periods. Further study is recommended to investigate the effect of *in vitro* sperm activation from non–liquefied semen on successful rate of artificial insemination husband.

Key words: Liquefaction, Oligoasthenozoospermia, In vitro activation.

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Introduction

Infertility is the inability or reduced ability to produce a pregnancy within a reasonable period of trying, usually 12 months (1). Primary infertility is a term used

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to designate those couples who have never conceived, whereas secondary infertility indicate that at least one conception has occurred but that the couple is currently not able to achieve pregnancy (2). Male factor is the sole or contributing factor in roughly half of these cases, and no identifiable cause can be found in over 25 % of the infertile males (3). An estimated 6 % of adult males are thought to be infertile (4).

It was known that the spermatozoa are smallest cells in the body and have to fulfill their functional extracorporeal. So it is mandatory to have a highly efficient mechanism to generate motility and hence, sperm motility is the only natural way through which sperm meet the ova during fertilization (5). Asthenozoospermia means reduce sperm motility or progressive motility or both (6), and considered one of the most important factors related to male infertility (7). Asthenozoospermia may result from a defect in the sperm structure, presence of antisperm antibodies (AsAb), genital tract infection, idiopathic causes, prolonged abstinence period, and partial ductal obstruction (8). Sometimes, drug therapy may result in decreased motility and this can be improved by the removal of the drug, or introduction of specific culture media to the sperm washing procedure (9). Asthenozoospermia may be managed by in vitro sperm activation using different types of stimulators (10), and then IUI (11, 12).

The aim of sperm preparation for ARTs including artificial insemination husband (AIH) is to maximize the chances of fertilization (13). Basically the culture media used for ARTs are modification of balanced salt solution (14), and it is apparent that spermatozoa of mammalian species including human can acquire the ability to fertilize after a short incubation in defined culture media (15). Sperm processing and isolation of highly motile spermatozoa from the whole semen specimen have been tried with variable success prior to its use for ARTs (16). Therefore, the objective of this study was to evaluate the efficacy of *in vitro* sperm activation (ISA) using non–liquefied versus liquefied asthenozoospermic semen samples for improvement of sperm parameters.

Subjects, Materials and Methods *Patients*

Fifty six oligoasthenozoospermic (OA) patients (age range: 22–44 years; mean: 32.089 years) were enrolled in this study during their attendance at IVF Institute of Embryo Research and Infertility Treatment / Al–Nahrain University. Mean duration of infertility was 4.55 years (range: 2–10 years). The OA patients

have been seen previously by urologists and endocrinologists for the presence or absence of varicocele, hernia, cryptorchidism and other hormonal problems.

Seminal fluid analysis

Fifty six semen samples were collected directly into wide open, clean, dry, sterile disposable Petri dish containers by masturbation in a private room near the Semen Analysis Laboratory at IVF Institute. Prior to the collection of the semen specimen, 3 to 5 days of abstinence was recommended. The container was labeled with name of patient and his wife, time of collection and date of collection. The semen specimens were kept warm in an air incubator at 37 °C to allow normal liquefaction. The standard criteria for spermiogram of WHO (17) were applied to record the results of seminal fluid analysis (Table 1).

Seminal fluid analysis involving macroscopic and microscopic examinations were performed according to WHO methodology mentioned in details in manual of NAFA–ESHRE (18) and developed from manual of WHO (17). Macroscopic parameters were semen volume, liquefaction time, viscosity and acidity (pH). Microscopic parameters of spermatozoa were sperm concentration, sperm motility (%), progressive sperm motility (%), normal sperm morphology (%), sperm agglutination (%), round cells count, and sperm hypo– osmotic swelling (HOS) test (%).

In vitro sperm activation (ISA) technique

In the present study, we used direct swim–up technique for ISA to separate the motile spermatozoa from seminal plasma. This technique utilizes the capacity of motile spermatozoa to migrate or "swim–up" from semen layer to the upper layer of culture medium.

Minimum Essential Medium Eagle (MEME; Sigma –Aldrich Co. LTD., Irvine, UK) enriched with 5% Human serum albumin (HSA, Human Albumin 20% low salt content, Biotest Pharma GmbH, Dreieich, Germany) was used for sperm preparation.

One mL of seminal plasma was layered beneath 1 mL of MEME enriched with 5% HSA within graduated Falcone conical tube, and placed for incubation in an air incubator at 37 °C for 30 minutes. Then one drop (10 μ L) from upper layer of culture medium was taken using automatic pipette (Eppindorf, volume 10–20 μ L, Germany) to be examined under high power field (40X) for assessment of microscopic sperm parameters (19).

