Investigation of the Effect of Different Concentrations of Sildenafil Citrate (Viagra) ™ On Sperm Parameters during In Vitro Human Sperm Activation in Asthenozoospermic Patients

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Abstract

This study was designed to investigate the effect of different concentration of sildenafil citrate on sperm parameters during *in vitro* human sperm activation in asthenozoospermic patients. Forty five infertile patients were involved in this study. Modified Earl's medium (MEM) was used only as a control group (G1:15 patients) and two treated group with different concentrations of sildenafil citrate (G2: 15 patients; MEM supplemented with 100 ngm/ml) and (G3: 15 patients; MEM supplemented with 200 ngm/ml) for *in vitro* sperm processing. spermatozoa were prepared by using direct layering technique. Furthermore, sperm concentration, sperm motility, progressive sperm motility, sperm agglutination, and normal sperm morphology were evaluated according to standard WHO criteria (1999). For preparation technique, sperm prepared and incubated for 30 minute in 5% CO2 at 37C˚ after sperm processing. The Results of the present study indicated a significant differences ($P<0.05$) in sperm motility (%) for *in vitro* post-preparation when compared with the control group. In addition, a lower concentration (100 ngm/ml) of sildenafil citrate showed a significant enhancement in progressive sperm motility as compared with the control group. It was concluded that a low dose of sildenafil citrate concentration give the best results in sperm preparation by increase motility and progressive motility. Also, further studies are recommended to assess the effect of sildenafil citrate on in vitro assisted reproductive technologies results (ART) and intrauterine insemination (IUI).

1- Introduction

Sildenafil citrate is known chemically as 1- 4- ethanoxy-3- (6,7-dihydro-1-methyl-7-oxo-3-propyl-1- H- pyrazolo- (4,3-D) pyrimidin-5-yl- phenyl sulphonyl-4 methyl piperazine citrate (1). The mechanistic analysis of solubility and permeability at different pHs will facilitate to optimize transmucosal delivery of sildenafil and other ionizable drugs with low aqueous solubility (2). As well, absorption of sildenafil after oral administration is rapidly absorbed from gastrointestinal tract. The peak plasma concentrations of sildenafil are reached in less than 1 hour and bioavailability of sildenafil about 41%. Sildenafil is approximalely 96% bound to plasma protein, leaving 4% of the total drug circulating in plasma as unbound free drug available for interaction with PDE5 at its intracellular receptor (3). PDS5 is found in skeletal muscle, visceral smooth muscle, vascular smooth muscle, and corpus cavernosum. It is not found in the myocardium but is present in coronary smooth muscle tissue. The major event of PDE5 inhibitor decrease the muscular tone and prolonged the relaxations and enhance No-cGMP pathway to occur erection (4). Erection is largely a

hemodynamic event, which is regulated by vascular tone and blood flow balance in the penis. Therefore, it augments the sexual pleasure and performance (5). In general, pharmacological agents that bring about rises in intracellular levels of cAMP can stimulate poorly motile sperm samples. It is therefore, Human spermatozoa can obtain energy from both glycolysis and mitochondrial oxidative phosphorylation. Thus, motility of fresh and cryopreserved spermatozoa significantly increases after stimulation with caffeine, and 2-deoxyadenosine due to amplified glycolysis and fructolysis (6). Bicarbonate (HCO_3) and calicium contributed in the sperm motility regulation, when bicarbonate and calicium added significant increases in motility were observed (7). In human spermatozoa, the potent (cGMP) dependent phosphodiesterase blocker sildenafil markedly increases tyrosine phosphorylation of same proteins belonging to the fibrous sheet, increasing sperm velocity, lateral head displacement and sperm capacitation. When capacitation is stimulated in presence of NO releasing compounds, overall tyrosine phosphorylation level in human spermatozoa significantly increases (8).

Sildenafil citrate is a phosphodiesterase-5 (PDE5) inhibitors and is used in treatment of erectile dysfunction with achieve and maintain a sufficient erection for satisfactory sexual performance (9). There are eleven different types of the phosphodiesterase (PDEs) which are distributed throughout body (10). PDEs vary in their substrate specificity for cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) including PDE5, PDE6 and PDE9 and specific for cGMP while PDE4, PDE7 and PDE8 are specific for cAMP (11).

