Mice embryonic development from cryopreserved epididymal sperm activated by pentoxifylline and L-carnitine: experimental model for human obstructive azoospermia

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Abstract:

Background

Many studies has been found that the pentoxifylline (PX) and L-carnitine (LC) as motility stimulants showed a positive effect on the activation of sperm in vitro and improve the forward movement after cryopreservation

Objectives

The present study aim to investigate the possibility of using the motility stimulants(i.ePX ,LC and mixture of PX with LC medium) for in vitro sperm activation after cryopreservation of epididymal sperms and their effects in vitro on fertilization rate (FR) and early embryonic development (ED) following in vitro fertilization (IVF) process as a model for obstructive azoospermia in men.

Methods

Fifty mature male and Fifty mature female mice, 8-12 weeks old were used. Epididymal sperms were obtained from caudal region and in vitro direct activation technique was done after cryopreservation by using four media namely; PX alone, LC alone, a mixture of PX with LC and Ham's F-12 (as control media). The four media were used for IVF of mature ova and FR with ED were recorded.

Results

In vitro activation of epididymal sperms with PX , LC and mixture of PX with LC has shown positive effect on sperm concentration, sperm motility, and grade activity of progressive forward movement after cryopreservation. There was a significant ($P<0.05$) increase of FR in treated media compared with the control medium after 24 hours of insemination. The study showed that the quality and quantity of embryos

Conclusion

It is concluded from the results of the present study that adding PX, LC and mixture of PX with LC medium to the culture medium of cryopreserved epididymal sperms and IVF leads to an improvement in certain sperm function parameters, supports the FR and early ED rate .

Key word: pentoxifylline ,L-carnitine, embryonic development

Introduction

Infertility is defined as the inability to achieve conception despite one year of regular, unprotected intercourse (1) .For healthy young couples, the probability of achieving pregnancy within the first year of fertility focused sexual activity is 85%(2) . Approximately 15% of couples do not achieve pregnancy within one year of unprotected sexual intercourse. A male infertility factor is identified in about 50% of these cases and is solely responsible in 20% of couples (3). Male infertility has been attributed to a variety of causes including lifestyle factors, gonadotoxin exposure, hormonal dysfunction, chromosomal disorders, varicocele, testicular failure, ejaculatory disorders, and obstruction . One of male infertility factor is azoospermia. On the other hand, many substances have been proposed for stimulation of human sperm function in vitro. These include poorly defined biologic materials, (ex: serum, peritoneal fluid, and follicular fluid) as well as defined agents, such as adenosine analogues, progesterone and methylxanthines (ex: caffeine and pentoxifylline) (4) .

Pentoxifylline is a methylxanthine that acts as a phosphodiesterase inhibitor and subsequently increases intracellular cyclic adenosine monophosphate (cAMP) levels. L-carnitine considering the chemical structure, the choline-like metabolite LC is (3-Hydroxy-4-N-trimethylammonium butyric acid) (5) . Is a quaternary amine compound biosynthesized from amino acids lysine and methionine (6) . Carnitine exists in two stereosomers : its biologically active form is LC whiles its nantiomer , D-carnitine, is biologically inactive (7) .

Cryopreservation is widely used in many assisted conception units to preserve male fertility, for example before cytotoxic chemotherapy, radiotherapy (8), or certain surgical treatments that may lead to testicular failure or ejaculatory dysfunction. Unfortunately, the use of cryopreserved spermatozoa limits the success of these techniques because the freeze - thaw process results in reduced motility, viability and fertilization potential of human semen (9) . Freezing and thawing involve damage to the plasma membrane and acrosome of human spermatozoa as evidenced by significant ultra structural changes demonstrated by electron microscopy (10) . Thus the aim of this study is to investigate the possibility of using a motility stimulants namely PX ,LC and mixture of PX with LC medium for in vitro sperm activation after cryopreservation and to found out their effects on fertilization rate (FR) and early embryonic development (ED) following in vitro fertilization (IVF) process.

Materials and Methods

Animals Management: Fifty mature male and Fifty mature females mice with age of 8-12 weeks old and 25-30 gm body weight were obtained from the animal house at the High Institute of Infertility Diagnosis and ART / AL-Nahrain University. They were kept in an air conditioned room (~25 ?C) with a photoperiod (13+2) hours. The animals were housed in a box cage isolated females from males in opaque plastic measuring (29×15×12) cm. Its floor covered with wooden shave. Each cage contains four animals and Tap water and diet were freely available for the animals.

