A new simple medium for in vitro sperm activation of asthenozoospermic patients using direct swim-up technique

Assist. Prof. Dr. Muhammad-Baqir M-R. Fakhrildin [1*] and Nisreen Khazal Flayyih [2]
[1] Dept. of Clinical Reproductive Physiology, [2] Dept. of Applied Embryology, Institute of Embryo Research and Infertility Treatment, Al-Nahrain University, Baghdad, IRAQ.
([*] e-mail: art_mbmrfd@yahoo.com; 07901752676).

وسط زرعي جديد لتنشيط النطف خارج الجسم للمرضى المصابين بوهن النطف باستخدام تقنية سباحة النطف للأعلى مباشرة

أ.م.د.محمد باقر محمد رشاد فخرالدين[١] وم.م.نسرين خزعل فليح[٢]
[١] قسم فسلجة التناسل السريري -[٢] قسم الاجنة التطبيقي- معهد ابحاث الاجنة وعلاج العقم- جامعة النهرين

الخلاصة:

صممت هذه الدراسة للمقارنه بين نتائج متغيرات النطف للمرضى اللذين يعانون من وهن النطف قبل وبعد تنشيط النطف خارج الجسم باستخدام وسط زرعي جديد وبسيط وتقنية سباحة النطف للأعلى مباشرة.

اشترك خمسين مريضا مصابا بوهن النطف في هذه الدراسة الحاليه وبعمر يتراوح من ٢٢ـ ٤١ سنه وفترة عقم ٢ـ ١٠ سنه . اخذت عينة السائل المنوي من كل مريض واجري لها فحص السائل المنوي. حضر وسط زرعي بسيط وجديد بالاعتماد على محلول رنكر (Ringer solution) واضافة بيروفت الصوديوم (Sodium pyruvate) و زلال المصل البشري (Human serum albumin ؛تركيز ٥% او ١٠%) وقد اصطلح له مختصر وسط سمارت (SMART medium ؛مختصر وسط زرعي بسيط للتقنيات المساعده على الأنجاب). استخدم و سطي سمارت (SMART) و ايرلز (Earl's) لتنشيط النطف خارج الجسم باستخدام تقنية سباحة النطف للأعلى مباشرة. تم تقييم تركيز النطف والنسبة المئويه للنطف المتحركه والنسبة المئويه لنشاط النطف والنسبة المئويه لشكلياء النطف الطبيعي والنسبة المئويه لتكتل النطف. كما تم تحليل النتائج الأوليه احصائياً.

أظهرت نتائج الدراسة الحاليه تحسن معنوي ($P<0.05$) في أغلب متغيرات النطف بعد التنشيط خارج الجسم عند المقارنه مع قبل التنشيط باستخدام وسط سمارت وتقنيه سباحة النطف للأعلى مباشرة. حصلت زياده معنويه ($P<0.05$) بعد تنشيط النطف في النسب المئويه لكل من الحركة التقدميه (A) للنطف والحركة التقدميه الكلية (A + B) للنطف وشكلياء النطف الطبيعي لكلا مجموعتي المعامله (وسط سمارت) عند المقارنه مع مجموعتي السيطرة (وسط ايرلز). وعلى اي حال ظهرت علاقات عالية المعنويه ($P<0.01$) وايجابيه لكل من تركيز النطف وشكلياء النطف الطبيعي لكل مجاميع السيطرة والمعاملة بين قبل وبعد تنشيط النطف خارج الجسم .

يمكن الاستنتاج ان وسط سمارت مناسباً لتحسين متغيرات النطف للمرضى المصابين بوهن النطف باستخدام تقنية سباحة النطف للأعلى مباشرة. هنالك حاجه لأجراء دراسات اضافية لتقييم تاثيرات وسط سمارت على معدل نجاح التنميه داخل الرحم.

Abstract

This study was designed to compare the results of sperm parameters for asthenozoospermic patients pre- and post-activation *in vitro* using a new simple medium and direct swim-up technique.

Fifty asthenozoospermic patients were involved in the present study with age within range 22-41 years and duration of infertility 2-10 years. From each patient, semen sample was taken and seminal fluid analysis (SFA) was done. A new simple medium was prepared based on Ringer solution supplemented with additives sodium pyruvate and human serum albumin (5% or 10%) which referred as SMART medium (Simple Medium for Assisted Reproductive Techniques; SMART). SMART and Earl's media were used for *in vitro* sperm activation by direct swim-up technique. Sperm concentration, sperm motility (%), sperm grade activity (%), normal sperm morphology (%) and sperm agglutination (%) were assessed. Crude data were statistically analyzed.

