Impact of *in vitro* Preparation with *Citrullus Colocynthis* on DNA Normality of Human Asthenozoospermic Semen

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Abstract

Background:

Citrullus colocynthis (CC) is an herbal medicine used in the treatment of a wide range of diseases. Recently, It has been studied on *in vitro* activation of mice sperms with no research concern the effect of this plant on the DNA normality following *in vitro* preparation of human semen samples.

Objectives: The present study was aimed to examine the effect of adding CC in the medium used for *in vitro* sperm activation of asthenospermic men and related factors and to found out the effect of *CC* on the DNA status following the activation.

Materials and Methods: *Citrullus colocynthis* aqueous extract (0.01mg/ml culture medium) was used for *in vitro* sperm activation . One hundred and ten semen samples were divided into two groups, the first group activated *in vitro* by adding CC extract to the culture medium Ham's F-12 (the treated group) and, the second group sperm activated *in vitro* with Ham's F-12 alone (the control group).

Results: *In vitro* activation of sperms with aqueous extract of CC has shown positive effect on sperm concentration, sperm motility, and grade activity of progressive forward movement. There was a significant ($P<0.05$) increase in certain sperm function parameters of asthenozoospermic in treated group compared with the control group .The study showed that the DNA fragmentation resulted from treated group were lower than that of control group.

Conclusions: According to the results of present study, adding aqueous extract of CC to the human sperms activation culture medium leads to an improvement in certain sperm function parameters of asthenozoospermic men with no harmful effect on the human sperms DNA with decrease in DNA denaturation.

Key words: ***Citrullus colocynthis*, asthenospermic , DNA normality**

Introduction:

Male factor infertility plays a role in approximately 50% of infertile couples [1]. A number of etiologies have been identified as potential causes of male infertility, which include gene mutations, aneuploidies, infectious diseases, ejaculatory duct occlusion, varicocele, radiation, chemotherapy and erectile dysfunction [2].The importance of sperm DNA has become more obvious in the context of assisted reproductive technologies (ART), which has gained tremendous importance in the treatment of infertile couples [3]. The abnormalities in the male genome that characterized by damaged DNA may be indicative of male sub fertility regardless of the routine semen parameters [4]. Moreover, poor semen quality has been associated with an increase in the proportion of sperm with DNA fragmentation [5]. Although the use of herbal plants and plant products as medicines could be traced as far back as the beginning of human civilization. The earliest mention of medicinal use of plant in Hindu culture is founds in "Rig Veda", which is said to have been written between 1600-4500 B.C [6, 7]. One of these medicinal herbal plants is *Citrullus colocynthis* .The CC is perennial herbs usually trailing. Commonly found wild in the sandy lands of North West, the Punjab, Sind, and Central and southern India. Also found indigenous in Arabia, West Asia, Africa and in the Mediterranean region (e.g. Iraq) [8]. This plant is known to exhibit many pharmacological actions, including antioxidant, anti-inflammatory and analgesic activities and others [9, 10]. However in our knowledge there is a very few researches regarding the effect of the CC medium on *in vitro* sperm activation of asthenospermic men and to explore the effect of *CC* on the DNA *normality* following the activation.

Materials and Methods:

1. Seminal fluid analysis:

The husbands included were examined by a consultant urologist in the Institute. Semen sample (100%) was collected by masturbation after 2-5 days of abstinence directly into a clean, dry and sterile disposable Petri dish in a especially allocated room for this purpose in the Institute. The sample was transported to the semen examination laboratory immediately and allowed to liquefy in an incubator at 37ºC. After complete liquefaction, it was analyzed by macroscopic and microscopic examinations using the standardization of WHO,(1999).

