In vitro sperm activation using culture medium supplied with oxytocin in asthenozoospermic patients

Adnan S. Al-Janabi Ferial A. Al-Mehdawi and Makarim Q. Al-Lami
1 Al-Nahrain University
2 Department of Biology-College of Science- Baghdad Universit

Abstract:

Background
Pretreatment of the semen samples of the asthenozoospermia patients with certain sperm stimulant agents prior to use for assisted reproductive techniques (ART) has been reported to have beneficial effects on fertilization rates.

Objectives
Investigating the influence of addition of different concentrations of oxytocin (OT) to semen samples of asthenozoospermic patients for *in vitro* sperm activation as a new method for stimulation of sperm motility in these patients and exploring the concentration of OT that harvests a maximal number of motile sperms.

Methods:
Simple layer technique for *in vitro* sperm activation using culture medium enriched with OT was studied in asthenozoospermic patients (No. 90). Semen samples were examined before sperm activation, and then they were divided into three equal groups. In the first group, three concentrations of OT (2, 4, and 6 IU/ml) were applied. Based on the results of the mentioned work, OT at the concentration of 2 IU/ml was applied with sperm preparation medium (SPM) and without in the second and third groups, respectively.

Results
Results showed that there was a significant improvement in the percentage of sperm motility and sperm grade activity of the OT-treated samples at the concentration of 2 IU/ml as compared to the control samples. Moreover, there was a tendency for a decrease in sperm grade activity with increase OT concentration. The outcome of sperm activation in presence of SPM revealed a significant increase in the sperm motility characteristics (the percentage of sperm motility and sperm grade activity) in the control and OT- treated samples and the best improvement in these sperm motility characteristics was observed in the OT- treated samples. When SPM was absent, a significant improvement in the sperm motility characteristics was observed only in the OT-treated samples.

Conclusion
The results of this study indicate that OT at the concentration of 2 IU/ml has got a stimulatory effect on sperm motility characteristics; also, the use of SPM succeeds to support this effect.

Keywords: Sperm activation, Oxytocin, Asthenozoospermia.

Introduction

Male infertility is responsible in about 20% of infertile couples and is contributory in another 30% to 40% (1). Asthenozoospermia is considered one of the most frequent causes of male infertility (2). The last two decades witnessed a progress in the field of assisted reproduction to provide a more hope for many infertile couples (3). In assisted reproductive techniques (ART), sperm preparation techniques were developed to maximize the chances of fertilization and to provide as many artificially fertilized oocytes as possible (4). Pretreatment of the ejaculate with certain sperm stimulant agents such as: caffeine, pentoxifyllin and progesterone prior to use for ART has been reported to have beneficial effects on fertilization rates (5; 6).

Oxytocin (OT) is produced in the male mammal, in similar quantities as in the female, yet for the male little is known about the physiology associated with this hormone. In the female, the role of OT in the processes of lactation and parturition is well documented (7). In the male, majority of the investigations were

performed *in vivo to* study the physiological role of this hormone. Most of the previous studies have shown no effect of OT, when it was injected intravenously, on sperm output or seminal parameters of the studied cases (8; 9). While in another study on azoospermic or severely oligozoospermic patients, OT given before a second ejaculation improved emptying of spermatozoal stores (10).

Materials and Methods

Ninety asthenozoospermic patients were involved in this study during their attendance to the High Institute for Infertility Diagnosis & ART, Al-Nahrain University from April 2004 to March 2005. The patients were asked to provide semen by masturbation after an intercourse abstinence period ranging between 3-4 days. The medicult sperm preparation medium (SPM) was used for in vitro sperm activation. Oxytocin used in this study was provided in 50 ml vials [(10 IU OT/ml), Kela laboratoria, Belgium]. Normal saline (NS) was used for preparation of three concentrations of OT (2, 4, and 6 IU/ml).

