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DNA Normality Following in Vitro Sperm Preparation with Pentoxifylline and L-Carnitine for Asthenozoospermic Infertile Men

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Objective: The goal of this study is to found out the optimum medium can increase the active motility percentage and reduce sperm DNA damage for asthenozoospermic men using the motility stimulant substances, PX and LC for this purpose.

Methods: Semen was collected from 100 infertile men involved in the current study. Each semen sample was divided into four portions. One part was considered as a control group and in vitro activated by using culture medium only. The other portions were considered as treated groups and in vitro activated by adding PX (1mg) and/or LC (0.5mg) to the culture medium. Certain sperm function parameters were examined before and following in vitro activation using layering technique. Sperms DNA damage was detected by using acridine orange (AO) test.

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Results: The results revealed a highly significant ($P<0.01$) increment in the mean of sperm concentration (m/ml) percentage of progressive sperm motility grade (A), grade (B) and grade (A+B) and the percentage of morphologically normal sperm (MNS) after using PX and/or LC medium in comparison with control medium .As well as, a highly significant ($P<0.001$) increase in the percentage of sperm normal DNA by using AO test.

Conclusion: It was concluded from the results of the present study that using layering technique with adding PX and/or LC to the culture medium resulting in an improvement in certain sperm function parameters and in the percentage of sperm normal DNA of asthenozoospermic men.

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I. Introduction

Infertility is the inability of a sexually active non-contraception couple to achieve pregnancy in one year (WHO ,2010) . A male factor is solely responsible in about 20% of infertile couples and contributory in another 30- 40%, if a male infertility factor is present, it is almost always defined by the finding of an abnormal semen analysis for the assessment of male fertility (Agarwal et al.,2003). Problems with the production and maturation of sperm are the most common causes of male infertility. Sperm may be immature, abnormally shaped, or unable to move properly. But, normal sperm may be produced in abnormally low numbers (oligozoospermia) or seemingly not at all (azoospermia) (Diemer et al., 2000).

Asthenozoospermia is one of the major causes of infertility or reduced fertility in men (4). Motility is the prime functional parameter that determines the fertilizing ability of spermatozoa, the cause underlying loss of sperm motility may be either hormonal, biochemical, immunological or infection (Henkel and Schill, 2003; Twigg et al., 1998).

Semen preparation techniques for assisted reproduction were developed to concentrate progressively motile, functional and morphologically normal spermatozoa, and to remove defective and non vital sperms as well as cells e.g. spermatogenic cells and leukocytes. Leucocytes, bacteria and dead spermatozoa produce oxygen radicals that negatively influence the ability to fertilize the egg (Aviad and Dettelbache, 1984). Layering technique is used for normospermic and asthenospermic semen, which allow self-selection of motile sperm.Two factors protect the sperm DNA from oxidative insult: the characteristic tight packaging of the DNA; and the antioxidants present in seminal plasma (Stanic et al, 2002).

On the other hand, pentoxifylline is dimethylexanthine derivative designated chemically as 1-(5-oxohexyl)-3, 7-dimethylexanthine (Okada,1997).The PX prevents cAMP breakdown by inhibiting the activity of the cAMP phosphodiesterase and presumably, stimulates sperm motion (Steiber et al.,2004). Moreover, PX has a protective effect on sperm membranes (i.e.it would preserve functional membrane integrity of sperm tail) as it scavenges ROS and then reduces lipid peroxidation (Muller et al 2002).

Moreover, L-carnitine is a quaternary ammonium compound biosynthesize from the amino acids lysine and methionine (Claudette and Lawrence, 1996). In human body, the primary L-carnitine function is to carry fatty acids into the mitochondria where they

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can be broken down with the ultimate production of energy (Oosterhuis et al., 2000). In the epididymis, the sperm use fatty acids as a source of metabolic energy and scientists believe that one of the functions of LC in sperm is to carry fatty acids into the sperm mitochondria, thereby assisting in the production of energy. Secondly, the conversion of some of the LC to ALC in the mature sperm facilitates the continuation of energy production within the sperm and the newly formed ALC serves as a readily available source of acetyl groups, i.e. energy, for the sperm (Sills, 2004).