The mechanism of action of sildenafil citrate is when nitric oxide (NO) is an important neurotransmitter in autonomic nervous system. When sexual stimulated parasympathetic nervous system, release of NO from non cholinergic, non adrenergic neurons in the penis, as well as from endothelial cells (12). Where, it activates soluble guanylate cyclase (sGC), the enzyme that converts gaunosine triphosphate (GTP) to (cGMP) lead to smooth muscle relaxation (vasodilation) in the corpus cavernosum resulting erection (13). Finally, sperm motility appears to be affected by guanylate cyclase activation mainly through an increase in intracellular c AMP, wherase the acrosome reaction depends more directly on cGMP synthesis. The accumulation of cGMP and cAMP is enhanced by phosphodiesterase inhibitors (14). Therefore, This study were designed to evaluate effect of different concentration of sildenafil citrate on sperm parameters during *in vitro* human sperm activation in asthenozoospermic men.

2. Subjects, Materials and Methods

2.1. Subjects

Fifty asthenozoospermic patients were included in this study and obtained at laboratory section/ Al-Hussein Teaching Hospital/ Thi-Qar Health directorate. Each semen sample was divided into three aliquots. The first aliquot put beneath (1:1 v:v) of modified Earl's medium (MEM) in falcon tube. The second aliquot put in a falcon tube under of MEM supplemented with 100 ngm sildenafil and the third aliquot put under of MEM with supplemented 200ngm of sildenafil citrate. The samples put in an

incubator in oblique position and sperm parameters were assesssed after 30 minute. The selection of infertile patients was based on physical examination and assessment was by using questionnaire and each infertile patient was to have baseline semen samples including the parameters of sperm function.

2.2. Clinical and laboratory investigation

2.2.1. Seminal fluid collection and analysis

Semen samples were collected by masturbation after 3-5 days abstinence into a dry, clean, and sterile Petri-dish labeled with name and age of patient, period of abstinence and time of collection. The specimens were placed in the incubator at 37ºC to allow the semen liquefaction. WHO criteria for normal semen values were applied (WHO, 2010).

2.3. Preparation of culture medium and sildenafil citrate solution

Modified Earl's medium (MEM) used with earl's salts (0.88gm), Ampencilline (0.88gm), sodium pyrovate (0.001 gm), and sodium bicarbonate (0.21gm). MEM 0.88 gm was dissolved in 100 ml of deionized distilled water and 5% of human serum albumin was added. After that, pH of solution was adjusted between (7.2-7.4). Then, the prepared culture medium was filtrated by using Millipore filter (0.22µm) and stored in refrigerator until use. Sildenafil citrate powder was dissolved in culture medium to get (100 and 200 ngm /ml). Sildenafil citrate solution was prepared by dissolving completely a crushed one tablet (100 mg) in 2 ml of normal saline to obtain different doses of sildenafil citrate.

2.4. Sperm preparation technique (direct layering technique)

The semen was prepared by using a direct layering technique. However, 1mL of prepared MEM culture medium (Sigma, Aldrich, UK) was added to the test tube either alone or supplied with one of two concentration of sildenafil citrate, and then 1ml of the liquefied semen was layered beneath a culture medium. After incubation for 30 minute in 5% CO_2 at 37C˚, one drop (10µL.) was aspirated by pasture pipette from upper layer and examined under light microscope at 400X magnification for assessment sperm parameters.

2.5. Statistical analysis

Statistical analysis was performed with the SPSS (Statistical Package for Social Sciences software version 12.00). Crude data analysis was done using student's t-test so called paired samples t-test for table with mean and standard error of mean (S.E.M) to compare between pre and post preparation for sperm parameters. As well, ANOVA test was applied to compare among mean groups of different sildenafil citrate concentrations. (P Value < 0.05) used as a level of statistically significance.

3. Results

3.1. Parameters of SFA and in vitro sperm processing

Table (1) shows the effect of modified Earl's medium (MEM) only or supplemented with different concentrations of sildenafil citrate (100 or 200 ngm/mL) on sperm parameters during in vitro sperm activation after 30 minute of incubation by direct layering technique. In general, it was noticed that sperm concentration and sperm agglutination (%) was significantly reduced (P<0.05) as compared to pre-activation, while sperm motility (%), progressive sperm motility (%), and normal sperm morphology (%) were increased significantly (P<0.05). However, percentage of sperm motility was increased significantly (P<0.05) after direct layering technique using MEM supplemented with 100 ngm or 200 ngm SC as compared with the control group. Also, a highly significant increase (P<0.001) was seen in progressive sperm motility when using MEM+ 100 ngm SC and significant increase (P<0.05) by using MEM+ 200 ngm SC as compared to MEM only. However, a highly significant decrease (P<0.001) was noticed in the progressive sperm motility (%) by using MEM+ 200 NGM SC versus MEM+100 ngm SC after direct layering technique. In addition, percentage of normal sperm morphology showed non significant (P>0.05) differences in both treated groups compared to MEM only.

