In vitro sperm activation with pentoxifylline and L-carnitine for infertile men semen using layering and sedimentation techniques

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Abstract:

Background:
Pretreatment of semen samples of the infertile patients and *in vitro* preparation techniques with the certain sperm stimulant agents prior to use for assisted reproductive techniques (ART) has been reported to have beneficial effects on pregnancy rates.

Objectives:
The objective of this study is to improve certain sperm function parameters *in vitro* of infertile patients semen by using novel combination motility stimulants substances that improved medium to be used for ART in future.

Patients and Methods:
One hundred infertile men were involved in the current study. They were divided into four groups, and each semen sample was divided into four portions. One portion was considered as a control and *in vitro* activated by using culture medium only. The other three portions were considered as treated groups and activated *in vitro* by adding pentoxifylline (PX,1mg/ml) and/or L-carnitine (LC,0.5mg/ml) to the culture media. Certain sperm function parameters were examined before and following *in vitro* activation using layering and sedimentation techniques.

Results:
The results showed a highly significant (P<0.001) improvement in the sperm concentration and percentage of progressive sperm motility grade (A), grade (B) and grade (A+B) with a highly significant (P<0.001) increase in the percentage of morphologically normal sperm (MNS) after addition of PX and/or LC to the culture medium in comparison with control medium using layering and sedimentation (layering under paraffin) techniques in the infertile patients…

Conclusion:
The results of this study indicated that adding PXand/or LC to the culture medium for *in vitro* sperm activation using layering and sedimentation techniques leads to an improvement in certain sperm function parameters.

Keywords: Pentoxifylline, L-carnitine, *in vitro* activation, sperm preparation techniques.

Introduction:

Infertility has to be a worldwide problem, affecting an estimated 60 -80 million women and men worldwide, a vast majority of whom live in resource poor countries(1) and affecting approximately 15% of all couples in their reproductive age(2). Male infertility refers to the inability of a male to achieve a pregnancy in a fertile female, and it is commonly due to deficiencies in the semen quality that is used as a surrogate measure of male infertility(3). Male infertility is due to the complete absence of sperm in the ejaculate and is relatively uncommon. Male subfertility can be due to low numbers of sperm, a low percentage of sperm with effective progressive movement or abnormalities in the sperm>s ability to fertilize an egg(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(5). Asthenozoospermia is therefore one of the major causes of infertility or reduced fertility in men(6).

A detailed semen analysis became the most important examination to be performed in the approach to the infertile couples(7). Basically, sperm count, motility and percentage of MNS are conventional criteria for semen quality(8).

Semen preparation techniques for assisted reproduction, including intrauterine insemination (IUI), were developed to concentrate progressively motile, functional and morphologically normal spermatozoa, and to remove defective and non vital sperms as well as cells (polygonal epithelial cells from urethral tract), spermatogenic cells and leukocytes(9) Sedimentation technique was used when the counts and poor motility of sperms are very low, and/or high percentage of debris exist in the sample. Whereas layering technique is used for normospermic and asthenospermic semen, which allow self-selection of motile sperm,(10).

There are different motility stimulant agents. One of these is PX which is dimethylexanthine derivative designated chemically as 1- (5- oxohexyl) -3, 7-dimethylexanthine(11). PX prevents cAMP breakdown by inhibiting the activity of the cAMP phosphodiesterase and presumably, stimulates sperm motion(12). Moreover, PX has a protective effect on sperm membranes as it scavenges ROS and then reduces lipid peroxidation(13).

Other stimulant agent is L-carnitine. The LC is a quaternary ammonium compound biosynthesize from the amino acids lysine and methionine(14). L-carnitine play important role in sperm metabolism by providing readily available energy for use by spermatozoa, which positively affects sperm motility, maturation and the spermatogenic process. This beneficial effect is mediated by the transport of long chain fatty acids across the inner membrane of the mitochondria for utilization in metabolism(15). The LC decreases DNA damage and improves the *in vitro* blastocyst development rate in mouse embryos(16). Therefore the aim of the present study is to examine the effect of adding PX and LC to the culture medium on certain sperm functions of asthenozoospermic men.