A number of studies have investigated the relationship between human sperm DNA damage and semen parameters, such as concentration, motility and morphology (Gil-Guzman et al, 2001; Sikka, 2001). Oxidative stress (OS) may develop as a result of an imbalance between ROS generation and antioxidant scavenging activities (Cocuzza et al., 2007). Sperm preparation techniques can be used to decrease ROS damage production to enhance and maintain sperm quality after ejaculation (WHO, 1999). Therefore the aim of the present study was to use PX and LC for stimulate certain sperm functions and protect the male germ cell from the influence of free radicals.

II. Material and Methods

Semen samples were obtained from 100 infertile men during their attendance to the IVF High Institute, Al-Nahrain University through the period from November 2011 to October 2012. The samples of seminal fluid were collected after 3 to 5 days of abstinence directly into a clean, dry and sterile disposable Petri-dishes by masturbation in a room near the laboratory. After liquefaction time, macroscopic and microscopic analysis of semen samples was don using standardization of WHO (1999) to determine certain sperm function parameters namely; sperm concentration (million/ml), percentage of sperm motility and morphologically normal sperm (MNS) percentage. The DNA denaturation was examined by using acridine orange test according to Tejada, et al. (1984).

a) Preparation of Pentoxifylline Stock Solution

This solution was prepared by dissolving 10 mg from PX powder (sigma, USA) in 10 ml of PBS (0.1%) then stirring until dissolve. These concentrations prepared daily under sterile condition using UV light and Millipore filter (0.45μM).

b) Preparation of L-Carnitine Stock Solution

This solution was prepared by adding 0.5mg of LC powder (Natrol,USA) to 10 ml of phosphate buffer solution in plastic test tube . Then it was filtered by using Millipore 0.45 μM and have been fixed at pH 7.4- 7.8 at 25˚C.

c) In vitro activation technique

After liquefaction of human semen, layering activation technique was used according to Hall, et al. (1995), each semen sample was divided into four portions, one portion was considered as a control group by using Hams F-12 medium (Sigma, Germany) and the other three portions of semen is considered as treated groups by adding the following substances: Pentoxifylline (PX) 1mg/ml, L-Carnitine (LC) 0.5 mg/ml and both equally added PX + LC. Certain sperm function parameters were examined following in vitro activation according to WHO (1999) too.

Statistical analysis: Data from treated and control media groups were expressed as mean ± SEM and statistically analyzed using analysis of variance (ANOVA) 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 (Sorlie, 1995).

III. Results

Tables 1,2,3 and 4 shows that no significant ($P< 0.05$) difference in the mean of sperm concentration between control and treated media groups after activation in most infertile patients (asthenozoospermic men,table-1,oligoasthenozoospermic men, table-2, astheno- teratozoospermic men ,table-3 and oligoasth-enoteratozoospermic men ,table-4). The activation of human sperm in vitro with both control (Hams F-12) and treated (PX and/or LC) media caused a significant ($P<0.05$) and a highly significant ($P<0.001$) increase in the percentage of progressive sperm motility grade (A,B,A+B) compared to before activation by layering activation technique in all treated infertile groups(Tables 1,2,3,4). There was a highly significant ($P<0.001$) increment in the mean of sperm concentration, percentage of progressive sperm motility grade (A), grade (B) and grade (A+B) with the percentage MNS after using mixing PX+LC medium in comparison with using PX and LC alone and with control medium in the asthenozoospermic patients (Table-1) and other mild male factors infertility (Tables 2, 3, 4). Activation of human sperm caused a highly significant ($P<0.001$) improvement in the MNS in both control and treated group when compared to before activation and between treated semen samples when compared to control semen samples in asthenoteratozoospermic patients(Table-1) and oligoasthenoteratozoospermic (Table-4) patients following layering technique. There was a significant ($P<0.05$) and a highly significant ($P<0.001$) decrease in the round cells in both control and treated groups when compared to before activation of all infertile men. Also the results show a highly significant ($P<0.001$) improvement in the percentage of normal DNA sperms after activation with PX and/or LC by layering technique in most infertile patients.

