Effect of Vitamin C on *In Vitro* Sperm Activation of Asthenozoospermic Infertile Patients

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Abstract

One hundred and fifty asthenozoospermic patients were involved in the present study at age ranging between 20 to 62 years during their attendance at Al-Sadder Teaching Hospital in Al-Najaf/Iraq in a period from November 2012 to July 2013. The objective of this study was to determine the effect of vitamin c on sperm activation of asthenozoospermic infertile patients. The results of the study showed that there was a significant decrease ($P<0.05$) in the sperm concentration that assessed post-activation *in vitro* in both treated groups and control groups as compared to pre-activation groups using fertiCult medium by direct mixing technique, but there were no significant differences in the assessed value with different concentrations of vitamin C that used. Also, it was appeared that, the sperm motility percent, and grade of motility were significantly increased ($P<0.05$) in both treated groups and control groups as compared to pre-activation groups using fertiCult medium by direct mixing technique, but there were no significant differences in the assessed values with different concentrations of vitamin C that used. However, best *in vitro* sperm activation results regarding the improvement of the sperm motility percent, and grade of motility were observed with fertiCult medium supplemented with (0.06 mg/ml) vitamin C.

Keywords: Vitamin C, FertiCult medium, *In vitro* sperm activation, Asthenozoospermia

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Introduction

The World Health Organization (WHO) defines the infertility as an inability of a couple to achieve conception or bring a pregnancy to term after one year or more of regular, unprotected sexual intercourse. Conception is normally achieved within 12 months in

80%–85% of couples using no contraceptive measures (WHO, 2010). Infertility is a major clinical concern, affecting about 15% of all reproductive age couples. Male factors which including decreased the semen quality are responsible for 25% of these cases.

Reactive oxygen species (ROS) are highly reactive oxidizing agents belonging to the class of free radicals, which are a group of highly reactive chemical molecules with one or more unpaired electrons that can oxidatively modify biomolecules they encounter. Superoxide anion, hydroxyl radical and hydrogen peroxide are major reactive oxygen species present in seminal plasma. Cells living under aerobic conditions require oxygen to support life. However, metabolites such as ROS might modify the cell functions and endanger cell survival. Male germ cells at various stages of differentiation have the potential to generate ROS, and low physiologic levels are needed to regulate sperm capacitation, acrosome reaction and sperm–oocyte fusion (Agarwal et al., 2004; Agarwal Ashok and Lucky, 2010). An excess ROS must be continuously inactivated by seminal plasma antioxidants to maintain normal cell function. Oxidative stress (OS) arises when excess free radicals overwhelm the antioxidant defence of the male reproductive system (Dinesh et al., 2012). The levels of ROS are normally limited by antioxidant defense mechanisms such as vitamin C and E that are present within the seminal plasma and sperm plasma membrane (Magda, 2012). However, the supplementing of infertile males with antioxidant vitamin C and E suggested as a potential treatment for idiopathic male infertility (Ko and Edmund 2012).

Vitamin C basically secreted from seminal vesicles during ejaculation to protect sperm from endogenous oxidative DNA damage (Greco et al., 2005). It is acts as a scavenger of a wide range of ROS which explains its ability of successfully counteract the effects the DNA damage and ROS production (Eskenazi et al., 2005). It has previously been shown to be the major antioxidant in seminal plasma of fertile men contributing up to 65% of the total chain-breaking antioxidant capacity. In addition, concentration of vitamin C in seminal plasma is 10 times greater than the concentration found in blood plasma (Greco et al., 2005). The etiology of suboptimal semen quality is poorly understood, and many physiological, environmental, and genetic factors, including oxidative stress, have been implicated (Mohammad and Moslemi, 2011). Therefore, the objective of this study was to determine the effect of vitamin C on *in vitro* sperm activity of asthenozoospermic infertile patients.

Materials and Methods

Patients

One hundred fifty semen specimens obtained from asthenozoospermic infertile patients at age ranging between 20 to 62 years-old during their attendance at Al-Sader Teaching Hospital in Najaf/Iraq. Duration of study was from November 2012 to July 2013. Complete history of age, blood group, type of infertility, duration of infertility and assessment for the presence or absence of varicocele was obtained. The patients with diabetic, hypertensive or leukospermic were excluded in this study.