Seminal Fluid Analysis

Seminal fluid analysis (SFA) was performed according to World Health Organization (WHO) guidelines (11) to evaluate semen quality of the patients.

Sperm Activation by Oxytocin

Simple layer technique was used to separate the motile sperm from seminal plasma (12). Three concentrations of OT (2, 4, and 6 IU/ml) were applied and 10 semen samples were studied for each concentration. A culture tube was prepared for each application and 0.5 ml of SPM was mixed with 0.5 ml of each concentration of OT, then 1 ml of semen sample was layered beneath each of the prepared medium. After incubation for 30 minutes in 5% CO2 incubator at 37 C, a drop from upper layer was aspirated by micropipette to be examined under 40X objective lens to study the effect of OT at these concentrations on the semen parameters. Based on the outcome of this experiment which involved 30 semen samples (1st group), OT at 2 IU/ml was tried, as shown above, with SPM (2nd group) and without (3rd group); each group involved 30 semen samples. All results were compared with semen samples treated with NS as control (OT = 0 IU/ml).

Statistical Analysis

Statistical analysis was done using SPSS, version 10.5. All results were expressed as mean ±SD. Statistical comparisons between groups were made using student's t-test. Differences between groups were analyzed using ANOVA. $P<0.05$ was regarded as statistically significant (13).

Results

Seminal Fluid Analysis

The results of SFA (Table 1) revealed that mean of liquefaction time was on the maximal side of the normal value, while mean of semen volume was on the minimal side of the normal value. All semen samples had normal alkaline pH, which was within the normal limit. Sperm concentration was within the normal limit, while the percentage of motile sperm was below the normal limit; also, mean of sperm grade activity was below the normal range of Macleod scale. The percentage of sperm abnormality was on the maximal side of the normal limit. The percentage of sperm agglutination and concentration of leukocytes and phagocytes were above WHO normal limit.

Table 1: Semen quality of the patients before oxytocin addition.

Semen Character	Value (Mean ± SD)	Normal Values
Liquefaction time (minute)	35.21 ± 8.50	< 30
Semen volume (ml)	2.52 ± 0.95	2-6 ml
pH	7.86 ± 0.15	7.4-8.2
Sperm concentration (million/ml)	44.40 ± 20.42	> 20
Sperm motility (%)	33.26 ± 11.44	> 60
Grade of activity (Macleod Scale)	1.66± 0.43	3
Sperm abnormality (%)	48.52 ± 8.44	< 50
Sperm agglutination (%)	3.24 ± 3.12	Negative
Leukocytes concentration (million/ml)	2.50 ± 1.20	< 1
Phagocytes concentration (million/ml)	2.78 ± 1.50	< 0.5

Effect of Oxytocin on Sperm Parameters

Results shown in table 2 revealed that there were no significant differences ($P>0.05$) in sperm concentration and the percentage of sperm abnormality between the treated samples with different concentrations of OT and their control samples, while a significant increase ($P<0.05$) was seen in the percentage of sperm motility and grade of activity in the OT-treated samples at the concentration of 2 IU/ml. Also; there was a tendency for a decrease in sperm grade activity with increase OT concentration.

Table 2: Effect of different concentrations of oxytocin (2, 4, and 6 IU/ml) on sperm parameters using simple layer technique.

Sperm parameters	Control	OT-treated (2 IU/ml)	Control	OT-treated (4 IU/ml)	Control	OT-treated (2 IU/ml)
Sperm concentration (million/ml)	30.90 ± 19.72	30.50 ± 19.47	30.80 ± 17.71	30.20 ± 18.12	30.50 ± 15.62	30.80 ± 15.68
Sperm motility (%)	81.20 ± 6.21	84.20 * ± 5.22	80.50 ± 5.99	81.42 ± 5.30	79.65 ± 4.82	80.00 ± 4.54
Grade of activity	2.94 ± 0.21	3.24 ± 0.22	2.80 ± 0.14	2.88 ± 0.18	2.78 ± 0.11	2.72 ± 0.12
Sperm abnormality (%)	38.30 ± 5.65	38.70 ± 4.75	40.60 ± 5.88	39.80 ± 6.61	39.70 ± 6.40	39.30 ± 6.45
Number of samples	10		10		10	