Material and Methods:

-Patients

The study was carried out in the High Institute of Embryo Research and Infertility treatment, Al-Nahrain University through the period from October 2012 to July 2013. One hundred with mild asthenozoospermia and/or other male factor infertility were involved in this research. The clinical examination performed by a consultant urologist in charge of male infertility Unit in the Institute including presence or absence varicocele, cryptoorchidism, hydrocele, hernia and others.

-Seminal fluid analysis

Semen samples were collected by masturbation into wide-mouth containers, supplied by the laboratory, after 3 -7 days of sexual abstinence in a room near the laboratory, immediately placed in an incubator at 37 C° till complete liquefaction. After liquefaction time, the semen samples were analyzed by a macroscopic and microscopic examinations using standardization of WHO(17).

-Preparation of Pentoxifylline stock solution

This solution was prepared by dissolving 10 mg from PX powder (Sigma, Germany) in 10 ml of phosphate buffered saline (PBS). This concentration was prepared daily under sterile condition using UV light and Millipore filter (0.45µM).

-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.

-*In Vitro* activation technique

After liquefaction of human semen, two techniques for *in vitro* sperm activation were used: layering technique(18) and sedimentation (layering under paraffin oil) technique(9). Each semen sample was divided into two parts. One part activated with layering technique and the other part with sedimentation technique. Each part was divided into four portions. One portion was considered as a

control by using Hams F-12 medium (Sigma, Germany) and the other three portions of semen considered as treated groups by adding the following substances: Pentoxifylline (PX) 1mg/ml, L-Carnitine (LC) 0.5 mg/ml and PX + LC. Certain sperm function parameters were examined following *in vitro* activation as described by WHO(17) too.

Statistical analysis:

Data from treated and control groups were expressed as mean ± SEM and using Students t-test to compare value between experimental and control groups. Differences between values were considered significant at P< 0.05. Analysis of variance (ANOVA) was used 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(19).

Results:

a) Effect of *in vitro* activation with Hams F-12, Pentoxifylline, L-carnitine on certain sperm function parameters of infertile patients using layering technique and sedimentation technique.

Tables 1,2,3 and 4 shows that the activation of human sperm *in vitro* with both control (Hams F-12) and treated (PX and/or LC) 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 in both sperm activation techniques in all treated infertile groups (asthenozoospermic men-table-1,Oligoasthenozoospermic, table-2,Oligoasthenozoospermic-table-3 and oligoasthenoteratozoospermic –table 4). There was no significant (P< 0.05) difference in the mean of sperm concentration between control and treated group after activation by both methods in most infertile patients. There was a highly significant (p<0.001) increment in the mean of sperm concentration and percentage of progressive sperm motility grade (A), grade (B) and grade (A+B) with the percentage of MNS by using a medium containing both PX+LC in comparison with using PX and LC each alone and with control medium after both activation techniques in the asthenozoospermic patients and other mild male factors infertility. Activation human sperm caused a significant (P<0.05) and a highly significant (P<0.001) improvement in the MNS in both control and treated groups when compared to before activation and between treated semen samples when compared to control semen samples after both *in vitro* activation techniques. There was a significant (P<0.05) and a highly significant (P<0.001) decrease in the round cells after both *in vitro* activation techniques in both control and treated group when compared to before activation of all infertile men.

b) Comparison between layering and sedimentation methods using four activation media (Hams F12, PX,LC, mixing PX +LC) in the infertile patients semen.

There was a highly significant (p<0.001) increment in sperm concentration following *in vitro* activation with PX and LC media comparison with control medium of oligoasthenozoospermic patients by using sedimentation technique rather than layering technique as showed (Table-2). The results revealed a highly significant (p<0.001) improvement after activation with PX and LC media comparison with control medium in the percentage progressive sperm motility grade (A) and grade (A+B) with the percent of MNS when using sedimentation technique in comparison with layering technique of asthenozoospermic patients (Table-1), while a highly significant (p<0.001) improvement in the percentage of MNS in asthenoteratozoospermic patients (Table-3) and oligoasthenoteratozoospermic patients (Table- 4) after both activation techniques.

