

1 DNA Normality Following in Vitro Sperm Preparation with 2 Pentoxifylline and L-Carnitine for Asthenozoospermic Infertile 3 Men

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6 **Abstract**

7 Background: Sperm cells from infertile patients have poor motility and defected DNA.
8 Pentoxifylline (PX) and L-carnitine (LC) are added in the medium for in vitro activation to
9 increase sperm motility and improve the pregnancy outcome. However, there are few studies
10 about the effect of PX with LC medium on sperm DNA normality. Objective: The goal of this
11 study is to found out the optimum medium can increase the active motility percentage and
12 reduce sperm DNA damage for asthenozoospermic men using the motility stimulant
13 substances, PX and LC for this purpose. Methods: Semen was collected from 100 infertile men
14 involved in the current study. Each semen sample was divided into four portions. One part
15 was considered as a control group and in vitro activated by using culture medium only. The
16 other portions were considered as treated groups and in vitro activated by adding PX (1mg)
17 and/or LC (0.5mg) to the culture medium. Certain sperm function parameters were examined
18 before and following in vitro activation using layering technique. Sperms DNA damage was
19 detected by using acridine orange (AO) test.
20

21 *Index terms*— pentoxifylline, l-carnitine, in vitro activation, layering preparation technique, male infertility.

22 **1 Introduction**

23 Infertility is the inability of a sexually active noncontraception couple to achieve pregnancy in one year ??WHO
24 ,2010) . A male factor is solely responsible in about 20% of infertile couples and contributory in another 30-40%,
25 if a male infertility factor is present, it is almost always defined by the finding of an abnormal semen analysis for
26 the assessment of male fertility (Agarwal et al.,2003). Problems with the production and maturation of sperm
27 are the most common causes of male infertility. Sperm may be immature, abnormally shaped, or unable to move
28 properly. But, normal sperm may be produced in abnormally low numbers (oligozoospermia) or seemingly not
29 at all (azoospermia) (Diemer et al., 2000).

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

34 Semen preparation techniques for assisted reproduction were developed to concentrate progressively motile,
35 functional and morphologically normal spermatozoa, and to remove defective and non vital sperms as well as
36 cells e.g. spermatogenic cells and leukocytes.

37 Leucocytes, bacteria and dead spermatozoa produce oxygen radicals that negatively influence the ability to
38 fertilize the egg (Aviad and Dettelbache, 1984). Layering technique is used for normospermic and asthenospermic
39 semen, which allow self-selection of motile sperm. Two factors protect the sperm DNA from oxidative insult: the
40 characteristic tight packaging of the DNA; and the antioxidants present in seminal plasma (Stanic et al, 2002).

41 On the other hand, pentoxifylline is dimethylxanthine derivative designated chemically as 1-(5oxohexyl)-3,
42 7-dimethylxanthine ??Okada,1997). The PX prevents cAMP breakdown by inhibiting the activity of the cAMP
43

7 RESULTS

44 phosphodiesterase and presumably, stimulates sperm motion (Steiber et al.,2004). Moreover, PX has a protective
45 effect on sperm membranes (i.e.it would preserve functional membrane integrity of sperm tail) as it scavenges
46 ROS and then reduces lipid peroxidation (Muller et al 2002).

47 Moreover, L-carnitine is a quaternary ammonium compound biosynthesize from the amino acids lysine and
48 methionine (Claudette and Lawrence, 1996). In human body, the primary L-carnitine function is to carry fatty
49 acids into the mitochondria where they can be broken down with the ultimate production of energy (Oosterhuis
50 et al., 2000). In the epididymis, the sperm use fatty acids as a source of metabolic energy and scientists believe
51 that one of the functions of LC in sperm is to carry fatty acids into the sperm mitochondria, thereby assisting
52 in the production of energy. Secondly, the conversion of some of the LC to ALC in the mature sperm facilitates
53 the continuation of energy production within the sperm and the newly formed ALC serves as a readily available
54 source of acetyl groups, i.e. energy, for the sperm (Sills, 2004).

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

61 2 II.

62 3 Material and Methods

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

71 4 a) Preparation of Pentoxifylline Stock Solution

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

75 5 b) Preparation of L-Carnitine Stock Solution

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

78 6 c) In vitro activation technique

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

85 Statistical analysis: Data from treated and control media groups were expressed as mean \pm SEM and
86 statistically analyzed using analysis of variance (ANOVA) to compare the differences between the four prepared
87 media. When F values reach the significant level at 5%, least significant difference (LSD) test was used (Sorlie,
88 1995).III.