From the results of present study, it was appeared that most sperm parameters were significantly ($P<0.05$) enhanced post-activation *in vitro* as compared to pre-activation using SMART medium by direct swim-up technique. After *in vitro* sperm activation, the percentages of progressive sperm activity with grade A, total progressive sperm motility (grades A and B) and normal sperm morphology were significantly increased ($P<0.05$) in both treated groups as compared to control groups. However, highly significant ($P<0.01$) and positive correlations for sperm concentration and normal sperm morphology (%) were observed among groups of pre- and post-activation.

It was concluded that the SMART medium was suitable for enhancement of sperm parameters of asthenozoospermic patients using direct swim-up technique. Further studies are needed to evaluate effects of SMART medium on successful rate of intrauterine insemination.

Keywords: Asthenozoospermia, sperm, activation, SMART medium.

Introduction:

The treatment of infertile couples has made substantial progress over the last two decades. The rapid increase of assisted reproductive techniques (ART) as treatment modalities for infertility during the last two decades has led to the development of a wide range of different sperm preparation methods (1,2). With the advancement in the techniques of ART in humans, the need to improve sperm processing and provision of actively motile spermatozoa has increased tremendously (3).

Several available sperm separation methods are applied with or without centrifugation (4,5). All of these techniques are capable of effectively separating sperm from the seminal plasma, but to varying degree. Recovery rates, motility, morphology and degree of DNA damage vary greatly between procedures (6). However, three alternative methods for washing spermatozoa without centrifugation have been reviewed by Mortimer (7). The first involves direct migration of the sperm cells from the seminal plasma into suspending medium. The second depends on the observation that intact motile spermatozoa differ in density from other cells and from damaged spermatozoa. The third utilizes columns of beads or glass wool, to which the unwanted cells and plasma components adhere.

While washing of human sperm cells by centrifugation and resuspension is a procedure in widespread use, there have been indications that this procedure *per se* may be harmful to the sperm cells (8). For the isolation of functionally normal spermatozoa, direct swim-up or sperm migration technique remains the most popular method, which

separates the sample into motile and non-motile fractions (2). In addition, an ideal sperm preparation method should be cost-effective and allow for processing of a large volume of the ejaculate, which in turn maximizes the number of spermatozoa available (4).

Culture media (CM) provide the spermatozoa with needs that maintain optimal function of spermatozoa to give excellent results during preparation.

Culture media are isotonic with semen to prevent any osmotic shock to spermatozoa and developing embryos during *in vitro* manipulation steps. It provides optimal buffering capacity, and maintains the pH within physiological levels to ensure sperm survival (9). Therefore, in the present study, we compared the efficacy of a new simple culture medium based on Ringer solution enriched with human serum albumin (referred as SMART medium) and Earl's medium using direct swim-up technique.

Materials and Methods:

1- Infertile males:

This study was conducted in the Institute of Embryo Research and Infertility Treatment, Al-Nahrain University from January to March - 2009, and fifty asthenozoospermic patients were included. The range of ages was between 22-41 years and duration of infertility 2-10 years.

2- Seminal fluid analysis (SFA):

Most of seminal fluid samples were collected, after 3-5 days of sexual abstinence, directly into clean dry and sterile dish by masturbation in especially room near the laboratory of semen examination. The normal semen specimen was liquefied within 60 minutes at 37 ºC. Macroscopic parameters of seminal fluid were evaluated (10). Then, sperm parameters were assessed microscopically (11).

3- Preparation of culture media and *in vitro* sperm activation technique:

In present study, two types of culture media were used involving SMART and Earl's media. Ringer solution (Pharmaceutical solution industry, Jeddah, KSA; chemical composition per liter is bicarbonate 29 mmol/ l, sodium lactate3.2 g, sodium chloride 6.0g, potassium chloride 0.4 g and calcium chloride 0.27g) was taken within special non toxic bottle and specific additives were added including 0.5 g phenol red (Panreac Quimica SA, Spain) and sodium pyruvate (0.01g; Prolabo, France). Human serum albumin bottle (HSA, 20%; Biotest company) contains 100 mL solution (each mL contains 0.2g serum protein) ready to use. Just before *in vitro* sperm activation process, either 5% or 10% was added for treated 1 or treated 2 groups; respectively.