Experimental Design: Male mice were sacrificed by cervical dislocation and dissect the epididymis, the sperms were obtained by flashing method with 1 ml culture medium (Ham's F-12).

Epididymal Sperm Cryopreservation: The cryopreservation occur by using 5%DMSO as cryoprotectant was mixed with epididymal sperm sample and put in cryovial (Nunc Tm, Denmark)(capacity 1.8 ml). Each cryovial was hold by cryovial holder (Nunc Tm, Denmark) and leaf for 10 minutes at room temperature. After that the sample was hanged in the liquid nitrogen vapor for 30 minutes and then embedded the sample in the liquid nitrogen.

Thawing: After one month of cryopreservation the thawing of samples were done by rapid transfer cryovial from liquid nitrogen to the water bath (Kotterman) 37 ?C until melting ice, for at least 5 minutes Each sample was mixed with 1:1 ratio culture medium (Ham's F-12) and incubated for 10 minutes as described by Al-Dujaily, et al. (11) and then divided into four media :

1- Medium (1): control medium (free-treated with PX and LC) .The epididymal sperms were in vitro activated after cryopreservation by Ham's F-12 (0.5 ml) medium.

2- Medium (2): The epididymal sperms were in vitro activated after cryopreservation by adding (0.5 ml) from PX medium. 3- Medium (3): The epididymal sperms were activated after cryopreservation by adding (0.5 ml) from LC medium . 4- Medium (4): The epididymal sperms were in vitro activated after cryopreservation by adding (0.5 ml) from PX and LC medium. then certain sperm function parameters were recorded. In vitro Fertilization Procedure: The IVF procedure was performed according to Nagy,et al. (12) Superovulation Induction: Superovulation program starts by injecting female mice intraperitoneally with 7.5 I.U. of pregnant mare serum gonadotropin (PMSG) followed by 7.5 I.U. of hCG 48 hours later. Oocytes Collection: Female mice were sacrificed by cervical dislocation at approximately 13 hours post-hCG injection, the oviducts were isolated.

The oocytes were tested under inverted microscope to observe the fertilization with the degree of oocyte mitosis and maturation (13) .Then, the fertilized oocytes incubated overnight again to identify embryonic development . Fertilized ova were diagnosed by the observation of two pronuclear and two polar bodies. The morphology of 2 to 8 blastomeres of each embryo was divided into 4 grades and scored according to the criteria of Khalili and Anvari, (14) .

Statistical Analysis: For the treatment medium (PX alone or with LC) and for the control medium (free-treated with PX and LC) group data of mice sperm analysis were expressed as mean ±SEM and analyzed by using paired sample t-test. While Chi-square test was used to compare values from treatment and control group at fertilization rate, embryonic development rate and early embryo scoring after 24 and 48 hours of insemination. P-value<0.05 was considered significant in this study (15).

Results -Effect of in vitro activation on certain function parameters of cryopreserved epididymal sperms by PX medium , LC medium , medium containing both PX and LC and Ham's F-12 medium before cryopreservation.

There was a highly significant (P<0.001) decrease in sperm concentration and active sperm motility grade A, B and grade A+B in treated media by PX ,LC and medium containing both PX and LC compared with Ham's F-12 medium. Activation of epididymal sperm in vitro showed no significant (P>0.05) difference in the percentage MAS between treated media by PX ,LC and medium containing both PX and LC compared with Ham's F-12 medium as shown table (1).

-Effect of in vitro activation on certain function parameters of cryopreserved epididymal sperms by PX medium , LC medium , medium containing both PX and LC and Ham's F-12 medium after cryopreservation.

There was no significant (P<0.001) in sperm concentration and active sperm motility grade A and MAS in treated media by PX ,LC and medium containing both PX and LC compared with Ham's F-12 medium but showed a significant at (P<0.001) increase active sperm motility grade B ,grade A+B in treated media by PX ,LC and medium containing both PX and LC compared with Ham's F-12 medium as shown table (2).

-Fertilization Rate: The result of treated medium by PX alone was a highly significantly at (P<0.05) improved compared to another treated media as LC, PX and LC and control medium Ham's F-12 as shown table (3).