2. Assessment of human sperm chromatin structure by acridine test (AOT)

According to Tejada *et al* [13], the sperm chromatin structure was examined by using Acridine Orange test as the following steps preparation:

3. Preparation of the Acridine Orange stain.

The stain of Acridine orange test (AOT) was prepared by dissolved one gram of AO, (Sigma, Deisenhofer, Germany);in 1000 mL of distilled water and stored in the dark at 4°C as a stock solution.(10mL) of it was added to 40mL of (0.1M) Citric acid, (Panreac, Spain); and 2.5mL of (0.3M) $Na_2HPO4.7H_2O$, (BDH, England). Then pH adjusted to (2.5) before staining. All stocks solutions kept at room temperature except the acridine orange that kept in refrigerator the other solutions maintained at room temperature. [13].

3.1. Fixative Solution (Carnoy's Solution): This solution consists of three parts of Methanol, (BDH, England); and one part of Glacial Acetic acid (Scharlau, Spain). Carnoy's solution was prepared daily. [13].

3.2. Acridine orange protocol: Smears were prepared from each sample on the slides and allowed to air-dry for about 20 minutes. Then, the slides were fixed in Carnoy's solution for at least 3 hours to overnight at 4ºC. After that, the slides, were removed from the fixer and allowed to air-dry for a few minutes before staining. All solutions were prepared at room temperature under dim light and final pH of the stain was adjusted to 2.5. After being placed on a slide holder, 2-3 ml of the stain was spread over each slide for 5 minutes. The slides were covered by 22x50 mm glass cover slip . Slides were read on the same day of staining with a (40X) objective

lens on a fluorescence microscope (BEL Italy) image (3-4), which was equipped with an excitation filter of 460-490 nm and barrier filter of 520 nm. The nuclei of spermatozoa from each sample were examined and scored as fluorescing green, red or yellow. Spermatozoa displaying green fluorescence were recorded as normal, whereas sperm heads displaying yellow-red fluorescence were considered as abnormal[13].

3.3. *In vitro* sperm activation technique:
After liquefaction of semen sample, one drop of semen was examined under the microscope for certain sperm function parameters. Thereafter, sperm activation was performed using simple layer activation technique as described by [12].

3.4. Statistical analysis : Data of present study were expressed as mean ± SEM. The result of *in vitro* activation technique before and after activation .The data of the asthenozoospermic groups were analyzed by using analyzer of variance ANOVA (one tail).The beast signified difference was used to determined the significant at (P <0.05) between the groups. While Chi- square test was used to compare values of DNA fragmentation treatment and control group. (P<0.05) was considered significant in this study [14].

Results:

1. Effect of *in vitro* activation by Ham's F-12 and Ham's F12 + *CC* media on certain sperm function parameters of asthenozoospermic group:

Table (1) shown that the mean of sperm concentration (million/ml) following *in vitro* activation by Ham's F-12 +*CC* medium (24.860±2.05) and Ham's F-12 media (21.740±1.92) was highly significantly (P<0.05) decrease compared with before activation (53.620 ±3.22).
The percentage of progressive sperm motility (grade A) was significantly (P<0.05) improved by activation *in vitro* with Ham's F-12 +*CC* (28.180±1.66) more than that of sperms activated with control Ham's F-12 medium (16.420±1.63).While, a highly significant (P <0.001) increase after activation was observed by Ham's F-12 and Ham's F-12 +*CC* media compared with before activation (2.5±0.41).No significant (P>0.05) changes were recorded in active sperm motility Grade B after activation between Ham's F-12 +*CC* medium (49.04±1.57) and Ham's F-12 (46.9±1.94) .A highly significant (P<0.05) increment was statistically found after activation by Ham's F-12 and Ham's F12+*CC* media compared with before activation (35.6±0.84).
There was a highly significant (P<0.05) reduction in active sperm motility Grade C+D after activation by Ham's F-12 (25.9±1.63 and 10.680±1.13) and Ham's F12 +*CC* medium (16.760±1.12 and 6.020±0.61) compared with before activation (29.6±0.81 and 32.3±1.29). Activation of sperms *in vitro* caused a highly significant (P<0.05) increase in the percentage of morphologically normal sperm (MNS) following the activation with Ham's F12 +*CC* medium (66.700±1.35) when compared to the control Ham's F-12 medium (57.0±1.60). There was a highly significant (P<0.05) improvement after activation when compared the results of the two media and the result of before activation (36.380±0.92) as shown in table (1).