Powder of Earl's balanced salts (8.7g/L; Sigma Chemical Company, USA) dissolved in one litter of Milli-Q distilled water and special additives were involving sodium pyruvate 0.01g, sodium bicarbonate 0.21g and ampicillin 0.08g. The pH of prepared Earl's medium was adjusted between 7.2-7.4. Then, prepared Earl's medium was sterilized using Millipore filter (0.22 µ;Ministar, Sartorius AG, Goettingen, Germany) and divided into equal parts (10 mL) placed in a graduated conical falcon tubes. one mL of the prepared culture medium alone was added to the test tube,and then 1 mL of the liquefied semen was layered beneath a culture medium .After incubation for 30 minute at 5% CO_2 and 37Cº ,one drop (10µL) of the mixture was aspirated by pasture pipette from upper layer which separate semen than culture media .Then ,examined under light microscope at (400X) magnification (12).

Each semen sample was divided into 4 equal parts and 1 mL of 4 types of prepared culture media was added according to four groups including 2 control groups and 2 treated groups.

4- Experimental design:

In this study, fifty asthenozoospermic patients were shared in the present study, and one semen sample was taken from patient. Each semen sample was divided into 4 aliquots placed in a graduated conical glass tube. One mL of each 4 types of prepared media added according to 4 groups as:

Group 1: control 1; prepared Earl's medium.

Group 2: control 2; prepared Ringer solution without HSA (SMART medium).

Group 3: treated 1; prepared SMART medium enriched with 5% HSA.

Group 4: treated 2; prepared SMART medium enriched with 10% HSA.

5- Statistical analysis:

Perform with SPSS (Statistical package for social sciences) versions 12. The analysis done using paired t-test to compare pre-and post activation values of the study in which the data expressed as mean ±SEM. MANOVA was applied to compare among different groups of both *in vitro* sperm activation techniques. Also, correlation and regression test was used (13). Differences between values of means were considered statistically significant at ($P<0.05$).

Results:

Results of the present study appeared that the most sperm parameters were significantly ($P<0.05$) enhanced post-activation *in vitro* as compared to pre-activation using direct swim-up technique (Table 1). Results of *in vitro* sperm activation observed that percentages of progressive sperm activity with grade A, total progressive sperm motility (grades A and B) and normal sperm morphology were significantly increased ($P<0.05$) in both treated groups as compared to control groups. Also, sperm concentration and percentage of sperm motility for treated-2 group were improved significantly ($P<0.05$) as compared to both control groups (Table 1). From same table, percentage of sperm immotility for treated-2 group was decreased significantly ($P<0.05$) as compared to both control groups.

Table (2) shows the correlations for sperm parameters among different groups of pre- and post-activation using direct swim-up technique. Highly significant ($P<0.01$) and positive correlations for sperm concentration and normal sperm morphology (%) were assessed among groups of pre- and post-activation, between both control groups ($r = 0.934$; $P<0.001$), between treated-1 group and both control groups ($r = 0.919$; $P<0.001$ and $r = 0.885$; $P<0.001$; respectively), between treated-2 group and between both control groups ($r = 0.931$; $P<0.001$ and $r = 0.896$; $P<0.001$; respectively). However, percentages of sperm motility, sperm grade activity with grades A, C and D have highly significant ($P<0.01$) and positive correlations between both control groups, between treated-1 group and both control groups, between treated-2 group and between both control groups (Table 2). From same table, percentages of grade B sperm activity and total progressive sperm motility have positive and highly significant ($P<0.01$) correlations were evaluated for control-2 group and pre-activation group ($r = 0.858$; $P<0.01$ and $r = 0.928$; $P<0.001$; respectively), between both control groups ($r = 0.913$; $P<0.001$ and $r = 0.925$; $P<0.001$; respectively), between treated-1 group and both control groups, between treated-2 group and between both control groups.