-Embryonic Development:

1 .Embryonic Development after 24 hours:There was a significant (P<0.05) improvement in the total number of embryos in treated media by PX ,LC and PX+LC compared to the control medium Ham's F-12 at two cell and three-four cell stage of embryos.Also, there were variations in the ED between treated media was best PX and LC medium at two cell stage, and PX medium significant at 3-4 cell stage of embryos as shown table (4).

2. Embryonic Development after 48 hours: There were a significant differences (P<0.05) between treated media by PX, LC and PX+LC in the total number of two cell stage, three-four cell and five - eight cell stage of embryos after 48 hour compared control medium Ham's F-12. Also, there were variations in the ED between treated media was best LC alone and PX and LC medium at two cell stage, PX medium at 3-4 cell stage and 5-8 cell stage of embryos as shown table (5).

-Embryos Grading Score:

*Two and Three - Four Cell Stage Embryos after 24 Hours: There was a significant (P<0.05) increase in the number of normal ED in grade A and B in the treated media by PX, LC and PX+LC compared to control medium Ham's F-12. While, there was a highly significant (P<0.05) decrease in grade C and D in the treated media by PX, LC and PX+LC compared to control medium Ham's F-12. Also, there was a variations in the ED between treated media by PX, LC and PX+LC no significant (P<0.05) difference between them in grade A and B, But there was a difference between them in grade C and D at two cell stage as shown table (6).

Two, Three - Four and Five -Eight Cell Stage Embryos after 48 Hours: A significant (P<0.05) differences were observed between the treated media by PX, LC and PX+LC and the control medium Ham's F-12with respect to the different grades of development .Also there was a significant (P<0.05) difference in the number of ED between treated media PX, LC and PX+LC in grades A ,C and D .The best result in treated medium with PX alone in grade A and C. But there was no difference between them in grade B at 5-8 cell stage of embryos as shown table (7).

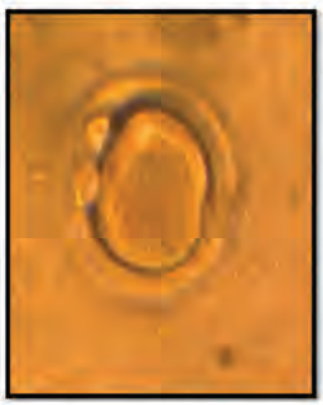


Figure (1): fertilized oocyte (Ham?s F-12 medium, 40X)

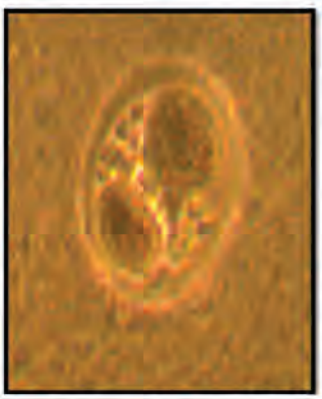
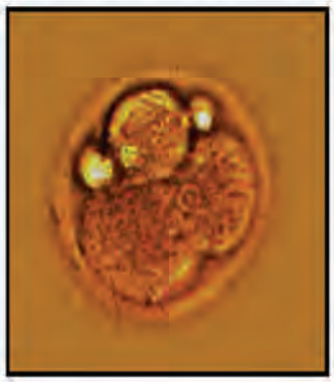


Figure (2): Two cell stage embryo. (Ham's F-12



Figure(3):Three cell stage embryo (Ham' F-12 medium, 40X)

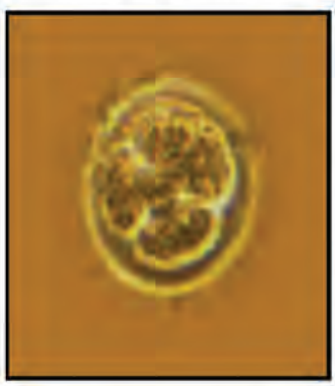


Figure (4): Four cell stage embryo.medium, 40X) (Ham's F-12 medium,

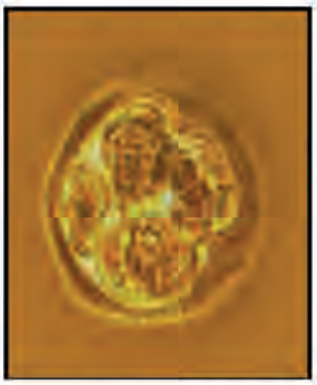


Figure (5): Five cell stage embryo. (Ham's F-12 medium, 40X)