Semen Specimen Collection

Semen specimens were collected by masturbation after 3-5 days of abstinence, in wide mouth disposable plastic container. The semen specimens were incubated at 37^{o}C to liquefy (Pal et al., 2006). *In vitro* parameters of semen specimens included appearance, volume, pH, color, viscosity, liquefaction time, sperm concentration, sperm motility percent, sperm grade activity, sperm morphology percent and agglutination were assessed using fertiCult medium by direct mixing techniqueas compared to pre-activation group (WHO, 1999).

Methods

The Semen specimens were divided into four fractions. First part was control, 0.5ml of liquefied semen mixed with 0.5ml fertiCult medium and incubated at 37^{o}C for 30 minutes. Second part (Vit. C1), 0.5ml liquefied semen was mixed with 0.5ml fertiCult medium supplemented with 0.02 mg/ml vitamin C and incubated at 37^{o}C for 30 minutes. Third part (Vit. C2), 0.5ml liquefied semen was mixed with 0.5ml fertiCult medium supplemented with 0.04 mg/ml vitamin C and incubated at 37^{o}C for 30 minutes. Fourth part (Vit. C3), 0.5ml liquefied semen was mixed with 0.5ml fertiCult medium supplemented with 0.06 mg/ml vitamin C and incubate at 37^{o}C for 30 minutes. After semen specimen treated in all fractions, they were examined and assessed for macroscopical and microscopical changes. Statistical analysis was performed with the SPSS (Statistical package for social sciences) version 17. The data analysis was done using paired sample t-test to assess the statistical differences in the results. Mean and standard error of mean (SEM) obtained from crude data to compare between pre-and post activation for semen parameters. P value < 0.05 was considered significance as a level of statistical analysis.

Results

Results of this study showed that there was a significant decrease at P<0.05 in the sperm concentration that assessed post-activation *in vitro* in both treated and control groups, while there were no significant differences in the sperm concentration showed among treated groups. The sperm motility percent and grade of motility were significantly increased at P<0.05 in both treated groups and control group compared to pre-activation, while there were no significant differences in the assessment values among vitamin C groups. However, the best *in vitro* results showed in the sperm motility percent and grade of motility were observed with fertiCult medium supplemented with vitamin C in a concentration of 0.06 mg/ml (Table 1 and figures 1-4).

Table 1: Effect of different concentrations of vitamin C on spermiogram of asthenozoospermic infertile patients following *in vitro* activation technique

Spermiogram	*In vitro* activation				
	Before activation	After activation			
		Control	Vit.C1	Vit.C2	Vit.C3
	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM	Mean±SEM
Sperm concentration (million/ml)	32.76±6.56 A	23.98±6.32 b	23.92±6.56 b	22.32±6.61 B	23.0±6.75 B
Sperm motility (%)	23.42±11.12 A	35.1±10.1 b	40.18±10.61 c	40.81±11.03 C	41.50±11.21 C
Grade of motility	1.42±0.18 A	1.80±0.20 b	2.08±0.22 c	2.13±0.24 C	2.16±0.24 C
Sperm motility index	49.54±10.04 A	81.69±9.74 b	97.18±13.01 c	106.43±12.7 D	123.5±13.20 E

Vit.C1=0.02mg/ml, Vit.C2=0.04mg/ml, Vit.C3=0.06mg/ml, FertiCult medium used as control. Different letters indicate a significance differences between related groups (P <0.05).

