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In Vitro Sperm Preparation by Progesterone, Pentoxifylline and *Glycyrrhiza Glabra* for Asthenozoospermic Men

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Abstract – Background: Results of artificial insemination (AI) still does not exceed 20-25% because of different factors one of them is male factor infertility. Therefore, the global companies concern on the culture media that used for activation of semen samples of infertile men. However, till now, there are many semen samples did not response to these media leading to failure in AI. The objective of this study is to improve certain sperm function parameters *in vitro* of asthenoteratospermic patients by using novel combination motility stimulants substances that improved medium to be used for assisted reproductive technologies (such as artificial insemination) in future.

One hundred asthenozoospermic patients are involved in this study. They are divided randomly into equal four groups. Each semen sample had been divided into two parts. One part was considered as a control by using Ham's F10 medium and the other part is considered as treated group by adding the following substances in combination; Pentoxifylline (PF) 1mg/ml, Progesterone (P) 0.409 mg/ml and *Glycyrrhiza glabra* (Gg) 1mg/ml in the following groups. Group 1:PF and Gg, Group2: PF and P,Group3: Gg and P, and Group4:PF,Gg and P. Sperm function parameters are examined before and following *in vitro* preparation using wash and spin technique.

Keywords : pentoxifylline, glycyrrhiza glabra, progeste-rone, in vitro sperm preparation.

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Keywords : pentoxifylline, glycyrrhiza glabra, progesterone, in vitro sperm preparation.

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I. Introduction

Since about half of the infertility cases are due to male factors, a detailed sperm analysis became the most important examination to be performed in the approach to the infertile couples (Karpuz *et al.*, 2007). Basically, sperm count, motility and percentage of normal sperms are conventional criteria for semen quality (Check *et al.*, 1992). Among these parameters, sperm morphology and motility are the best criteria for demonstrating the fertilization capacity of a male (Bonde *et al.,* 1998). Sperm motility gives a measure of the integrity of the sperm axoneme and tail structures as well as the metabolic machinery of the mitochondria, and sperm morphology is a surrogate measure of the integrity of DNA packaging and the quality of spermatogenesis (Pacey, 2006).

One of the major causes of male factor infertility is related to asthenozoospermia which is characterized by reduced forward motility (WHO grade a+b sperm motility <50% or a <25%) or absent sperm motility in fresh ejaculate (Mehrannia *et al.,* 2009). Although there is a general consensus that ultrastructural anomalies underlie severe asthenozoospermia (Chemes *et al.*, 1998), the etiology and pathogenesis of temporary and/or mild asthenozoospermia remains, for the most part, undefined. (Yunes *et al.,*2003).

Sperm motility is an important factor to consider in couples pursuing assisted reproductive technologies (ART). Several studies have demonstrated the influence of sperm motility on the outcomes of various ARTs, including intrauterine insemination (IUI) (Yalti *et al.,* 2004), conventional *in vitro* fertilization (IVF) (Repping *et al.*, 2002), and IVF with intracytoplasmic sperm injection (ICSI) (Stalf *et al.,* 2005).

Over the last decade, it have been noticed the increasing use of assisted reproductive technologies (ARTs) to overcome the problems of infertile couple (Katz *et al.*, 2002). ARTs are now routinely proposed in the cases of male partner of infertile couple who wish to have a child (Kaewnoonual *et al.*, 2008). Even that, the result of AI still do not exceed 20% as a result of different factors one of them is male factor through activation technique. Therefore, the objective of the present study is to investigate the possibility of using different

components to enhance the certain sperm functions *in vitro*. The study will be achieved by selection of certain substances such as Pentoxifylline, Progesterone and medical plants as *Glycyrrhiza glabra* for *in vitro* activation of infertile human semen.

II. Materials and Methods

a) Patients

The study was carried out in the High Institute for Embryo Researches and Infertility Treatment, AL-Nahrain University. One hundred with mild asthenozoospermia and/or other male factor infertility were involved in this research. The patient's age ranged between 24-50 years. The clinical examination performed by a consultant urologist in the charge of male infertility Unit in the Institute including presence or absence varicocele, cryptoorchidism, hydrocele, hernia and others.

b) Seminal Fluid Analysis

Semen samples were collected by masturbation into wide-mouth glass or plastic containers, supplied by the laboratory, after 3–7 days of sexual abstinence. The sample was transported to the laboratory immediately and placed in an incubator at 37 C^{o} till complete liquefaction, then semen samples were analyzed by a macroscopic and microscopic examination using standardization of WHO (1999).