Figure (6): Five-Eight cell stage embryo (Ham's F-12 medium, 40X)

Table (1):Comparison the effect of in vitro activation on certain function parameters of cryopreserved epididymal sperms between pentoxifylline (PX) medium , L-carnitine(LC) medium and medium containing both pentoxifylline (PX) and L-carnitine(LC) with Ham's F-12 medium (control) before cryopreservation.

Certain sperm function parameters		Cryopreservation status			
		Before cryopreservation free medium (Ham's F-12)	After cryopreservation and activation by PX medium	After cryopreservation and activation by LC medium	After cryopreservation and activation by PX and LC medium
Sperm concentration(10^6/ml)		(30.375±1.618) A	(16.8±0.925) B	(17.15±0.549) B	(17.75±0.575) B
Active sperm Motility (%)	Grade A	(20.230±0.992) A	(2.581±0.232) B	(1.809±0.157) B	(1.781±0.179) B
	Grade B	(22.356±1.074) A	(10.875±0.699) B	(10.18±0.530) B	(11.135±0.665) B
	Grade A+B	(42.587±1.609) A	(13.456±0.799) B	(11.989±0.776) B	(12.916±0.788) B
Morphologically abnormal sperm (%)		(3.383±0.222) A	(3.1±0.203) A	(3.29±0.178) A	(3.15±0.208) A

Values are expressed as mean ±SEM ANOVA Test capital letters means a highly significant at P<0.001 .
" No . samples before cryopreservation=60.
" No. sample treated by PX=20.
" No. sample treated by LC =20.
" number sample treated by PX and LC =20.

table (2): Comparison the effect of in vitro activation on certain function parameters of cryopreserved epididymal sperms between pentoxifylline(PX) medium, L-carnitine(LC) medium and medium containing both pentoxifylline(PX) and L-carnitine(LC) with Ham's F-12 medium (control) after cryopreservation.

Certain sperm function parameters		Cryopreservation status			
		After cryopreservation free medium (Ham's F-12)	After cryopreservation and activation by PX medium	After cryopreservation and activation by LC medium	After cryopreservation and activation by PX and LC medium
Sperm concentration(10^6/ml)		(18.083±0.454) A	(16.8±0.925) A	(17.15±0.549) A	(17.75±0.575) A
Active sperm Motility (%)	Grade A	(1.247±0.039) A	(2.581±0.232) A	(1.809±0.157) A	(1.781±0.179) A
	Grade B	(6.95±0.228) A (a)	(10.875±0.699) B	(10.18±0.530) B	(11.135±0.665) b
	Grade A+B	(8.197±0.257) A	(13.456±0.799) B	(11.989±0.776) B	(12.916±0.788) B
Morphologically abnormal sperm (%)		(4.033±0.144) A	(3.1±0.203) B	(3.29±0.178) A	(3.15±0.208) A

Values are expressed as mean ±SEM

Different capital letters means a highly significant at P<0.001
" Different small letters means a significant at P<0.05.
" No. samples after cryopreservation=60.
" No. sample treated by PX=20.
" No. sample treated by LC=20.
" No. sample treated by PX and LC=20

Table (3) : In vitro Fertilization rate by using control medium and treated media after 24 hours of insemination.

Parameters	Control medium (Ham' s F-12)	Treated media		
		Medium with PX	Medium with LC	Medium with PX and LC
Collected oocyte	362	241	458	321
Fertilized oocyte after 24 hours	137	129	96	126
Fertilization rate	37.84% B	53.52% A	20.96% C	39.25% B
Different capital letters means a significant at P<0.05				

Table (4) : Comparison of embryonic development after 24 hours following in vitro fertilization process between Ham?s F-12 medium (control) and media containing pentoxifylline medium , L-carnitine medium and medium containing both pentoxifylline and L-carnitine.