Table 1 : Effect of in vitro activation with Hams F-12, Pentoxifylline , L-carnitine on certain sperm function parameters of asthenozoospermic patients using layering technique

Certain sperm function parameters		Before activation	hams F12	hams F12 +PX	LC	PX + LC
Sperm Concentration (Million/ml)		53.96±3.77^{a}	50.52±3.23^{a}	56.29±5.34^{a}	58.13±5.49^{a}	59.78±4.62^{a}
Active sperm motility (%)	Grade A	4.62±0.89^{A}	19.13±2.21^{B}	30.86±4.62^{B}	29.13±4.2^{B}	32.83±3.3^{B}
	Grade B	34.35±1.17^{A}	39.43±1.85^{A}	42.86±3.34^{b}	42.4±2.4^{b}	44.52±2.5^{B}
	Grade A+B	38.96±1^{A}	58.57±3.39^{B}	73.71±5.98^{Ba}	71.53±4.88^{Ba}	77.35±4.13^{B}
Morphologically Normal sperm (%)		35.92±0.81^{A}	39.13±1.3^{a}	45.57±2.31^{b}	46.2±2.42^{B}	51.35±2.34^{B}
Round cells (cell/HPF)		7.31±1.2^{a}	5±0.94^{ab}	3.21±1.28^{b}	3.67±1.2^{b}	3.96±0.99^{b}
Green sperm %		50.14±6.51^{a}	68.87±6.06^{b}	77.74±7.1^{b}	66.47±6.99^{ab}	73.12±6.46^{b}
Orange sperm %		49.86±6.51^{a}	31.12±6.06^{b}	22.25±7.09^{b}	33.77±6.92^{ab}	26.87±6.46^{b}

Values are expressed as Mean± SEM.
Different small letters mean significant difference at P<0.05.
Different capital letters mean significant difference at P<0.001.

Table 2 : Effect of in vitro activation with Hams F-12, Pentoxifylline, L-carnitine on certain sperm function parameters of oligoasthenozoospermic patients using layering technique

Certain sperm function parameters		Before activation	hams F12	hams F12 +PX	LC	PX + LC
Sperm Concentration (Million/ml)		10.81±1.22^{a}	16.92±3.83^{a}	22.70±2.30^{Ab}	26.15±2.96^{Ab}	32.94±4.16^{B}
Active sperm motility (%)	Grade A	0.94±0.50^{A}	6.42±2.23^{A}	28.90±5.54^{B}	22.31±3.64^{Ab}	29.94±3.04^{B}
	Grade B	25.63±1.91^{A}	23.67±1.96^{A}	35.80±2.10^{b}	38.00±3.30^{B}	42.19±2.28^{B}
	Grade A+B	26.56±1.85^{A}	30.08±3.63^{A}	64.70±7.20^{B}	60.31±6.55^{B}	72.13±4.79^{B}
Morphologically Normal sperm (%)		33.44±0.94^{a}	37.92±1.82^{a}	44.90±1.79^{b}	30.00±2.89^{a}	45.63±2.77^{B}
Round cells		4.63±0.61^{a}	3.33±0.85^{a}	2.40±0.58^{b}	1.85±0.60^{b}	1.88±0.66^{b}
Green sperm %		7.39±1.19^{A}	35.75±6.85^{B}	81.03±4.56^{B}	77.42±3.54^{B}	84.69±4.52^{B}
Orange sperm %		92.60±1.19^{A}	64.24±6.85^{B}	18.96±4.56^{B}	22.57±3.54^{B}	15.30±4.52^{B}

Values are expressed as Mean±SEM.
Different small letters mean significant difference at P<0.05.
Different capital letters mean significant difference at P<0.001.