c) Preparation of Glycyrrhiza Glabra Extract

This extract was provided in a powder from which is water –soluble.The extract was obtained by crushing the root of plant and extracted as described by Chakravarty, (1976). Storage of Glycyrrhiza was done in well –closed container protected from light and moisture (Maisonneave, 1971).

d) Preparation of Glycyrriza glabra for Human Sperm Activation In Vitro

The concentration of *Glycyrrhiza glabra* of 0.1% was prepared by adding 10 mg from Gg extract to 10 ml PBS in plastic test tubes contained broad spectrum antibiotic (Ampicillin 0.004g) to prevent bacterial growth, (Al-Dujaily and Al-Shammary, 2008). The solution was filtered using Millipore (0.45 μM).

e) Preparation of Pentoxifylline Stock Solution

Pentoxifylline powder (Sigma, Germany) 10 mg was dissolved in 10 ml of Phosphate buffered saline (PBS). These concentrations prepared daily under sterile condition using Millipore filter, 0.45 μM.

f) Preparation of Progesterone

The medroxyprogesterone acetate (150 mg/mL vial, Depo Provera ®, Pharmacia) was used in a dose of 0.409 mg/ml and mixed with one ml of PBS. These concentrations prepared daily under sterile condition using Millipore filter, 0.45µM.

All the media were fixed at a pH 7.5-8, temperature 22-25 C^{O} and Osmolarity 285-300 mOsmol/L.

g) In Vitro Activation Technique

After liquefaction of human semen using wash and spin method (Avery and Elder, 1992).

Each semen sample had been divided into two parts. One part was considered as a control by using Ham's F10 medium(Sigma,Germany) and the other part is considered as treated group by adding the following substances in combination; Pentoxifylline (PF) 1mg/ml, Progesterone (P) 0.409 mg/ml and *Glycyrrhiza glabra* (Gg) 1mg/ml in the following groups. Group 1:PF and Gg ,Group2: PF and P,Group3: Gg and P, and Group4:PF, Gg and P. Certain sperm function parameters are examined following *in vitro* activation as described by WHO (1999) too.

h) Statistical Analysis

Data from treatment and control groups were expressed as mean ±SEM and using Student's t-test to compare values from experimental and control groups. Differences between values were considered significant at $P<0.05$. Analysis of variance (ANOVA) was used to compare the differences between the four prepared media. When F values reach the significant level at 5%, least significant difference (LSD) test was used, (Sorile, 1995).

III. Results

a) Effect of pentoxifylline glycyrrhiza glabra and progesterone on human sperm in vitro

Tables 1, 2, 3 and 4 shows that the activation of human sperm *in vitro* with both control (Hams F-10 medium) and treated (PF and Gg medium, Table -1; PF and P medium, Table -2; *Gg* and P medium, Table-3 and *PF, Gg* and P medium Table-4) groups caused a significant ($P<0.05/P<0.01$) decrease in sperm concentration compared to before activation. There is no significant ($P>0.05$) difference in the mean of sperm concentration between control and treated groups. Regarding sperm motility, although there is highly significant ($P<0.01$) increase in sperm motility (grade a) in both control and treated groups compared to before activation, there is highly significant ($P<0.01$) increase in sperm motility (grade a) in treated group when compared to control. A highly significant ($P<0.01$) improvement in sperm motility (grade b) in treated semen samples is recorded compared to before activation, while there is a significant ($P<0.05$) increase in sperm motility (grade b) in control when compared to before activation. Activation of human sperm caused a highly significant ($P<0.01$) increase in the percentage of morphologically normal sperm in both control and treated group when compared to before activation, while there is a significant ($P<0.05$) increment in morphologically normal sperm of treated semen samples when compared to control semen samples.

Table 1 : Effect of pentoxifylline and *glycyrrhiza glabra* on certain sperm function character of asthenospermic patient following *in vitro* activation technique

Certain Sperm Function Characters	*In vitro* activation		
	Before Activation	After Activation Control	After Activation PF+Gg
	Mean± SEM	Mean ±SEM	Mean±SEM
Sperm Concentration (million/ml)	43.12± 3.69	^{A} 20.28± 2.77	^{A} 22.64 ± 2.55
Sperm Motility Grade a(%)	2.8 ±0.752	^{b} 16.72 ±1.21	^{a} 27.08±1.10 ^{c}
Sperm Motility Grade b(%)	31.48 ±1.99	^{B} 40.0 ±0.913	^{a}50.80 ±1.72 ^{C}
Sperm Motility Grades a+b(%)	34.28 ±2.55	^{B} 56.72 ± 3.88	^{a}7.08 ±3.72 ^{c}
Morphologically Normal Sperm (%)	47.64 ±1.62	^{b} 78.0 ±1.66	^{a}84.60 ±1.98 ^{C}