Table 1: Sperm parameters for infertile patients complaining from asthenozoospermia pre- and post-activation *in vitro* by direct swim-up technique using Earl's and SMART media and different additives

Sperm parameters		Pre – activation	Post-activation			
			Control – 1; Earl's medium	**Control – 2;** SMART medium	**Treated - 1;** SMART medium + 5% HSA	**Treated - 2;** SMART medium + 10% HSA
Sperm concentration (million/mL)		46.30 ± 2.26	29.90 * ± 1.69	29.74 * ± 1.64	31.02 * ± 1.70	31.14 * $ ± 1.68
Sperm Motility (%)		41.65 ± 1.86	81.30 * ± 1.19	80.94 * ± 1.29	82.80 * ± 1.50	89.48 * $ ± 1.13
Sperm grade activity (%)	A	5.18 ± 1.53	16.18 * ± 1.15	17.02 * ± 0.97	18.72 * # ± 0.97	19.46 * $ ± 0.91
	B	24.44 ± 1.73	48.32 * ± 1.35	47.04 * ± 1.59	48.70 * ± 1.52	49.03 * ± 1.37
	C	12.14 ± 1.22	17.42 * ± 1.22	17.06 * ± 1.25	15.74 * ± 1.02	15.48 * ± 1.06
	D	58.34 ± 1.76	18.56 * ± 1.16	19.08 * ± 1.30	17.92 * ± 1.56	15.86 * $ ± 1.80
Total progressive sperm motility (%)		37.64 ± 2.01	64.12 * ± 1.66	64.40 * ± 1.67	67.84 * # ± 1.66	68.36 * $ ± 1.36
Normal sperm morphology (%)		38.68 ±1.76	42.76 * ± 1.19	44.38 * ± 1.36	46.20 * # ± 1.63	47.64 * $ ± 1.55

* : Significant difference (P<0.05) between pre- and post-activation.
# : Significant difference (P<0.05) between treated – 1 and control groups.
$: Significant difference (P<0.05) between treated – 2 and control groups.
a : Significant difference (P<0.05) between control – 1 and control – 2 groups.
b : Significant difference (P<0.05) between treated – 1 and treated – 2 groups.

Table 2: Correlations among sperm parameters for infertile patients complaining from oligoasthenozoospermia pre- and post-activation *in vitro* by direct swim-up technique using Earl's and SMART media and different additives

Sperm parameters		Pre – activation	Post-activation			
			Control – 1; Earl's medium	**Control – 2;** SMART medium	**Treated - 1;** SMART medium + 5% HSA	**Treated - 2;** SMART medium + 10% HSA
Sperm concentration (million/mL)		46.30 ± 2.26	**a**	**a ; b**	**a ; c**	**a ; d ; e**
Sperm motility (%)		41.65 ± 1.86		**b**	**c**	**d ; e**
Sperm grade activity (%)	A	5.18 ± 1.53		**b**	**c**	**d ; e**
	B	24.44 ± 1.73		**a ; b**	**c**	**d ; e**
	C	12.14 ± 1.22		**b**	**c**	**d ; e**
	D	58.34 ± 1.76		**b**	**c**	**d ; e**
Total progressive sperm motility (%)		37.64 ± 2.01		**a ; b**	**a ; c**	**d ; e**
Normal sperm morphology (%)		38.68 ±1.76	**a**	**a ; b**	**a ; c**	**a ; d ; e**

a : Highly significant (P<0.01) positive correlation between pre- and post-activation.
b : Highly significant (P<0.01) positive correlation between control groups.
c : Highly significant (P<0.01) positive correlation between treated – 1 and control groups.
d : Highly significant (P<0.01) positive correlation between treated – 2 and control groups.
e : Highly significant (P<0.01) positive correlation between treated groups.

Discussion:

Assisted reproductive techniques (ART) have become the treatment of choice in many cases of male and female infertility. The quality of semen samples is one of the factors determining successful assisted reproduction. A variety of sperm preparation techniques are available to select motile spermatozoa (14).

It is noticed that the use of *in vitro* culture media increases sperm motility. The reason is that the seminal fluid with high viscosity obstructs sperm progressive motility so that the uses of *in vitro* media with aqueous nature lead to decrease the viscosity of the seminal fluid and as a result sperms move more freely. This is why sperm motility increases with the use of *in vitro* media (15). In addition to the high viscosity, the seminal fluid contains substituents which obstruct sperm forward progressive motility, as antisperm antibodies, bacteria, leukocytes, and damaging secretion from the seminal vesicles, thus the use of there *in vitro* media decrease the damage occurring by these

substituents (16). Also, when spermatozoa are free of seminal plasma within culture medium have capacity to achieve capacitation as a results of removal of both decapacitation factor and acrostatin "acrosin inhibitor" which prevents oocytes fertilization (17).