Experimental design

This study was performed on semen specimens were

Daramatars	Seminal fluid	Oligoasthenozoospermic patients (n= 56)		WHO criteria
	(n=56)	Primary infertility (n= 46)	Secondary infertility (n=10)	(1999)
Semen volume (mL)	3.076 ± 0.086	2.812 ± 0.098	4.102 ± 0.651 ns	2–5 mL
Semen liquefaction time (min.)	38.696 ± 0.972	38.215 ± 1.108	39.087 ± 2.006 ^{ns}	Within 60 min
Semen viscosity (cm)	1.832 ± 0.037	1.902 ± 0.0395	1.741 ± 0.0318 ^{ns}	< 2 cm
Semen pH	7.693 ± 0.105	7.414 ± 0.139	7.869 ± 0.179 ^{ns}	7.2–8.0
Sperm concentration (X10 ⁶ sperm/mL)	12.383 ± 0.597	12.054 ± 0.671	13.911 ± 1.233 ^{ns}	$\geq 20 \text{ X } 10^6$ sperm/ mL
Sperm motility (%)	21.964 ± 1.506	20.510 ± 1.689	28.013 ± 2.431 ^a	≥ 50 %
Progressive sperm motility (%)	5.482 ± 0.886	4.673 ± 0.933	9.206 ± 2.230^{a}	\geq 50 % with forward progressive motility
Normal sperm morphology (%)	40.571 ± 1.059	39.173 ± 0.982	47.008 ± 3.266 ^{ns}	\geq 30 %
Sperm agglutination (%)	4.339 ± 0.710	4.413 ± 0.811	4.071 ± 1.453 ^{ns}	< 10
Sperm HOS test (%)	35.125 ± 1.780	33.391 ± 2.003	43.104 ± 2.750 ns	≥ 50 %

 Table 1. Parameters of seminal fluid analysis classified according to type of infertility for oligoasthenozoospermic patients*

* = Total number of patients 56.

a = significant (P<0.05) difference between OA patients with primary and secondary infertility.

ns = non significant difference between OA patients with primary and secondary infertility.

obtained from OA males to investigate the impact of semen liquefaction period on outcome of sperm parameters after ISA. After 3 to 5 minutes of taken of semen specimen by masturbation, 1 mL of mixed seminal plasma was aspirated using fixed automatic pipette (Eppindorf, 1 mL, Germany) and placed beneath 1 mL of MEME enriched with 5% HSA, and placed for incubation in an air incubator at 37 °C for 30 minutes, and therefore was considered non–liquefied seminal plasma. While the another part of this study, after semen liquefaction totally, 1mL liquefied seminal plasma was taken beneath 1 mL of MEME enriched with 5% HSA, and placed for incubation in an air incubator at 37 °C for 30 minutes. At end period of *in vitro* incubation, microscopic sperm parameters were assessed.

Statistical analysis

Crude data were collected and analyzed using SPSS (Statistical program for social studies, Version 12, Illinois, USA) for descriptive statistics involving means and standard error of mean (SEM) and for compare means using paired *t*- test and ANOVA to detect the significant differences of pre- and post-activation and between groups of liquefied and non-liquefied semen samples; respectively. *P* value less than 0.05 was considered as statistic-cally significant.

Results

Table 1 shows the results of SFA for all OA males (no. 56) were enrolled in this study. It was clear that the sperm concentration and percentage of sperm motility were reduced when compared to the normal criteria of WHO (17), and therefore, considered oligoastheno-zoospermic patients. Also, sperm HOS test (%) was reduced as compared to normal value of WHO (17).

According to type of infertility, infertile patients were classified into patients with either primary infertility (1°; no. 46; 82.15 %) or secondary infertility (2°; no. 10; 17.85 %). Mean age and duration of infertility for OA patients with 1° infertility was 31.173 years (range: 22–44 years) and 4.521 years (range: 2–10 years); respectively. While, mean age and duration of infertility for OA patients with 2° infertility was 34.306 years (range: 28–44 years) and 4.732 years (range: 3–9 years); respectively.