89 7 Results

90 Tables 1,2,3 and 4 shows that no significant ($P < 0.05$) difference in the mean of sperm concentration between
91 control and treated media groups after activation in most infertile patients (asthenozoospermic men,table-
92 1,oligoasthenozoospermic men, table-2, astheno-teratozoospermic men ,table-3 and oligoasthenoteratozoospermic
93 men ,table-4). The activation of human sperm in vitro with both control (Hams F-12) and treated (PX and/or
94 LC) media caused a significant ($P < 0.05$) and a highly significant ($P < 0.001$) increase in the percentage of
95 progressive sperm motility grade (A,B,A+B) compared to before activation by layering activation technique
96 in all treated infertile groups(Tables 1,2,3,4). There was a highly significant ($P < 0.001$) increment in the mean
97 of sperm concentration, percentage of progressive sperm motility grade (A), grade (B) and grade (A+B) with
98 the percentage MNS after using mixing PX+LC medium in comparison with using PX and LC alone and with

99 control medium in the asthenozoospermic patients (Table-1) and other mild male factors infertility (Tables 2, 3,
100 4). Activation of human sperm caused a highly significant ($P<0.001$) improvement in the MNS in both control
101 and treated group when compared to before activation and between treated semen samples when compared to
102 control semen samples in asthenoteratozoospermic patients(Table-1) and oligoasthenoteratozoospermic (Table-
103 4) patients following layering technique. There was a significant ($P<0.05$) and a highly significant ($P<0.001$)
104 decrease in the round cells in both control and treated groups when compared to before activation of all infertile
105 men. Also the results show a highly significant ($P<0.001$) improvement in the percentage of normal DNA
106 sperms after activation with PX and/or LC by layering technique in most infertile patients. Values are expressed
107 as Mean \pm SEM. Different small letters mean significant difference at $P<0.05$. Different capital letters mean
108 significant difference at $P<0.001$.

109 IV.

110 8 Discussion

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

125 This study believed that the medium contains both LC and PX gave excellent improvement in progressive
126 sperm motility grade (A) and grade (A+B). stated that in vitro administration of LC and PX to extracted
127 testicular sperm samples led to increased sperm motility. Whereas giving a highly improvement results in the
128 percent of MNS may be resulting from the important effect of both PX and/or LC that works as antioxidant
129 ROS scavengers to reduce sperm DNA damage after activation (Menezo et al., 2007).

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

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

151 It was concluded that LC and PX can be added for the medium as activator substances to stimulate certain
152 sperm function parameters in vitro of asthenozoospermic patients with or without other male infertility factors
153 to reduce DNA damage in sperms. This results can be utilized for in vitro activation medium used in the ART
154 centers. ¹

8 DISCUSSION

1

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			
	GradA 4.62±0.89 A A	19.13±2.21 B	30.86±4.62 B	29.13±4.2 B	32.83±4.2 B			
Active sperm motility (%)	GradB 34.35±1.17 B A 38.96±1	39.43±1.85 A	42.86±3.34 b	42.4±2.4 b	71.53±4.88 B			
	GradA 58.57±3.39 A+B	73.71±5.98 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±2.42 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±0.96 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±6.42 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±6.42 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.

Figure 1: Table 1 :

2

Certain sperm function parameters	Before activation	hams	hams	LC	PX
Sperm Concentration (Million/ml)	F12	F12	+PX	+LC	
	10.81±1.2 a	16.92±2.30 a	30±2.20 b	15±2.90 b	4.16 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.

Figure 2: Table 2 :

3

Certain sperm function parameters	Before activation	hams F12	hams LC
Sperm Concentration (Million/ml)	31.15±2.85 a	40.96±3.89 a	36.50±5.82 a
	Grad 6.58±1.33 A	18.39±2.10 B	21.71±3.06 B
Active sperm motility (%)	Grad 24.31±1.98 B	37.61±2.63 B	36.14±4.62 Ba
	Grad 30.88±2.48 A+BA	36.60±3.13 Ba	56.00±4.20 B
Morphologically Normal sperm (%)	19.96±1.29 Aa	31.26±2.23 b	33.36±4.06 B
Round cells(cell/HPF)	7.46±1.44 a	5.74±1.30 a	3.07±1.03 b
Green sperm (%)	62.92±6.44 a	63.77±6.42 a	80.88±6.51 b
Orange sperm (%)	37.12±6.43 a	36.24±6.42 a	19.12±6.51 b
		25.26±6.72 a	76.73±6.72 a

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.

Figure 3: Table 3 :

4

Certain parameters	sperm function	Before activation	hams F12	hams +PX
Sperm Concentration (Million/ml)	12.75±0.76 a	18.24±2.59 ab		20.38±2.33 b
	Grade A	2.60±0.97 A	15.59±1.55 B	23.50±2.63 E
Active sperm motility (%)	Grade B	19.55±1.46 A	35.18±2.13 B	22.15±1.94 A
	Grade A+B	50.76±2.99 B	37.31±2.80 E	
Morphologically Normal sperm (%)	21.90±1.17 A	33.47±1.15 B		34.38±2.11 H
Round cells(cell/HPF)	6.65±0.66 A	2.41±0.49 B		2.38±0.93 B
Green sperm (%)	21.02±4.65 A	51.82±6.52 B		62.43±6.56 E
Orange sperm (%)	78.97±4.65 A	48.17±6.52 B		37.56±6.56 H

Figure 4: Table 4 :

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8 DISCUSSION

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