In the present study, we applied direct sperm technique for sperm preparation from asthenozoospermic patients. In an attempt to prevent damage by centrifugation and generation of reactive oxygen species (ROS), the method of sperm preparation has been developed using direct swim-up from original sperm sample (18). The letter method has recently been recommended for sperm preparation (19). There was no significant difference between DNA fragmentation in the original sample and spermatozoa prepared by the direct swim–up method. Although the DNA fragmentation in the spermatozoa processed by the direct method was lower than that in the original semen, it was higher than that seen in the centrifugation swim-up procedure (20).

There is significant ($P<0.05$) increase in the sperm concentration, percentages of sperm motility, progressive sperm activity (grades A and B) was observed after activation. This was an expected result when using the activation media containing pyruvate (21). This study cleared that the use of modified Earl's medium in activation sperm quality *in vitro* fertilization. Similar results were reported using same medium. Modified Earl's medium contains what sperm need to stay alive and what *in vitro* activation process require because it contains inorganic ions and pyruvate as energy source (22).

Capacitation is an essential process that a spermatozoon must undergo in the female reproductive tract to become able to fertilize an egg. As a consequence of capacitation sperm become responsive to stimuli that induce the acrosome reaction and develop a particular pattern of motility known as hyperactivation (23). Capacitation can be achieved *in vitro* by incubation sperm in defined media. Two essential components of these media are serum albumin and bicarbonate is believe to facilitated the efflux of cholesterol from the sperm plasma membrane by acting as an acceptor for the lipid (24). Whereas entry of the bicarbonate ion into spermatozoa has been shown to be involve an increase in intracellular pH during capacitation (25).

Human serum albumin used in culture media acts as a powerful antioxidant that prevents oxidative stress-induced damage (26). That means the antioxidant effect of albumin play an important role in preserving sperms damage and makes its motility more easily. Furthermore, albumin is considered as nutrition media for sperms that supplied them, those proteins in form of albumin which is found in high concentration in seminal plasma that makes up about one third of the protein content of semen. Sperm motility appeared to be more negatively influenced when the medium lacked protein (27).

From the results of the present study, it was concluded that the SMART medium was suitable for enhancement of sperm parameters of asthenozoospermic patients using direct swim-up technique. Further studies are needed to evaluate effects of SMART medium on successful rate of intrauterine insemination.

References:

1. 1. Allamaneni, SR.; Agarwal, A.; Rama, S.; Ranganathan. P. and Sharma, R.K. (2005). Comparative study on density gradients and swim-up preparation Techniques utilizing neat and cryopreserved spermatozoa. Asian J. Androl.7: 86-92.
2. 2. Boomsma, C.M.; Heineman, M.J.; Cohlen, B.J. and Farquhar, C. (2007). Semen preparation techniques for intrauterine insemination. Systematic Reviews. 4: 1-16.
3. 3. Noah. L.; Jain, T.; Missmer, S.A. and Hornstein, M.D. (2004). Trends in assisted reproductive technology. N. Engl. J. Med. 351: 398-399.
4. 4. Inaudi, P.; Petrillinthei, S.; Joghtapour, A.; Trusso, P. and Petraglia, F. (2002). Reduction of steps preparation of motile sperm for intrauterine insemination does not reduce efficacy of the procedure: Simplified one step swim-up method versus classic swim-up. Hum. Reprod. 17: 1288-1291.
5. 5. Henkel, R. and Schill, W. (2003). Sperm preparation for ART. Reprod. Biol. & Endocrinol .1: 108-120.
6. 6. Vaught, L.K. and Dodge, S.T. (2007). Sperm processing different methods produce more dependable results for intrauterine insemination. Merion publications 2900 horizon drive, king of prussia, PA 19406.800-355-5627.publishers of advance news magazines http:/www.advanceweb.com.
7. 7. Mortimer, D. (2000). Sperm preparation methods. Andrology Lab Corner J. Androl. 21: 334-340.
8. 8. Alvarez, J.A.; Castilla, D. and Martinez, M. (2003). Biological variation seminal parameters in healthy subjects. Hum. Reprod.18: 2082-2088.
9. 9. Ranch, D.K. (2005). Methods for *in-vitro* fertilization. United States patent: http:/www.pharmcast.com./patent to sub web/patent Home.htm (Int.file).
10. 10. World Health Organization (WHO; 1992). Seminal fluid analysis. Mortimer D. Practical laboratory andrology. New York: Oxford University Press. Pp.:1-3.
11. 11. Fakhrildin, M-B. M-R. (2000). Materials and Methods. In: The Impact of Seminal Fluid Infections and Treatment on Sperm Functions and Fertilizing Capacity in Infertile patients. Ph. D. Thesis in Reproductive Physiology and Assisted Reproductive Technologies, College of Science, Al-Mustansiriya University. Pp: 72-85.
12. 12. Al-Zubaidi, U.I.M. (2008). Comparative study on the effects of Sildenafil Citrate treatment on parameters of Human and Mice spermatozoa. M. Sc. Thesis in Applied Embryology, Institute of Embryo Research and Infertility Treatment, Al-Nahrain University.
13. 13. Daneil, W.W. (1988). Multiple regression and correlation. In: Biostatistics: A Foundation for Analysis in the Health Sciences. Daneil, W.W.(Ed.). John Wiley and Sons Incorp. USA. 4th Edition. Pp:317-351.
14. 14. Said,T.M.; Grunewald, S.; Pasch, U.; Glander, H.J.; Baumann, T.; Kriegal, C.; Liang, L. and Agarwal, A. (2005). Advantage of combining magnetic.
15. 15. Makler, A.; Fisher, M.; Murillo, O.; Laufer, N.; Dechereny, A. and Naftilin, F. (1984). Factor affecting sperm motility. IX. Survival of spermatozoa in various biological media and under different gaseous compositions. Fertil. Steril. 41: 428-432.