Values are means ± SD.
***Significant difference (P<0.05).**

Addition of OT (2 IU/ml) to the semen revealed that, in presence of SPM (Table 3), a high significant decrease (P<0.001) was observed in the sperm concentration and the percentage of sperm abnormality associated with a high significant increase (P<0.001) in the percentage of sperm motility and sperm grade activity after activation in control and OT-treated samples. When results of OT-treated samples were compared with control samples, a high significant increase (P<0.001) was observed in the percentage of sperm motility and sperm grade activity in the OT-treated samples.

Table 3: Effect of oxytocin (2 IU/ml) on sperm parameters ollowing sperm activation with presence of sperm preparation medium

Sperm parameters	Before Activation	After activation	
		Control	Treated
Sperm concentration (Million/ml)	45.93 ± 20.58	22.20a** ± 15.77	22.05a** ± 14.80
Sperm motility (%)	35.20 ± 12.66	81.83a** ± 6.28	85.53b** ± 6.46
Grade of activity	1.85 ± 0.50	3.04a** ± 0.21	3.68b** ± 0.24
Sperm abnormality (%)	44.50 ± 7.51	30.80a**± 4.51	30.40a**± 4.52

Values are means ± SD.
** Significant difference (P<0.001).
Similar small letters indicate a non-significant difference (P>0.05).
Different small letters indicate a significant difference (P<0.001).

In absence of SPM (Table 4), a high significant decrease (P<0.001) was observed in sperm concentration after activation in the control and OT-treated samples, while the percentage of sperm motility and sperm grade activity increased significantly (P<0.05) after activation only in the OT-treated samples. The table also showed that there was a significant increase (P<0.05) in the percentage of sperm motility and sperm grade activity in the OT-treated sample.

Table 4: Effect of oxytocin (2 IU/ml) on sperm parameters following sperm activation with absence of sperm preparation medium.

Sperm parameters	Before Activation	After activation	
		Control	OT- Treated
Sperm concentration (Million/ml)	44.27 ± 20.35	22.37a** ± 11.60	22.63a** ± 11.56
Sperm motility (%)	37.83 ± 10.78	38.90a ± 12.63	42.78b* ± 12.56
Grade of activity	1.71 ± 0.47	1.82a ± 0.52	2.38b* ± 0.46
Sperm abnormality (%)	44.80 ± 8.35	42.70a± 6.17	43.12a± 6.29

Values are means ± SD. * Significant difference (P<0.05). ** Significant difference (P<0.001).
Similar small letters indicate a non-significant difference (P>0.05).
Different small letters indicate a significant difference (P<001).

Discussion

Seminal fluid analysis of ninety asthenozoospermic patients revealed a significant increase in the liquefaction time associated with a significant decrease in semen volume as compared to the reported values in the available literature (11). Inadequate liquefaction of ejaculated semen after 30 minutes of incubation may indicate a deficiency of prostatic enzymes (14). Low semen volume can be indicative of partial duct obstruction, and androgen deficiency (15). Although the mean of sperm concentration of the studied cases was within normal limit, some of them had sperm concentration below the normal limit. The decline in sperm concentration may be due to several reasons that interfere with spermatogenesis such as environmental factors including exposure to heat, radiation, and pollutions (16). The low percentage of sperm motility and sperm grade activity of the semen samples in the present study could be attributed to the presence of toxic agents in the environment and occupational exposure to toxic chemicals. De Celis et al. in their study reported a reduction in sperm motility in men exposed to environmental toxic substances and they suggested that these substances may cause changes in the physicochemical characteristics of the semen, resulting in increased viscosity and/or incapacity for liquefaction and inhibiting the rapid foreward progression of the sperm (17). Most of the patients had a range of percentage of sperm abnormality between 50 and 59%. This can be due to a variety insults, including: infection, testicular stress (e.g. varicocele, poor sperm production, environmental toxins), and hormonal imbalance (14). Presence of agglutinated sperm in the semen may attribute to the presence of antisperm antibodies in the seminal plasma or to infections of the male genital tract. It has been reported that antisperm antibodies bound to sperm surfaces causing a relatively high incidence of asthenozoospermia (18). Most of the semen samples showed elevated leukocytes and phagocytes concentrations. An association between a large number of leukocytes and decreased semen quality could be attributed to the generation of toxic products, i.e. reactive oxygen species in response to infection (19).