Table -1: Certain sperm function parameters before and after *in vitro* activation by adding Citrullus colocynthis to Ham's F-12 medium of asthenozoospermic group.

Certain sperm function parameters		Before activation	After *in vitro* activation		*P* -value
			Ham's F12	Ham's F12+ *CC*	
Sperm concentration (x10^6/ml)		53.620 ±3.22a	21.740±1.92 b	24.860±2.05 b	<0.001**
Sperm motility (%)	A	2.500 ±0.41 c	16.420 ±1.63 b	28.180 ±1.66 a	<0.001**
	B	35.600 ±0.84 b	46.900 ±1.94 a	49.040±1.57 a	<0.001**
	C	29.600 ±0.81 a	25.900 ±1.63 b	16.760 ±1.12 c	<0.001**
	D	32.300±1.29 a	10.680 ±1.13 b	6.020±0.61 c	<0.001**
Morphologically normal sperm (%)		36.380 ±0.92 c	57.000 ±1.60 b	66.700 ±1.35 a	<0.001**

Values are expressed as Mean±SE.
Different letters within each row mean a significant difference at (*P* <0.05).

2. Effect of *in vitro* activation by Ham's F-12+*CC* medium on certain sperm function parameters for all groups (asthenozoospermic, AO, AT, AOT) on this study:

Table (2) showed the result of *in vitro* activation with Ham's F-12 contains *CC* medium on certain sperm function parameters for all groups. The mean of sperm concentration after activation technique with Ham's F-12 for asthenozoospermic group (24.860±2.05) was significantly (*P*<0.05) increased compared with AO, AT AOT. While no significant (*P*>0.05) differences was found between AO group (5.9±0.67) and AOT group (5.0±0.71).

After activation by Ham's F12 contains *CC*, the percentage of progressive motility (grade A) in AOT group (45.0±5.53) was highly significant (*P*<0.05) accelerated compared with another groups (asthenozoospermic, AT, AO) (28.180±1.69, 23.500±5.33and 34.500±3.77 respectively). While no significant (*P*>0.05) changes was observed in asthenozoospermic group (28.180±1.66) compared with AO group (23.500±5.33).

The active sperm motility Grade A was significantly (*P*<0.05) increased in the AOT (45.000±5.53) when compared with another groups (asthenozoospermic, AO and AT) (28.180±1.66, 23.500±5.33and 34.500±3.37 respectively).There was no significant (*P*>0.05) recorded in active sperm motility Grade B between (asthenozoospermic, AO and AT) groups (49.04±1.57, 46.0±3.48 and 45.0±2.98, respectively). While significant (*P*<0.05) increased in AOT group (37.0±4.16) compared with (asthenozoospermic, AO, AT) after activation by Ham's F12+*CC*. There was no significant (*P*>0.05) alterations after activation by Ham's F-12+*CC* in active sperm motility Grade C in AO and AT (16.500±3.42 and 15.000±2.24) compared with asthenozoospermic and AOT (16.760±1.12 and 11.000±3.06) as shown in table (2).This table showed a significant (*P*<0.05) increased in immotile sperm grade D of AO group (14.0±3.14) compared with another groups (asthenozoospermic, AT and AOT).Activation of sperms *in vitro* caused a highly significant (*P*<0.05) decrease in the percentage of morphologically normal sperm (MNS) in AT (51.0±2.87) when compared to the (asthenozoospermic, AO

and AOT) after activation by Ham's F-12+*CC* (Table 2).

Table-2: Certain sperm function parameters after *in vitro* activation by Ham's F-12+*CC* for all groups on this study.