Table 3 : Effect of in vitro activation with Hams F-12, Pentoxifylline, L-carnitine on certain sperm function parameters of asthenoteratozoospermic patients using layering technique

Certain sperm function parameters		Before activation	hams F12	hams F12 +PX	LC	PX + LC
Sperm Concentration (Million/ml)		31.15±2.85^{a}	40.96±3.89^{a}	36.50±5.82^{a}	37.33±5.52^{a}	59.87±4.59^{B}
Active sperm motility (%)	Grade A	6.58±1.33^{A}	18.39±2.10^{B}	21.71±3.06^{B}	21.47±3.03^{B}	32.78±2.92^{B}
	Grade B	24.31±1.98^{A}	37.61±2.63^{B}	36.14±4.62^{Ba}	36.60±3.13^{Ba}	46.65±1.94^{B}
	Grade A+B	30.88±2.48^{A}	56.00±4.20^{B}	57.86±7.04^{B}	58.07±5.66^{Ba}	79.43±3.49^{B}
Morphologically Normal sperm (%)		19.96±1.29^{Aa}	31.26±2.23^{b}	33.36±4.06^{B}	36.87±2.97^{B}	52.22±2.56^{B}
Round cells(cell/HPF)		7.46±1.44^{a}	5.74±1.30^{a}	3.07±1.03^{b}	6.07±1.76^{a}	4.52±1.09^{a}
Green sperm (%)		62.92±6.44^{a}	63.77±6.42^{a}	80.88±6.51^{b}	76.73±6.72^{a}	71.22±6.72^{ab}
Orange sperm (%)		37.12±6.43^{a}	36.24±6.42^{a}	19.12±6.51^{b}	25.26±6.72^{a}	28.77±6.72^{ab}

Values are expressed as Mean±SEM .
Different small letters mean significant difference at P<0.05.
Different capital letters mean significant difference at P<0.001.

Table 4 : Effect of in vitro activation with Hams F-12, Pentoxifylline , L-carnitine on certain sperm function parameters of oligoasthenoteratozoospermic patients using layering technique.

Certain sperm function parameters		Before activation	hams F12	hams F12 +PX	LC	PX + LC
Sperm Concentration (Million/ml)		12.75±0.76^{a}	18.24±2.59^{ab}	20.38±2.33^{bB}	21.86±1.78^{b}	28.44±3.10^{b}
Active sperm motility (%)	Grade A	2.60±0.97^{A}	15.59±1.55^{B}	23.50±2.63^{B}	14.93±1.78^{b}	27.89±3.09^{B}
	Grade B	19.55±1.46^{A}	35.18±2.13^{B}	37.31±2.80^{B}	31.57±2.20^{Bb}	43.33±2.26^{B}
	Grade A+B	22.15±1.94^{A}	50.76±2.99^{B}	60.81±5.03^{B}	46.50±3.37^{B}	71.22±4.77^{B}
Morphologically Normal sperm (%)		21.90±1.17^{A}	33.47±1.15^{B}	34.38±2.11^{B}	33.79±2.18^{B}	41.78±2.74^{Ba}
Round cells(cell/HPF)		6.65±0.66^{A}	2.41±0.49^{B}	2.38±0.93^{B}	2.21±0.70^{B}	2.94±0.92^{B}
Green sperm (%)		21.02±4.65^{A}	51.82±6.52^{B}	62.43±6.56^{B}	68.91±4.27^{B}	74.71±5.55^{B}
Orange sperm (%)		78.97±4.65^{A}	48.17±6.52^{B}	37.56±6.56^{Ba}	31.08±4.27^{B}	25.29±5.55^{B}

Values are expressed as Mean±SEM.
Different small letters mean significant difference at P<0.05.
Different capital letters mean significant difference at P<0.001.