Non significant (P>0.05) differences in the macroscopic parameters of semen samples were reported between both types of infertility (Table 1). From same table, sperm concentration, normal sperm morphology (%), sperm agglutination (%) and sperm HOS test (%) have non significant (P>0.05) differences between both types of infertility. In contrast, significant (P< 0.05) reductions were noticed in the percentages of

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where sper in parameters	IIC-Activation	Liquefied semen	Non–liquefied semen
Sperm concentration (X10 ⁶ sperm/mL)	12.383 ± 0.597	1.866 ± 0.264 ^A	3.901 ± 0.357 ^{A a}
Sperm motility (%)	21.964 ± 1.506	31.910 ± 3.616 ^A	$48.482 \pm 2.805 \ ^{A a}$
Progressive sperm motility (%)	5.482 ± 0.886	11.160 ± 1.461 ^A	23.625 ± 1.712 ^{A a}
Normal sperm morphology (%)	40.571 ± 1.059	38.767 ± 4.067 ^{ns}	57.428 ± 2.471 ^{A a}
Sperm agglutination (%)	4.339 ± 0.710	0 ± 0	0 ± 0
Sperm HOS test (%)	35.125 ± 1.780	40.821 ± 4.400 ns	64.250 ± 3.083 ^{A a}

Table 2. Sperm parameters pre– and post–activation *in vitro* for non–liquefied and liquefied semen specimens of oligoasthenozoospermic patients*

* = Total number of OA patients 56.

Large letter means significant (P<0.001) difference between post- and pre-activation.

Small letter means significant (P<0.001) difference between liquefied and non-liquefied semen.

ns = Means non significant (P>0.05) difference.

sperm motility and progressive sperm motility for OA patients with 1° infertility as compared to OA patients with 2° infertility.

From the results of the present study, all sperm parameters were significantly (P<0.001) enhanced after *in vitro* activation of liquefied and non–liquefied semen samples as compared to pre–activation (Table 2). However, best results were achieved, in the present study, for non–liquefied semen samples as compared to liquefied semen samples. After *in vitro* sperm activation, it was observed that the sperm concentration, percentages of sperm motility, progressive sperm motility, normal sperm morphology and sperm HOS test were significantly (P<0.001) improved for non– liquefied semen samples when compared to liquefied semen samples (Table 2).

According to type of infertility, results of ISA appeared successful rate 27/46 (58.69 %) of semen samples were obtained from OA patients with 1° infertility compared with 9/10 (90 %) of semen samples were obtained from OA patients with 2° infertility. Also, the successful rate of ISA from non–liquefied semen samples was 53/56 (94.642 %), while from liquefied semen samples was 26/56 (46.428 %).

Discussion

In the present study, higher percentage of OA patients with 1° infertility (82.15 %) was calculated as compared to OA patients with 2° infertility (17.85 %). Different factors may be play major role to present this percentage.

Hormonal levels play a great role in the initiation and maintenance of gametogenesis and sexual activity (7). In males, levels of reproductive hormones have direct effects on sperm production, physiology and fertilizing capacity (20,21). Several studies reported that the parameters of SFA for infertile patients with 1° infertility complaining from severe deviation from normal criteria of WHO (17) when compared to patients with 2° infertility (22, 23). It was reported that the varicocele is observed by in 10–20 % of the general population, in 35–40 % of men with 1° infertility and 80 % of men with 2° infertility (24). Varicocele is remained the most common treatable causes of male infertility (25).

Therefore, those patients with 1° infertility were more interest for diagnosis, investigation and treatment than patients with 2° infertility. Additionally, patients with 1° infertility have no offspring when compared to most patients with 2° infertility have offspring. Also most of successful rate in ARTs including AIH was better in patients with 2° infertility than patients with 1° infertility (12, 23).

Only percentages of sperm motility and progressive sperm motility were significantly (P<0.05) reduced for OA patients with 1° as compared to OA patients with 2° (Table 1). Also in regard to successful rate of ISA, best result was achieved for OA patients with 2° (90 %), while for OA patients with 1° (58.69 %). These results certified the importance of sperm motility and grade activity to achieve fertilization and determine type of infertility (26). It was considered that the sperm motility as the most important parameter during evaluation of semen sample (27, 28), and cornerstone to undergo ICSI in the field of ARTs (29).

The results of the present study appeared enhancement of all sperm parameters were examined after ISA using direct swim–up technique only (Table 2).