Table1: Effect of *in vitro* activation with Hams F-12, Pentoxifylline, L-carnitine on certain sperm function parameters of asthenozoospermic patients using layering technique and sedimentation technique.

Certain sperm function		Before activation	In vitro activation							
			Hams F12		Hams F12 +PX		LC		PX+ LC	
			layering T.	Sedimen. T.	layering T.	Sedimen. T.	layering T.	layering T.	layering T.	Sedimen. T.
Sperm Concentration (Million/ml)		53.96 ±3.77^{a}	50.52± 3.23^{a}	58.85± 4.17^{a}	56.29 ±5.34^{a}	58.8 ±4.98^{a}	58.13± 5.49^{a}	58.13 ±5.49^{a}	59.78 ±4.62^{a}	62± 4.47^{a}
Active sperm motility (%)	Grade A	4.62± 0.89^{A}	19.13 ±2.21B	27.2± 3.09^{C}	30.86 ±4.62^{B}	28.47 ±3.34^{B}	29.13 ±4.2^{B}	29.13 ±4.2^{B}	32.83 ±3.3^{B}	34.58 ±3.59^{B}
	Grade B	34.35± 1.17^{A}	39.43 ±1.85^{A}	46.95 ±2.54^{B}	42.86± 3.34^{b}	49.27 ±2.72^{Bb}	42.4 ±2.4^{b}	42.4 ±2.4^{b}	44.52± 2.5^{B}	50.37± 2.89^{B}
	Grade A+B	38.96 ±1 ^{A}	58.57± 3.39^{B}	74.153.5± ^{C}	73.71± 5.98^{Ba}	77.73 ±3.89^{Bb}	71.53± 4.88^{Ba}	71.53± 4.88^{Ba}	77.35± 4.13^{B}	84.95 ±3.31^{B}
Morphologically Normal sperm (%)		35.92 ±0.81^{A}	39.13 ±1.3^{a}	46.95 ±2.24^{Bb}	45.57± 2.31^{b}	50.53± 3.08^{Bb}	46.2± 2.42^{B}	46.2± 2.42^{B}	51.35 ±2.34^{B}	52.84 ±2.78^{B}
Round cells Cell/ml Cee/m(cell/HPF)		7.31 ±1.2^{a}	5± 0.94^{a}	3.85± 1.53^{b}	3.21 ±1.28	2.4± 0.85^{Bb}	3.67 ±1.2 ^{b}	3.67 ±1.2^{b}	3.96± 0.99^{b}	1.79± 0.69^{Bb}

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 and sedimentation technique.

Certain sperm function		Before activation	In vitro activation							
			Hams F12		Hams F12 +PX		LC		PX+ LC	
			layering T.	Sedimen. T.	layering T.	Sedimen. T.	layering T.	layering T.	layering T.	Sedimen. T.
Sperm Concentration (Million/ml)		10.81± 1.22a	16.92 ±3.83a	35.92 ±5.77c	22.70± 2.30^{Ab}	51.80 ±8.20B	26.15± 2.96Ab	51.46± 7.03B	32.94 ±4.16B	37.93± 5.05B
Active sperm motility (%)	Grade A	0.94± 0.50A	6.42± 2.23 A	26.08 ±3.50B	28.90 ±5.54B	33.00± 2.71B	22.31± 3.64Ab	28.46 ±3.37B	29.94 ±3.04B	34.29± 3.85B
	Grade B	25.63 ±1.91A	23.67 ±1.96^{A}	43.77± 2.20B	35.80 ±2.10b	42.33± 2.17Bc	38.00 ±3.30B	42.54± 2.79B	42.19 ±2.28B	45.71± 1.03B
	Grade A+B	26.56± 1.85A	30.08 ±3.63A	69.85 ±4.75B	64.70± 7.20B	75.33 ±4.07B	60.31± 6.55B	71.00± 4.50B	72.13 ±4.79B	80.00± 3.44B
Morphologically Normal sperm (%)		33.44± 0.94^{a}	37.92 ±1.82^{a}	42.77 ±1.54b	44.90± 1.79b	47.13± 2.79Bb	30.00± 2.89a	43.00± 6.51a	45.63± 2.77B	46.86± 3.28B
Round cells Cell/ml Cee/m(cell/HPF)		4.63± 0.61^{a}	3.33± 0.85a	1.77± 0.73b	2.40± 0.58b	1.87 ±0.80b	1.85± 0.60b	1.23± 0.65b	1.88± 0.66b	1.64± 0.77Bb