IV. Discussion

In this study, there was a highly significant increase in the sperm motility grade (A) and grade (A+B), while there was a significant increase in sperm motility grade (B) in treated group. This finding is in agreement with studies that revealed a significant improvement in grade (A), hyperactivation and the acrosome reaction following activation by PX (Abid, 2005 and Al-Dujaily et al., 2007). PX has been demonstrated to increase testicular sperm motility when it added to culture media (Sato and Ishikawa, 2004). It inhibits the breakdown of cAMP and it is known that intracellular cAMP concentration plays a central role in cell energy which in turn sustain sperm motility. The increase of cAMP lead to increase progressive sperm motility. The cAMP plays an important role in the glycolytic path way of the sperm and, through its effect on glycolysis. It can influence the energy generation required for sperm motion (Steiber et al., 2004). The highly significant increase in most sperm parameters after adding LC to sperm activated medium which showed a highly elevation in active sperm motility was in agreement with other studies that reported a significant increase in the sperm motility when LC was added to ejaculated human spermatozoa (Al-Dujaily et al.,2012). The other positive effect of LC addition to the medium in current study may be its function to carry fatty acids into the sperm mitochondria to assisting the production of energy. (Agarwal and Said 2004).

This study believed that the medium contains both LC and PX gave excellent improvement in progressive sperm motility grade (A) and grade (A+B). This is in agreement with Aliabadi, et al. (2013) who

stated that in vitro administration of LC and PX to extracted testicular sperm samples led to increased sperm motility. Whereas giving a highly improvement results in the percent of MNS may be resulting from the important effect of both PX and/or LC that works as antioxidant ROS scavengers to reduce sperm DNA damage after activation (Menezo et al.,2007).

Further significant improvement in the percentage of MNS was recorded after activation. This finding may be related to the fast movement of normal spermatozoa from seminal plasma into upper layer of culture medium, and consequently elicited from impact of some seminal plasma components like leukocytes, round cell and others leading to kept the sperm out of stress factor and ROS production that responsible for DNA damage (Sharma et al., 2004). Thus, layering activation technique remove the immotile and dead cells from the sample. The same observation was noticed by other studies using culture for separation and activation of sperm in vitro (Al-Dujaily and Malik, 2013 and Al-Dujaily et al., 2006).

The results of the present study has found a highly significant reduction in abnormal (DNA damaged) sperms in the infertile semen after activation by all activation media compared with results before activation.This may be caused by the affection of antioxidants (including LC and PX every one alone or both of them)which added to the medium (HamsF12), PX has been oxygen-free radical scavenging capacities by reducing the superoxide release from human spermatozoa (McKinney et al.,1996).The PX has been shown to decrease ROS production. Moreover, the anti-oxidant property of LC may also have an influence on sperm motility. L-carnitine, as anti-oxidant (Solarska et al., 2010) may protect sperm plasma membrane with high level of unsaturated fatty acid content (Aitken and Clarkson, 1987). Free radicals can also decrease mitochondrial energy availability and impaired sperm motility (De-Lamirande and Gagnon, 1992). It has been emphasized that acridine orange staining of semen smears improve the information obtained by semen analysis with respect to sperm fertilizing capacity (Kosower et al.,1992). The cause of infertility in the infertile men with normal semen parameters could be related to abnormal sperm DNA (Menezo et al., 2007). Therefore, the present work depends on the evaluation of sperm DNA integrity to improve the positive effects of the activation technique and the motility stimulants and to add further information on the quality of spermatozoa that will be used in future on reproductive outcomes (Schulte et al.,2010).

It was concluded that LC and PX can be added for the medium as activator substances to stimulate certain sperm function parameters in vitro of asthenozoospermic patients with or without other male infertility factors to reduce DNA damage in sperms. This results can be utilized for in vitro activation medium used in the ART centers.

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