Certain sperm function parameters		After *in vitro* activation by (Ham'sF12+*CC*)				*P*-value
		Astheno	AO	AT	AOT	
Sperm concentration (x10^6/ml)		24.860 a ±2.05	5.900 c ±0.67	12.800 b ±3.31	5.000 c ±0.71	<0.001**
Sperm motility (%)	A	28.180 c ±1.66	23.500 c ±5.33	34.500 b ±3.37	45.000 a ±5.53	<0.001**
	B	49.040 a ±1.57	46.000 a ±3.48	45.000 a ±2.98	37.000 b ±4.16	0.071 ns
	C	16.760 a ±1.12	16.500 ab ±3.42	15.000 ab ±2.24	11.000 b ±3.06	0.148 ns
	D	6.020 b ±0.61	14.000 a ±3.14	5.500 b ±1.17	7.000 b ±2.00	0.056 ns
Morphologically normal sperm (%)		66.700 a ±1.35	62.000 ab ±1.70	51.000 c ±2.87	60.500 b ±3.69	<0.001**

Values are expressed as Mean±SE

- Different letters within each row mean significant different at (*P*<0.05).
- **AO**=Astheno-oligozoospermic
- **AT**=Astheno-teratozoospermic.
- **AOT**= Asthen-ooligo-teratozoospermic.

3. Effect of *in vitro* activation by Ham's F12 medium on certain sperm function parameters for all groups (asthenozoospermic, AO, AT, AOT) on this study:

Table (3) was revealed the results of certain sperm function parameters after activation by a Ham's F-12 medium for all groups. The mean of sperm concentration following activation technique in AO, AT and AOT (4.100±0.48, 10.50±2.35 and 4.000±0.47, respectively) was highly significant (*P*<0.05) decreased when compared with asthenozoospermic group (21.740±1.92).

After activation by Ham's F12, The percentage of progressive motility (grade A) was significantly (*P*<0.05) reduced in AO (12.000±3.27) compared with asthenozoospermic and AT (16.420±1.1.63and 17.000±2.60), While no significant (*P*>0.05) in AOT (13.500±3.80) when compared with another group.There was a significant (*P*<0.05) increased in active sperm motility Grade B after activation by Ham's F12 in AO (50.500±2.83) compared with asthenozoospermic and AOT groups (46.900±1.94 and44.500±3.02), While no significant (*P*>0.05) in AT (47.500±4.30) when compared with another group.

There was no significant (*P*>0.05) improvement in active sperm motility Grade C in all groups after activation by Ham's F12 as shown in table (3).

Regarding the results of Grade D, there was a highly significant (P<0.05) increased in the percentage of immotile sperms of AOT group (19.500±4.86) compared with asthenozoospermic, AO and AT (10.680±1.13, 13.000±3.35 and 12.500±4.67 respectively), While no significant (P>0.05) differences between AO (13.000±3.35) and AT groups (12.500±4.67) as shown in table (4).

Activation of sperms by Ham's F-12 *in vitro* cause a significant (P<0.05) increased in the percentage of morphologically normal sperm (MNS) in asthenozoospermic group (57.000±1.60) when compared to the (AO, AT, and ATO) groups (48.500±1.98, 37.000±1.86 and 44.500±3.20 respectively) as shown in table (3).

Table 3: Certain sperm function parameters after *in* Ham's F-12 medium for all groups.

Certain sperm function parameters.		After *in vitro* activation by(Ham's F12)				*P*-value
		Astheno	AO	AT	AOT	
Sperm concentration (x10^6/ml)		21.74 ±1.92a	4.1 ±0.48c	10.5±2.35 b	4±0.5 c	<0.001**
Sperm motility (%)	A	16.420±1.63 a	12.0±3.27 b	17.0±2.60 a	13.5±3.8 ab	0.091 ns
	B	46.9±1.94 b	50.5±2.83 a	47.5±4.30ab	44.5±3.0 b	<0.001**
	C	25.9±1.63 a	24.5±4.56 a	23.0±3.67 a	22.5±4.90 a	<0.001**
	D	10.680±1.13 c	13.0±3.35 b	12.5±4.67 b	19.5±4.86 a	<0.001**
Morphologically normal sperm (%)		57.0±1.60 a	48.5±1.98 b	37.0 ±1.86c	44.5 ±3.2b	<0.001**

Values are expressed as Mean±SE

- Different letters within each row mean significant difference at (P<0.05).
- **AO**=Asthenooligozoospermic.
- **AT**=Asthenoteratozoospermic.
- **AOT**=Asthenooligoteratozoospermic.