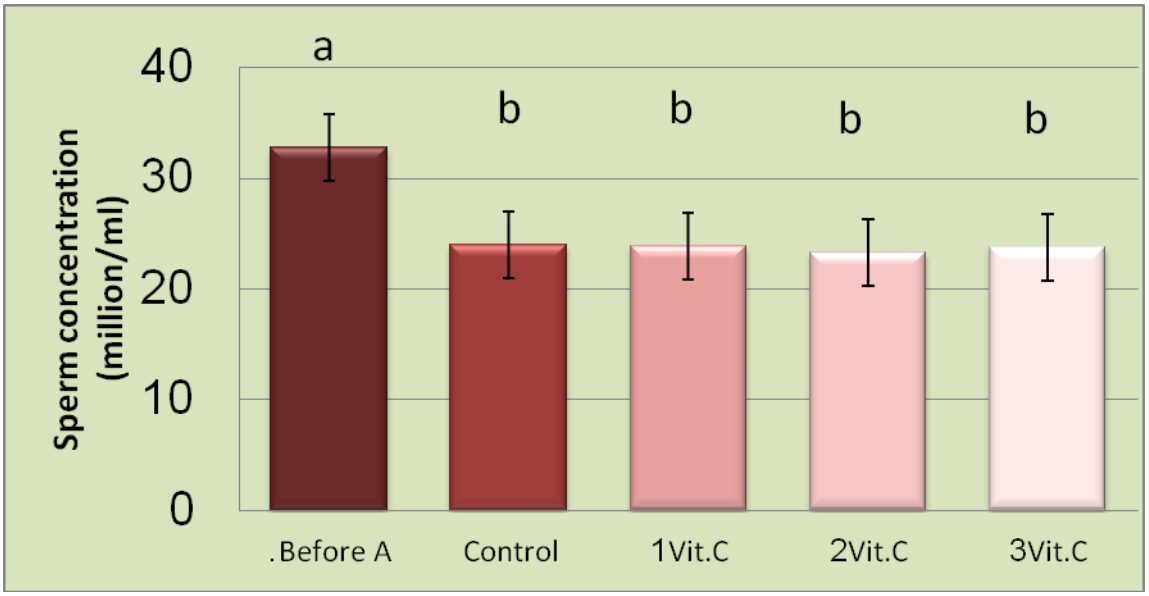

Figure (1): The mean sperm concentration in response to different concentrations of vitamin C. Before A=Before activation, Control = FertiCult medium, Vit. C1=VitaminC1 (0.02mg/ml), Vit. C2=Vitamin C (0.04mg/ml, Vit. C3= Vitamin C (0.06mg/ml), Bar = Standard error of mean. Different letters indicate a significance differences between related groups (P <0.05).

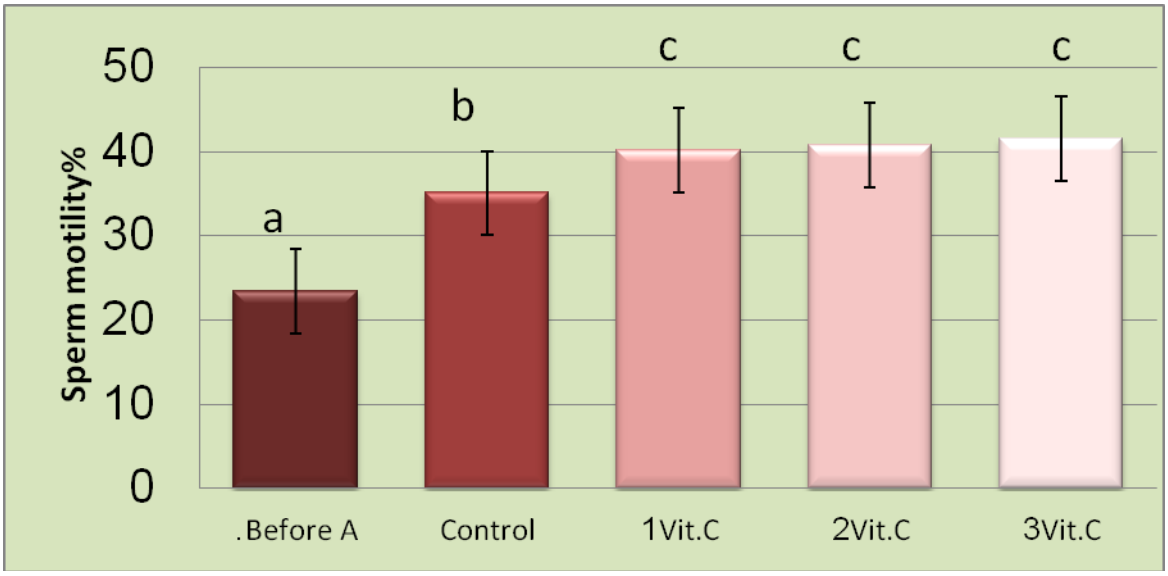

Figure (2): The mean sperm motility percent in response to different concentration of vitamin C. Before A= Before activation, Control= FertiCult medium, Vit. C1=VitaminC1 (0.02mg/ml), Vit. C2=Vitamin C (0.04mg/ml, Vit. C3= Vitamin C (0.06mg/ml), Bar = Standard error of mean. Different letters indicate a significance differences between related groups (P <0.05).