Table (1): *in vitro* sperm activation using direct layering technique and modified Earl's medium supplemented with different concentration of sildenafil citrate for asthenozoopermic patients using direct layering technique*.

Sperm parameters	Pre-activation No. 45	Post *in vitro* sperm activation using direct layering technique		
		Control	T1 100 ngm/ml SC	T2 200 ngm/ml SC
Sperm concentration ($\times 10^6$ sperm/ml)	52.55 ± 2.56	34.83 ± 1.66**	20.29 ± 1.06**	20.46 ± 1.32**
Sperm Motility (%)	58.18 ± 1.65	84.33 ± 0.44**	86.88 ± 1.61*a	86.79 ± 0.68*b
Progressive Sperm Motility (%)	34.53 ± 1.43	68.57 ± 2.06**	76.18 ± 1.47*A	72.15 ± 1.43*bB
Sperm agglutination (%)	8.52 ± 0.82	0.00 ± 0.00**	0.00 ± 0.00*	0.00 ± 0.00*
Normal sperm morphology (%)	37.60 ± 1.07	64.23 ± 1.54**	61.22 ± 1.34*	61.19 ± 1.50*

Values are Mean ± S.E.M

*** Total No. of infertile patients=45**

**** Means significant differences (P<0.05) between control, T1 and T2**

a Means significant differences (P<0.05) between T1 and control, B Means highly significant differences (P<0.001) between T1 and T2

A Means highly significant differences (P<0.001) between T1 and control b Means significant differences (P<0.001) between T2 and control

4-Discussion

In vitro sperm activation is a very important step in laboratory technique that plays an important role in determining the outcome of assisted reproductive technologies (15). In addition to the technique used in the sperm preparation and components of culture media, the properties of semen samples are considered one of

the important factors in the determination of successful *IVF* (16). *In vitro* human sperm activation technique using modified Earl's medium (MEM).

Markedly decreased sperm concentration and agglutination for all infertile men as compared to pre-activation. This result may be due to the beneficial effects of preparation by removal of dead, immotile spermatozoa and semen debris in such away only superior quality motile spermatozoa were harvested and unfortunate quality spermatozoa were absent in post-activation medium (17). The percentages of human sperm motility, progressive sperm motility, and normal sperm morphology were significantly enhanced ($P<0.05$) as compared to pre-activation (18). Really, improvement sperm parameters may be considered as normal response for sperm biology after removal of seminal plasma, pus cell, and agglutinated spermatozoa using sperm preparation technique. Furthermore, it was reported that only achieve motile sperms will swim up in the superior area or the equatorial zone during in vitro human sperm activation using direct layering technique (18). As well as, during sperm preparation technique the motile with the best morphologically normal sperm will swim up successfully (19). SC was considered a potent phosphodiesterase-5 (PDE-5) inhibitors and elevation cGMP amount in the cell leading to release and elevate cAMP level and protein phosphorylation (20). Another explanation on sperm motility stimulation using SC, spermatozoa themselves express a nitric oxide synthase (NOs) activity and are able to synthesize NO (21). Moreover, most studies have clearly demonstrated the presence of endothelial and neural NOs isoforms in human spermatozoa (22). However, second messenger system such as the cAMP/adenylate cyclase system and the cGMP/guanylate cyclase system were considered to have a high affinity with NO and appeared to regulate sperm function. Also, increased levels of intracystolic cAMP results in an enhancement of sperm motility and viability (23).

One of the most important explanation about sperm motility stimulation by SC was investigated that the inhibition of sperm PDE5 by sildenafil citrate was associated with a significant increase in cAMP and that sperm PDE activity measured with cGMP as a substrate was threefold lower than when measured with cAMP(24). There was also a dose dependent increase in cAMP levels in spermatozoa that were incubated with sildenafil citrate (25). SC probably acts on types 1 and 4 PDE because CAMP has been implicated in the regulation of sperm motility through activation of PKA. SC induced similar changes in sperm motility parameters as PDE inhibitors such as pentoxifylline, caffeine, and 3-isobutyl-1-methylxanthine and increase capacitation and acrosome reaction and associated tyrosine phosphorylation of p105/81 and two fibrous sheath proteins (26).