A significant improvement in both sperm motility and sperm grade activity after addition of OT at the concentration of 2 IU/ml indicates that OT has a dose-dependent stimulatory effect on sperm motility parameters. These findings are in agreement with the findings of Fuchs et al. (20). In that study, addition of OT at the concentration of 2 IU/ml to sperm of bull caused a significant increase in the percentage of sperm motility and sperm grade activity.

In presence of SPM, a significant reduction in sperm concentration after sperm activation in the control and OT - treated samples may be due to the elimination of dead and immotile sperm from the original semen, because dead and abnormal with poor motility spermatozoa were unable to swim-up and migrate from the sperm pellet to the upper layer of the culture medium. During in vitro sperm activation using layering technique, the more active spermatozoa selectively accumulated in the upper layering medium and the poor quality sperm were left behind in the activation medium (21). In agreement with a previous study (22), sperm motility percent and sperm grade activity improved significantly after activation in the control and OT-treated samples. It is well known that during layering, only good quality motile sperm were harvested in the upper layer of the medium, accordingly, sperm motility and sperm grade activity being improved in the final preparation. Reduction of abnormal sperm following activation in the studied cases may be explained on the ground that abnormal and more aged spermatozoa are unable to swim-up or migrate to upper layer. This finding agrees with a previous study (23) which indicated that the beneficial effect of layering for selecting morphologically normal mature active sperm.

A significant increase in the percentage of sperm motility and sperm grade activity in the OT-treated

samples as compared with the control samples indicates that OT at the concentration of 2 IU/ml induced best responses in sperm movement. In this study, we proposed several mechanisms by which added OT may improve the sperm motility. One of these mechanisms may be through cAMP intermediate. It has been reported that cAMP stimulates sperm motility by activating a cAMP-dependent protein kinase (24). This, in turn, is believed to induce enhanced sperm tail protein phosphorylation of a defined number of specific proteins, with subsequent enhancement of sperm motility (25). Another mechanism is that OT mediates the uptake of Ca^{2} into the sperm with resultant changes in the sperm movement. It has been proposed that an influx of calcium through the plasma membrane of the sperm activates membrane bound adenylate cyclase, resulting in an elevation in intracellular cAMP levels which, in turn, induces hyperactivation (26). Another possible mechanism of enhancing sperm motility may be through a loss of cholesterol from the plasma membrane. Lin and Kan showed that changes in the cholesterol level in the plasma membrane may increase lateral mobility of the membrane components, supporting hyperactivated motility of the sperm (27).

In absence of SPM, a significant increase in sperm motility percent and sperm grade activity in the OT-treated samples as compared with the control samples indicates that OT in the concentration of 2 IU/ml stimulated sperm motility even in the absence of SPM. On the other hand, the important role of SPM was obvious because when it was used, the outcome of sperm activation resulted in a significant improvement in the sperm function tests in both samples (control and OT-treated samples). It is well known that SPM contains proteins as well as carbohydrates and both are known to provide necessary requirement for sperm motility (28).

The results of this study indicate that OT at the concentration of 2 IU/ml has got a stimulatory effect on sperm motility characteristics; also, the use of SPM succeeds to support this effect.

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