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 C patients using layering technique and sedimentation technique.

Certain sperm function		Before activation	In vitro activation							
			Hams F12		Hams F12 +PX		LC		PX+ LC	
			layering T.	Sedimen. T.	layering T.	Sedimen. T.	layering T.	Sedimen. T.	layering T.	Sedimen. T.
Sperm Concentration (Million/ml)		31.15± 2.85a	40.96± 3.8 a	35.65± 5.47a	36.50± 5.82a	50.87± 3.21b	37.33± 5.52a	53.56± 5.12Bb	59.87± 4.59B	62.00± 4.47B
Active sperm motility (%)	Grade A	6.58± 1.33 A	18.39 ±2.10B	18.80± 2.28B	21.71 ±3.06B	24.93± 1.96B	21.47 ±3.03B	28.69 ±2.24B	32.78± 2.92B	34.58± 3.59B
	Grade B	24.31± 1.98 A	56.00± 4.20B	39.80± 2.86B	36.14± 4.62Ba	45.27 ±1.89Bb	36.60± 3.13Ba	49.88 ±1.92Bb	46.65± 1.94B	50.37 ±2.89B
	Grade A+B	30.88 ±2.48 A	58.57± 3.39^{B}	58.60 ±4.82B	57.86 ±7.04B	70.20± 3.02B	58.07± 5.66Ba	78.56± 3.19Bb	79.43± 3.49B	84.95 ±3.31B
Morphologically Normal sperm (%)		19.96± 1.29 Aa	31.26± 2.23b	35.85 ±4.03Bb	33.36± 4.06B	42.00± 3.16B	36.87 ±2.97B	42.88 ±2.86B	52.22 ±2.56B	52.84± 2.78B
Round cells Cell/ml Cee/m(cell/HPF)		7.46± 1.44 a	5.74 ±1.30a	6.00± 1.45a	3.07± 1.03b	4.07± 1.08a	6.07± 1.76a	4.81± 1.63a	4.52 ±1.09a	1.79± 0.69b

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 and sedimentation technique.

Certain sperm function		Before activation	In vitro activation							
			Hams F12		Hams F12 +PX		LC		PX+ LC	
			layering T.	Sedimen. T.	layering T.	Sedimen. T.	layering T.	Sedimen. T.	layering T.	Sedimen. T.
Sperm Concentration (Million/ml)		12.75± 0.76a	18.24 ±2.59ab	25.35± 2.87b	20.38± 2.33bB	41.20± 5.29B	21.86± 1.78b	29.47± 3.72b	28.44± 3.10b	32.47± 3.41b
Active sperm motility (%)	Grade A	2.60 ±0.97A	15.59 ±1.55B	17.65 ±2.27B	23.50± 2.63B	27.33± 2.56B	14.93± 1.78b	27.87± 2.99b	27.89± 3.09B	30.53± 2.98B
	Grade B	19.55 ±1.46A	35.18 ±2.13B	37.12± 2.14B	37.31± 2.80B	43.20± 3.12B	31.57± 2.20Bb	43.80± 2.85Bb	43.33± 2.26B	47.79± 2.60B
	Grade A+B	22.15± 1.94A	50.76 ±2.99B	54.76± 3.32B	60.81 ±5.03B	70.53± 3.61B	46.50± 3.37B	71.67± 5.16B	71.22± 4.77B	78.32± 4.53B
Morphologically Normal sperm (%)		21.90± 1.17A	33.47 ±1.15B	32.88± 1.22B	34.38± 2.11B	38.00± 2.53B	33.79± 2.18B	38.20± 1.72B	41.78± 2.74Ba	48.89± 2.72Bb
Round cells Cell/ml Cee/m(cell/HPF)		6.65 ±0.66A	2.41 ±0.49B	2.35± 1.25B	2.38± 0.93B	1.67± 0.70B	2.21± 0.70B	0.80± 0.30B	2.94± 0.92B	1.53± 0.59B