4. Results of DNA integrity by acridi-ne ne orange test after activation using g Ham's F-12 medium for all groups:

The results of DNA fragmentation after activation using Ham's F-12 medium for all groups on this study were demonstrated in Figure (1). A highly significant (P<0.05) increased in the mean of sperm fragmentation (%) were revealed in AOT group (28.30) when compared to another groups (Astheno=11, 70, AO=19.70 and AT=21.01) .But no significant (P>0.05) difference in DNA fragmentation was statistically recorded between AO (19.70) and AT (21.01).

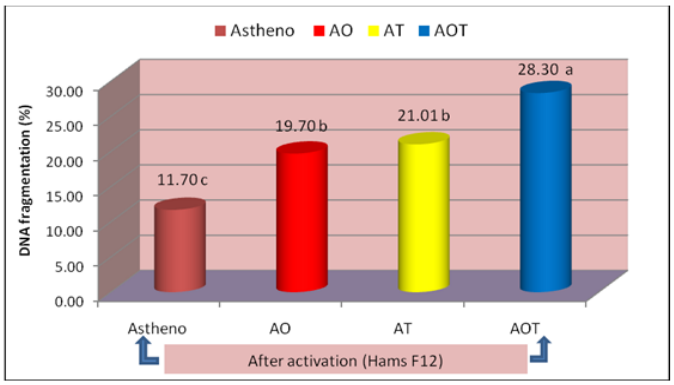

Figure-1: DNA fragmentation after activation using Ham's F-12 medium for all groups on this study.

5. Results of DNA integrity by acridine orange test after activation using Ham's F12+*CC* medium for all groups:

The results of DNA fragmentation after activation using Ham's F12+*CC* medium for all groups on this study were demonstrated in Figure (2). A significant (P<0.05) degrease in the means of sperm fragmentation (%) were revealed in Asthenozoospermic group (5.460) when compared to another groups (AO, AT and AOT) (10.80, 9.200 and 11.70, respectively).

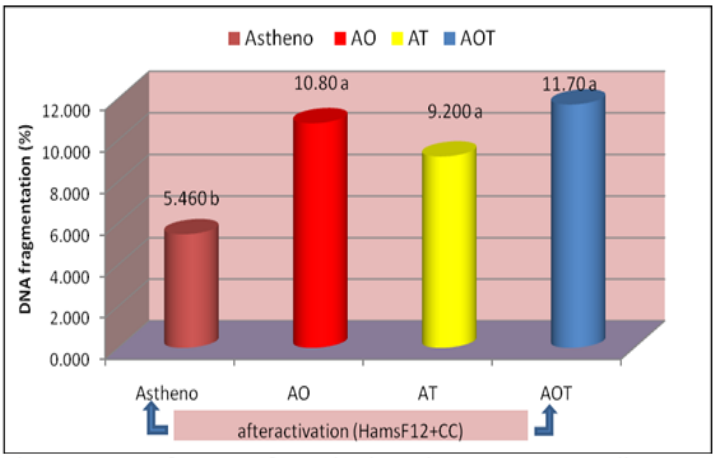

Figure 2: DNA of sperms after activation using Ham's F-12 medium containing *CC* for all groups on this study.

- Different letters mean a significant different at (P<0.05).
- **AO**=Astheno-oligozoospermic.
- **AT**=Astheno-teratozoospermic
- **AOT**= Astheno-oligo-teratozoospermic.

Discussion:

The results of all semen sample groups revealed that there is an decreased in the sperm concentration following *in vitro* activation by treated medium (Ham's F-12+CC)and control medium by (Ham's F-12).This finding may resulted from the effect of *in vitro* activation technique (swim up) that used in this work. In this technique the dead and immotile sperms were eliminated and only the active sperm swimming up and moving toward the upper layer of the medium [15].