Different small letters indicate a highly significance P<0.01
Different capital letters indicate a significance P<0.05
No. of sample used = 25
Ham's F-10 *medium* used as control

Table 2 : Effect of Pentoxifylline and Progesterone on certain sperm function characters of asthenospermic patient following *in vitro* activation

Certain Sperm Function Characters	*In vitro* activation		
	Before Activation	After Activation Control	After Activation PF+P
	Mean ± SEM	Mean ±SEM	Mean ±SEM
Sperm Concentration (million/ml)	43.24±+2.98	^{a} 19.84±1.94	^{a} 20.52±1.60
Motility Grade a(%)	3.96±0.524	^{B} 13.6±1.02^{B}	^{a} 21.40±1.40 ^{C}
Motility Grade b(%)	31.2±1.54	^{B} 49.2±0.85	^{a} 48.20±1.25
Motility Grades a+b(%)	35.16±2.97	^{B} 52.8±2.77^{B}	^{a} 69.6±3.55 ^{C}
Morphologically Normal Sperm (%)	44.84±1.75	^{B} 79.60±1.47	^{a} 80.80±1.57

Different small letters indicate a highly significance P<0.01
Different capital letters indicate a significance P<0.05
No. of sample used = 25 Ham's F-10 *medium* used as control

Table 3 : Effect of glycyrrhiza glabra and progesterone on certain sperm function characters of asthenospermic patient following in vitro activation

Certain Sperm function Characters	*In vitro* activation		
	Before Activation	After Activation Control	After Activation Gg+ P
	Mean ±SEM	Mean ±SEM	Mean ±SEM
Sperm Concentration (million/ml)	41.64+±2.5	^{a} 16.24±1.77	^{a} 13.4±1.37 ^{C}
Motility Grade a(%)	2.2±0.551	^{B} 13.4±0.945	^{a} 15.88±1.39 ^{c}
Motility Grade b(%)	37.36±0.9	^{B} 49.4±0.781	^{a} 46.12±1.38
Motility Grades a+b(%)	39.56±2.44	^{B} 62.8±4.25	^{a} 62.0±3.98
Morphologically Normal Sperm (%)	44.56±1.21	^{B} 78.6±1.62	^{a} 84.4±1.83 ^{C}

Different small letters indicate a highly significance P<0.01
Different capital letters indicate a significance P<0.05
No. of sample used = 25
Ham's F-10 *medium* used as control

Table 4 : Effect of *Glycyrrhiza glabra*, Progesterone and Pentoxifylline on certain sperm function character of asthenospermic patient following *in vitro* activation

Certain Sperm Function Characters	*In vitro* activation		
	Before Activation	After Activation Control	After Activation PF+Gg+P
	Mean±SEM	Mean ±SEM	Mean ±SEM
Sperm Concentration (million/ml)	41.64±2.2	^{a} 18.4±1.37	^{a} 16.08±1.28
Sperm Motility Grade a (%)	1.6±0.523	^{B} 12.8±0.961	^{a} 18.04±1.12 ^{C}
Sperm Motility Grade b (%)	36.64±0.991	^{B} 49.4±0.666	^{A} 47.4±0.918
Sperm Motility Grade a+b (%)	38.24±3.65	^{b} 61.12±3.88	^{a} 65.44±4.11
Morphologically Normal Sperm (%)	47.12±1.25	^{B} 79.8±2.88	^{a} 84.4±1.27 ^{C}

Different small letters indicate a highly significance P<0.01
Different capital letters indicate a significance P<0.05
No. of sample used = 25
Ham's F-10 *medium* used as control

b) Comparison the Results of Certain Sperm Function Characters following in vitro Activation between PF +GG, PF+P, GG+P and PF+P+GG Media

The sperm concentration following *in vitro* activation with PF and Gg medium is highly significant (P<0.01) increase compared with other media (Table-5). A significant (P<0.05) increase in sperm concentration is observed following *in vitro* activation with PF+P than that of Gg+P and PF+P+Gg media. The results revealed highly significant increment (P<0.01) in the percentage of total active sperm motility grades (A+B) and forward progressive sperm motility grade (A) with a significant improvement (P<0.05) in the percentage of progressive sperm motility grade (B) and morphologically normal sperm when using PF and Gg medium in comparison with control medium and other combination medium following 10, 30 and 60 minutes incubation.