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.

Discussion:

A significant improvement in the certain sperm function parameters was recorded after activation. This finding may be related to the fast movement of normal spermatozoa from seminal plasma into 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(20). The same observation was noticed by other studies (21,22). Regarding sperm motility, there was a highly significant increase in sperm motility grade (A) and grade (A+B), while there is a significant increase in sperm motility grade (B) in treated group. This in agreement with studies that revealed a significant improvement in grade (A), hyperactivation and the acrosome reaction following activation by PX (23,24). Pentoxifylline has been demonstrated to increase testicular sperm motility when it added to culture media(25). 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(12).

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(26). Peivandi(27) reported similar results when a significant correlation was noticed between seminal LC concentration and sperm concentration, sperm motility, rapid forward progression, live sperm count, membrane function, capacity for cervical mucus penetration, linearity of spermatozoal movement, amplitude of lateral sperm head movement and nuclear DNA integrity. 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, due to assisting the production of energy. This positive effect of LC on sperm motility is due to its role in sperm metabolism as well as its antioxidant properties(28). In present study, activation of human sperm with LC caused a significant increment in morphologically normal sperm. This result is compatible with Al-Dujaily(26) results. *In vitro* addition of LC may reduce the pathway of lipid peroxidation by transporting fatty acids into the mitochondria for ß-oxidation to generate adenosine triphosphate (ATP). Therefore, LC can protects the cell membrane and DNA against damage induced by ROS(29).

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(30) who stated that *in vitro* administration of LC and PX to extracted testicular sperm samples led to increased sperm motility and a highly improvement results in the percent MNS. This is may be resulting from the important effect of both antioxidant ROS scavengers to improves sperm DNA damage after activation(31).

The present study used two techniques (layering and sedimentation) were giving more effective in certain sperm parameters including progressive sperm motility specially grade (A), grade (B) and grade (A+B) which recoded highly significant elevation in sperm motility after activation with LC and PX alone and when both added to the medium used for asthenozoospermic and oligoasthenozoospermic patients semen. However by sedimentation technique the results of active motility sperm concentration was more improve than that of layering technique. This agreement with study of Sánchez(32) that showed after concentration of sperm cells in the ejaculate by sedimentation (layering under paraffin) technique, even in cases with severe oligo- and/or asthenozoospermia a sufficient number of motile spermatozoa were isolated after 2–3 hours of incubation. This author also demonstrated significantly better results for progressive motility, normal sperm morphology, chromatin condensation and reduction of the percentage of dead spermatozoa by using layering under paraffin technique due to the present of oil paraffin in sedimentation method which became more useful of rigidly maintaining fixed stable parameters of temperature pH and osmolarity. The use of an oil overlay also influences oxygen concentration in the medium, with resulting effects on the delicate balance of sperm metabolism; as mentioned previously, it can absorb and concentrate harmful volatile organic compounds. Oil overlays must be further equilibrated in the CO2 incubator for several hours (or overnight) before introducing media/gametes. As well as the prolonged exposure to the media and CO2 incubation may be causing improve the results of this method than others because of almost consumption more time than others(33).

It was concluded that the best combination of mixing PX and LC can be added for the medium as activator substances to stimulate sperm functions *in vitro* of asthenozoospermic patients with or without other male infertility factors and combination with both sperm separation methods to reduce DNA damaged sperms. This results can be utilized for *in vitro* activation medium used in the ART centers.

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