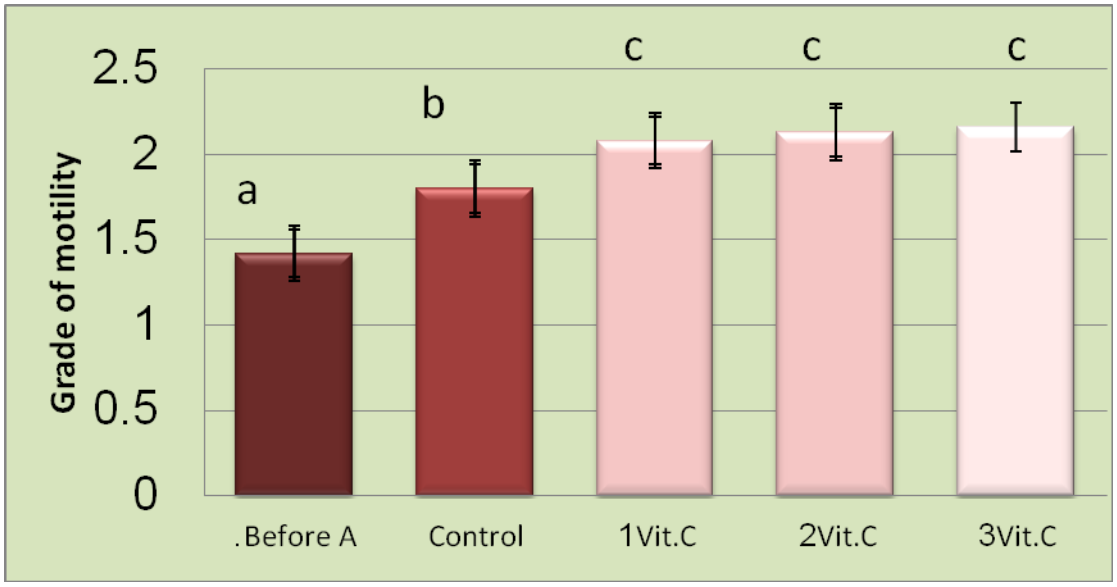

Figure (3): The mean grade of motility in response to different concentrations of vitamin C. Before A= Before activation, Control = Ferticult medium, Vit. C1=VitaminC1 (0.02mg/ml), Vit. C2=Vitamin C (0.04mg/ml, Vit. C3= Vitamin C (0.06mg/ml), Bar = Standard error of mean. Different letters indicate a significance differences between related groups (P <0.05).