Table 5 : Comparison results of certain sperm function characters following *in vitro* activation between PF +Gg, PF+P, Gg+P and PF+P+Gg media

Sperm Function Characters	In vitro Activation			
	After Activation PF+Gg	After Activation PF+P	After Activation Gg+P	After Activation PF+Gg+P
	Mean± SEM	Mean± SEM	Mean± SEM	Mean± SEM
Sperm Concentration (million/ml)	25.64 ±2.55**	20.52±1.60^{+}	13.4±1.37	16.08±1.28
Active Motility Grade a (%)	27.28±1.10**	21.40±1.40	15.88±1.39	18.04±1.12
Active Motility Grade b(%)	50.80 ± 1.72	48.20±1.25	46.12±1.38	47.4±0.918
Active Motility Grades a+b (%)	77.08 ±3.72*	69.6±3.55	62.0±3.98	65.44±4.11
Morphologically Normal Sperm (%)	84.60 ± 1.98	80.80±1.57	84.4±1.83	84.4±1.27

No. Samples per group=25
*P<0.05 significantly different from other media
**P<0.001 significantly different from other media
+P <0.05 significantly different from Gg+P and PF+P+Gg media.

IV. Discussion

There was a significant reduction in the concentration of spermatozoa in both control and treated groups following *in vitro* activation. This is due to the inability of the dead and abnormal sperms with poor motility spermatozoa to swim up and migrate from sperm pellet to the upper layer of culture medium. These results were in agreement with other studies using culture for the separation and activation of sperm *in vitro* (Check *et al.*, 1993 and Allaw, 1999).

Regarding sperm motility, there was highly significant increase in sperm motility (grade a and grades a+b), while there is a significant increase in sperm motility (grade b) in treated group. This in agreement with studies that revealed a significant improvement in the sperm motility percentage with forward progressive movement (grade a) and hyperactivation, following activation by PF and Gg, independently (Al–Dujaily *et al*., 2006 and Al–Dujaily *et al*.,2007). Adding PF at a concentration of 1mg/ ml usually results in the stimulation of various aspects of sperm functions ,including motility, acrosomal reaction ,penetration of zona free hamster oocyte and the binding of the zona pellucida in hemizona assay (Tesarik *et al.,* 1992a and Nassar *et al* .,1998). Thus, these observations were consistent with Al-Dujaily *et al.,* (2007) study that show the sperm motility percentage of mild asthenospermic semen was significantly improved by the addition of PF at a concentration of 1mg/ ml, at the same time Al-Dujaily and Al-Shammary, (2008) noticed that addition of Gg 1 mg/ml revealed a significant improvement in active sperm motility (grade a+b).

This study believed that the medium contains PF and Gg gave excellent improvement in sperm motility because PF may increase curvi-linear Velocity (VCL) , straight line velocity (VSL), ALH (lateral head displacement) , BCF (beat cross frequency) and sperm hyperactivation in both normospermic and asthenospermic specimen (Tesarik *et al* ., 1992b). In addition, an increased ALH may improve VCL by moving sperm head further from side to side. An increase in ALH and VCL may reflect an increase in energy consumption facilitated by pentoxifylline (Ward and Clissod, 1987) .On the other hand, Gg contents (glucose and fructose) are considered to be a source of energy of sperm motility (Al-Dujaily and Alsaadi, 2009). The sum of these may make the penetration power of sperm greater leading to improving fertilization rate (Sheena, *et al*., 1993; Al-Dujaily and Al-saadi, 2009). Furthermore, Gg contain proteins, vitamins (E, C, folic acid and others) and sugars like: glucose, sucrose, fructose and maltose (Taylor, 2004), all of these substances stimulate sperm motility and the grade activity of forward movement. Protein and amino acids, which maintain sperm osmolarity and in turn integrity of sperm cell membrane (AL- Dujaily *et al*., 2006). *In vitro* studies showed that vitamins E and C are major chain – breaking antioxidants in sperm membranes and appears to have a dose dependent protective effect (Agarwal and Prabakaran, 2005).

It is concluded that the best combination could be added as sperm motility stimulants for the culture medium used for *in vitro* activation of asthenoteratospermic patients alone or with other male infertility factors is PF and Gg. More researches are recommended to utilize this medium in the ART centers.

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