Swim-up or self migration resulted in the recovery of a significantly higher percentage of motile sperma-

tozoa compared with the traditional centrifugation (30). The swim–up technique can serve as predictive test for the *in vitro* fertilizing capacity of sperm (31). Steen *et al.* (32) reported that the centrifugation technique may cause damage to spermatozoa when used for ARTs. During sperm preparation for ARTs, defined culture medium (CM) was used and sometimes enriched with protein source and/or sperm stimulator (33). Improvement of sperm motility and grade activity was obtained as a result of special basic components of CM (34). The CM used for ARTs should contain protein source and buffers to promote sperm capacitation and hyperactivation (13). The acidity (pH) of CM is maintained by bicarbonate and CO₂ buffer system.

The CM should contain HEPES, which also maintains a stable pH (35). Sodium lactate and/or pyruvate along with glucose are added as a source of energy (14).

In the present work, we used human serum albumin (HSA) as protein source. It was known that the albumin play a major role in physiology and metabolism of spermatozoa (36). Albumin is protein present in the blood serum in humans and it comprises nearly half of the blood serum protein (37). It has been necessary to include some kind of proteins in the CM to support sperm capacitation and/or fertilizing ability (38). The stimulatory effect is most obvious in asthenozoospermic patients (39). Usually HSA is used for embryo culture.

The function of albumin in CM include limited buffering, the binding of various compounds including steroids and potentially toxic trace elements and capacitation of spermatozoa. Albumin was available as powder or sterile solution (40). HSA is the protein of choice for use as a tissue culture supplement in those applications requiring protein supplementation (38).

Significant (P<0.001) improvements in all sperm parameters for non–liquefied semen were assessed as compared to totally liquefied semen (Table 2). As well as, best successful rate of ISA was achieved for non– liquefied semen when compared to liquefied semen. According to our knowledge, this study the first time to achieve ISA from non–liquefied semen samples from OA patients. This enhancement in the sperm parameters may be related to the fast transfer of spermatozoa from seminal plasma into layer of culture medium.

Therefore, best conditions were prepared for spermatozoa within culture medium (MEME) supplemented with protein source (5% HSA). Culture media provide the spermatozoa with needs that maintain optimal function of spermatozoa to give rise excellent outcomes during *in vitro* sperm preparation (41).

It was reported that the seminal plasma contain different constituents in origin (42) and affects sperm physiology, metabolism and integrity (7,43). The accessory secretions coat the spermatozoa and may affect the functions of the sperm cells including their motility negatively (44). Low level of corrected fructose and hyperviscosity affects sperm motility (45). Both partial and complete lack of liquefaction cause complete or partial immobilization of spermatozoa (46). Seminal plasma contains decapacitation factors that prevent spontaneous capacitation.

Another factors in which prolonged exposure has adverse effects on sperm functions (47, 48). Reduced sperm motility is positively correlated with increased seminal zinc (48), infection (49), byproducts of leukocytes (50), acidity (17) and prolactin (21).

These factors mentioned above may be decrease sperm motility and progressive activity when spermatozoa remain within seminal fluid for long periods.

Table (2) showed significant (P<0.001) increased in the percentage of sperm HOS test post-activation when compared to pre-activation. Sperm HOS test evaluate an integrity of sperm plasma membrane (51). It was certified that the sperm plasma membrane integrity very important for normal sperm cell physiology, egg penetration and binding to oolemma, and lastly for decondensation to form male pronucleus and fertilization (52,53). Different factors may be cooperated and improved integrity of sperm plasma membrane. It was known that the treatment of spermatozoa with the protein digestive enzyme like chymotrypsin – galactose enhances HOS test score (54).

Further significant improvement in the percentage of HOS test was observed after ISA from non–liquefied semen as compared to results of HOS test from liquefied semen. This improvement may be related to the fast movement of spermatozoa from seminal plasma into layer of culture medium, and consequently removed from impact of some seminal plasma components. It was certified that the OA patients have high levels of reactive oxygen species, which are interacting with sperm plasma membrane (55,56). The human spermatozoa are rapidly losing their motility when incubation under high oxygen tension (57). Also, antisperm antibodies have direct harmful effect on sperm motility and plasma membrane integrity (58).

From the results of the present study, we concluded that the outcome of ISA using direct swim–up technique was enhanced in regard to sperm parameters when

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using non-liquefied semen of OA patients. Furthermore, some components of seminal plasma of OA patients may be have harmful effects on certain sperm functions when *in vitro* incubated for longer periods. Further study is recommended to investigate the effect of *in vitro* sperm activation from non-liquefied semen on successful rate of artificial insemination husband.

Acknowledgment

The author thanks assistant biologists Miss Sahar Rasheed and Miss Saja Ali in the Semen Analysis Laboratory at IVF Institute for valuable assistance. There is no conflict of interest.

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