16. 16. Makler, A. and Jacobi, P. (1981). Factor affecting sperm motility. V. Washing and resuspension of human spermatozoa in various artificial media. Fertil. Steril. 35: 442-446.

17. 17. Chen, Y.; Li, J.; Simkin, M.E.; Yang, X. and Foote, RH. (1989). Fertility of fresh and frozen rabbit semen inseminated at different times is indicative of male differences in capacitation time. Biol. Reprod. 41: 484-493.

18. 18. Mortimer, D. (1999). Sperm preparation techniques and iatrogenic failures of *in vitro* fertilization. Hum. Reprod. 6: 173-176.

19. 19. World Health Organization(WHO; 1999). Laboratory manual for the examination of the human semen and sperm-cervical interaction 4th edition. Cambridge University Press, Cambridge, UK. Pp:1-12.

20. 20. Younglai, E.V.; Holt, D.; Brown, P.; Jurisicova, A. and Casper, R.F. (2001). Sperm swim-up techniques and DNA fragmentation: Hum. Reprod. 9:1950-1953.

21. 21. Moreno, R.D.; Sonto, J.R.; Edward, K.L.; Wessel,G.M. and Schatten, J. (2000). The Golgi apparatus segregates from the lysosomal/ acrosomal vesicle during rhesus spermiogenesis: structural alteration. Deevelop. Bio. 219: 334-349.

22. 22. Al-Taji, H.A. (1994). Sperm activation and intrauterine insemination: The effect of sperm concentration and culture media on sperm activation potential *in vitro*. M.Sc. Thesis. College of Medicine, University of Baghdad.

23. 23. Yanagimachi, R. (1994). Mammalian fertilization. In: The physiology of reproduction. Knobil, E. and Neill, J.D. (Eds.). New York: Raven Press. Pp:189-317.

24. 24. Visconti, P.E.; Westbrook,V.A.; Chertinhin, O.; Demarko, I.; Sleigh, S.; Diekman, AB. (2002). Novel signaling pathways involved in sperm acquisition of fertilizing capacity. J. Reprod. Immuno. 53: 133-150.

25. 25. Zeng, Y.; Oberdorf, J.A. and Florman, H.M. (1996). pH regulation in mouse sperm: Identification of Na(+)-Cl(-) and HCO_3 (-) dependent and arylaminobenzoate-depending regulatory mechanism and characterization of their roles in sperm capacitation. Dev. Biol. 173: 510-520.

26. 26. Sikka, S.C. (2004). Role oxidative stress and antioxidants in Andrology and Assisted Reproductive Technology. J. Androl. 25: 786-799.

27. Owen, D.H. and Katz, D.F. (2005). A Review of the physical and chemical properties of human semen and the formulation of a semen stimulant. J. Androl. 26: 876-885.