The data of the present work found an increase in the percentage of active sperm motility grades (A&B) in all semen samples. This observation may be due to the effects of different compounds found in Hams F-12 and CC medium. The Hams F -12 medium provides the necessary salts, amino acids and vitamins which required for triggering of intact sperms leading to activation of sperms movement. Secondly, addition of CC to the Hams F-12 medium enhances different sperm function parameters following 30 minutes of activation, mainly total sperm motility percentage and grade activity of forward progressive movement as a result of CC components e.g. carbohydrates, amino acids, vitamins and others [15]. Furthermore, it has been reported that CC powder extract contains protein and amino acids [16], which sustain and maintain sperm osmolality and in turn integrity of sperm cell membrane. Therefore, the other possible effect of the CC to improve the sperm motility is that it may prevent free radicals formation, scavenging substance which has the ability to decrease the levels of free radicals leading to increase in sperm motility, grade activity of progressive forward movement. Decrease in the level of these free radicals is also associated with decrease in abnormal sperm forms [17] which is compatible with results of this study. The increase in sperm progressive motility after *in vitro* activation by medium contains Hams F-12 alone or with adding CC to the culture medium, resulted in a significant decrease in DNA abnormality of sperms in all patients men .This finding may contributed the effect of technique to collect only the active sperm motility with low sperm concentration and high MNS percentage. This in turn will increase the percentage of normal DNA sperms than before activation results [18].

However by using a medium contains CC the percentage of DNA fragmentation was significantly reduced than even by using a Hams F-12 medium alone. This result may

be due to the effect of constituents of CC that sustain a number of sluggish and immotile sperms with normal DNA to swim up to the upper layer of the medium leading to increase the sperm concentration than using Hams F 12 alone following *in vitro* activation, at the same time the percentage of DNA fragmentation was decreased when only the active and MNS were found in the last portion of activation. The decrease in DNA abnormality by using a medium contains CC may resulted from its protective effect on sperm membranes. CC may play a role in scavenging the reactive oxygen species(ROS) and reduces lipid peroxidation [19]. The CC has been shown to scavenge hydroxyl as well as superoxide radicals and inhibit their release [20]. Consequently, the *in vitro* activation of asthenozoospermic semen by Hams F-12 with CC showed a significant improvement than asthenooligozoospermic and asthenoteratozoospermic semen. This finding may related to oligozoospermia and teratozoospermic causes [21]. Several defects in the sperm forms and ultra-structure may founded in the asthenoteratozoospermic group of the current work leading to less response to activation medium contains CC .The correlation between asthenozoospermia and teratozoospermia associated with other syndromes was well known. It has been reported that immotile cilia syndrome (ICS) indicate a condition associated to sperm immotility and recurrent respiratory tract infections when all cilia and flagella functions are involved [22]. The impaired motility in ICS is due to defects of the axoneme[22].The results of DNA fragmentation of asthenospermic group following *in vitro* activation with CC were revealed a significant lower percentage than other groups. In normal status, the ROS known to affect the sperm genome, causing high frequencies of single- and double-strand DNA breaks [23] Both superoxide (O_2^-) and the hydroxyl radical (OH^-) are known to be mutagenic and cause chromosome deletions, dicentrics and sister chromatid exchanges [24].However, the activation with medium contains CC resulted in increase the number of intact DNA sperm than before activation and in comparison to other groups. This is because the CC has the ability to reducing the effects of ROS [25] by prevent free radicals formation, scavenging substance which has the ability to decrease the levels of free radicals leading to increase in sperm motility, grade activity of progressive forward movement [26] and concentration [27]. Finally, It was concluded from the present study that medium contains aqueous extract of CC have the components to improve the active sperm motility and increase DNA integrity of asthenozoospermic group which may emphasize that *Citrullus colocynthis* can be utilized for *in vitro* activation techniques in mammals.

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