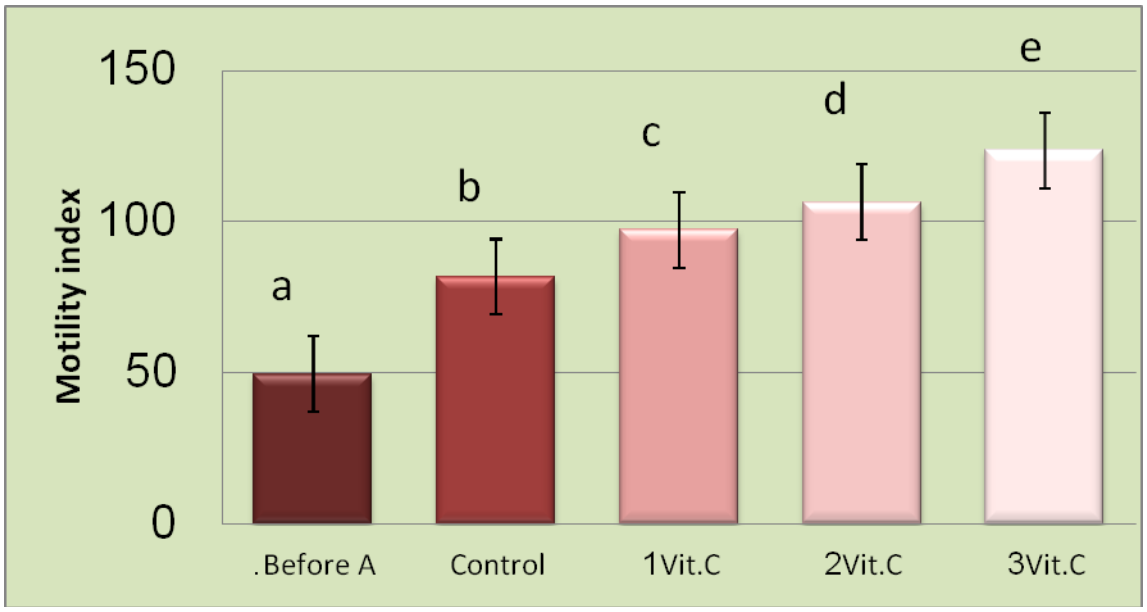

Figure (4): The mean sperm motility index in response to different concentrations of vitamin C. Before A= Before activation, Control=FertiCult medium, Vit. C1=Vitamin C1 (0.02mg/ml), Vit. C2=Vitamin C (0.04mg/ml, Vit. C3= Vitamin C (0.06mg/ml), Bar = Standard error of mean. Different letters indicate a significance differences between related groups (P <0.05).

Discussion

Assisted reproductive techniques (ART) recently became the treatment of choice in many cases of male and female infertility and one of the factors determining successful assisted reproduction is the quality of semen samples. A variety of sperm preparation techniques are available to select motile spermatozoa (Agarwal *et al*., 2005). In the present study, we applied direct mixing technique for sperm preparation from asthenozoospermic patients in an attempt to prevent damage by centrifugation and generation of reactive oxygen species (ROS). Agarwal *et al* (1994) reported that increased formation of ROS has been correlated with reduction of sperm motility. The selection of sperm preparation methods depend on the quality of the ejaculates, and ejaculates with high ROS production such as that of asthenozoospermic patients should not be separated by centrifugation method due to severely spermatozoa damage (Sills *et al.,* 2002).

The results of this study showed that among the three doses of vitamin C have been used, there was a significant decrease at $P<0.05$ in the sperm concentration that assessed post-activation *in vitro* in both treated groups and control groups as compared to pre-activation groups using fertiCult medium by direct mixing technique, but there were no significant differences in the assessed value among different concentrations of vitamin C, and this is an expected result because of dilution, since we have mixed 0.5ml of semen sample with 0.5ml of fertiCult medium supplemented with vitamin C, and this dilution might result in decreasing of the sperm concentration. Also, there were a significant increase at $P<0.05$ in the sperm motility percent and grade of sperm motility that assessed post activation in both treated groups and control groups but there is no significant differences in the assessed value between different concentrations of vitamin C that used. However, best *in vitro* sperm activation results regarding the motility percent and grade of sperm motility achieved with vitamin C (0.06) mg/ml. Our results agreed with the results of Lewis *et al*. (1997) who recommended adding low molecular weight antioxidants like ascorbate to sperm during preparation for ART, especially in asthenozoospermic patients, on the basis of low levels of ascorbate in such patients. They also indicated that it would be of more clinical benefit to add these antioxidants directly to the sperm rather than using dietary supplements, because of that defective seminal vesicle function may be one of the causes of decreased ascorbate levels in these patients.

Gomez *et al*. (1998) demonstrated that levels of ROS produced by spermatozoa were negatively correlated with the quality of sperm in the original semen. Vitamin C acts as a scavenger of a wide range of ROS, which explains its ability to counteract the effects of free radicals both in terms of induced DNA damage and ROS production. Mahmoud *et al*. (1999) have shown the protective role of vitamin C against endogenous oxidative DNA damage. Vitamin C enters the mitochondria through facilitated glucose transporter (glut 1) and protects it from oxidative injury (Sagun *et al*., 2005). Thiele *et al*. (1995) have found a positive correlation between vitamin C concentration in seminal plasma and number of morphologically normal spermatozoa. Prolonged *in vitro* incubation of period and repeated centrifugation of spermatozoa results in increased oxidative damage and addition of vitamin C to cryopreservation medium resulted in increased motility and fertilizing ability as evident by obtaining embryos from cryopreserved spermatozoa (Mirzoyan *et al.* 2006).

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