The superiority of a low concentration SC in the present work on the enhancement of human sperm motility may explain the biphasic response of sperm motility to sildenafil action that resembles nitric oxide action, which stimulate sperm motility at low concentration and acts as an oxidant at a higher dose (27). The overproduction of this free radical (NO) and the consequent excessive exposure conditions have a potential pathogenetics role in the reduction of sperm motility. When, an increased sildenafil citrate dose causes a decrease in the microenvironmental pH, it leads to suppress motility, in addition to other sperm parameters (28). Moreover,

the present data are supported by other researchers who suggested a dual mechanism for PDEs inhibition with a stimulatory effect on sperm motility when PDE5 is moderately inhibited. However, the extensive inhibition of PDE5 leads to decreased sperm motility (29). Sildenafil citrate increases intracellular NO levels through a stimulation of the synthesis and transcription of endothelial and inducible NO synthesis (eNOs and iNOs). Also, there are two isoforms eNOs and bNOs (brain NO synthetics) in human spermatozoa. However, eNOs and bNOs are expressed in blastocyst collected from the uterus of delayed implantation and the role of NO as messenger in a wide array of biological processes (30). In addition, the stimulation of NO generation is associated with the enhancement of tyrosine phosphorylation of sperm proteins and this activity is an essential component of the cascade of biochemical changes leading to sperm capacitation. Also, NO is capable of regulating cAMP concentration, consequently, capacitation via stimulation of adenylyl cyclase. This modulation could act directly by targeting the enzyme or by altering the action of distinct regulatory protein (31).

It was concluded that a low concentration of sildenafil citrate concentration give the best results in sperm preparation by increase motility and progressive motility.

References

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التحري عن تأثير سترات السلدينافيل (الفياغرا) على متغيرات النطف باستخدام تقنيات التنشيط في الزجاج لمرضى العقم المصابين بوهن حركة النطف.

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الخلاصة

تهدف هذه الدراسة الى التحري عن تاثير استخدام تراكيز مختلفة من سترات السلدينافيل (الفياغرا) على نشاط النطف في الزجاج للمرضى المصابين بوهن حركة النطف. تم اختيار المرضى وعددهم خمسة واربعون مريضاً وتقسيمهم الى ثلاثة مجاميع متساوية العدد (المجموعة الاولى: مجموعة سيطرة وكانت باستخدام الوسط الزرعي (MEM) فقط مع عينة السائل المنوي، المجموعة الثانية: مجموعة معاملة وكانت باستخدام الوسط الزرعي مع اضافة 100ملغم/مل من سترات السلدينافيل، المجموعة الثالثة: مجموعة معاملة وكانت باستخدام الوسط الزرعي مع اضافة 200ملغم/مل من سترات السلدينافيل لغرض التنشيط في الزجاج. عينات السائل المنوي تم فحصها بطريقة تقنية سباحة النطف المباشرة. اضافة الى ذلك, ان تركيز النطف, وحركة النطف, والحركة التقدمية للنطف, وتلازن النطف, والنسبة المئوية للنطف السوية حسب معايير منظمة الصحة العالمية (1999). تم حضن السائل المنوي مع الوسط الزرعي بدرجة ($37^{o}C$ و $5\%\ CO_2$) اثناء اجراء عملية التنشيط في الزجاج.

اظهرت نتائج الدراسة الحالية وجود فرقاً معنويًا ($P<0.05$) في حركة النطف بعد اجراء عملية التنشيط باستخدام كلا التركيزين مقارنة مع مجموعة السيطرة. اضافة الى ذلك, وباستعمال التركيز الاول/ الاوطى فان نتائج التنشيط تكون افضل فيما يتعلق بحركة النطف والحركة التقدمية للنطف. نستنتج من خلال هذه الدراسة ان التركيز الواطى لسترات السلدينافيل يعطي افضل النتائج في تقنيات تحضير النطف من خلال تحسن حركة النطف والحركة التقدمية للنطف السوية. لذلك نوصي بإجراء دراسة لمعرفة مدى تاثير سترات السلدينافيل على نتائج التقنيات المساعدة على الانجاب (ART) وتقنية التمنية داخل الرحم (IUI).