Embryonic development stages with and without adding pentoxifylline and L-carnitine medium		Embryonic development	
		No.	%
Total number of 2-cell stage of embryo	Ham' s F-12 medium	87/137	63.50% A
	pentoxifylline medium	70/129	54.25% B
	L-carnitine medium	62/96	64.58% A
	pentoxifylline and L-carnitine medium	96/126	76.19% C
Total number of 3-4 cell stage of embryo	Ham' s F-12 medium	50/137	36.50% A
	pentoxifylline medium	59/129	45.75% A
	L-carnitine medium	0/96	0% C
	pentoxifylline and L-carnitine medium	30/126	23.81% B
Different capital letters mean a significant difference at p<0.05			

Table (5) :Comparison of embryonic development rate after 48hours following in vitro fertilization procedure between Ham's F-12 medium (control) and media containing pentoxifylline medium , L-carnitine medium and medium containing both pentoxifylline and L-carnitine.

Embryonic development stages with and without adding pentoxifylline and L-carnitine medium		Embryonic development	
		No.	%
Total number of 2-cell stage of embryo	Ham' s F-12 medium	37/137	27.0% A
	pentoxifylline medium	30/129	23.30% A
	L-carnitine medium	62/96	64.58% B
	pentoxifylline and L-carnitine medium	83/126	65.85% B
Total number of 3-4 cell stage of embryo	Ham' s F-12 medium	62/137	45.25% A
	pentoxifylline medium	68/129	52.70% A
	L-carnitine medium	0/96	0% C
	pentoxifylline and L-carnitine medium	23/126	18.25% B
Total number of 5-8 cell stage of embryo	Ham' s F-12 medium	38/137	27.75% A
	pentoxifylline medium	31/129	24.00% A
	L-carnitine medium	0/96	0% C
	pentoxifylline and L-carnitine medium	20/126	15.90% B
Different capital letters mean a significant difference at p<0.05.			

Table (6): Comparison of embryo grading score between treated media and control medium after 24 hours of insemination by ***in vitro*** fertilization procedure.

Embryonic development stages with and without adding pentoxifylline and L-carnitine medium		No.	Embryonic grades			
			Grade A	Grade B	Grade C	Grade D
Total number of 2-cell stage of embryo	Ham's F-12 medium	67	15 17.24% A a	20 23% A b	25 28.73% A c	27 31.03% A c
	pentoxifylline medium	70	20 28.5% B a	21 30% B a	13 18.5% B c	16 23% B b
	L-carnitine medium	63	18 29.0% B a	16 26% A a	15 24% A b	13 21% C b
	pentoxifylline and L-carnitine medium	98	25 26.0% C a	31 32% B a	19 20% C b	21 22% B b
Total number of 3-4 cell stage of embryo	Ham's F-12 medium	50	10 20% A a	12 24% A b	13 26% A b	15 30% A c
	pentoxifylline medium	59	16 27% B a	19 32% B a	13 22% B b	11 19% C c
	L-carnitine medium	0	---	---	---	---
	pentoxifylline and L-carnitine medium	39	8 26.5% B a	11 37% C a	5 16.5% C c	6 20% B b
Different capital letters mean a significant difference at p<0.05 between the embryo grades.						
Different small letters mean a significant difference at p<0.05 between different media at each embryo grades.						

Table (7) : Comparison in embryo grading score between treated media and control medium after 48 hours of insemination by in vitro fertilization procedure

Embryonic development stages with and without adding pentoxifylline and L-carnitine medium		No.	Embryonic grades			
			Grade A	Grade B	Grade C	Grade D
Total number of 2-cell stage of embryo	Ham's F-12 medium	37	5 13.5% A c	11 29.7% A a	10 27% A b	11 29.8% A a
	pentoxifylline medium	30	6 20 % C b	9 30% A a	10 33.5% B a	5 16.5% C c
	L-carnitine medium	62	12 19.5% C b	18 29% A a	22 35.5% C a	10 16% C c
	pentoxifylline and L-carnitine medium	83	15 18 % B b	18 21.5% B b	27 32.5% B a	23 28% B a
Total number of 3-4 cell stage of embryo	Ham's F-12 medium	62	10 16% A a	15 24% A b	18 29% A c	19 31% A c
	pentoxifylline medium	68	13 19 % B b	20 29.5% B a	19 28% B a	16 23.5% B b
	L-carnitine medium	0	---	---	---	---
	pentoxifylline and L-carnitine medium	23	2 9% C c	6 26% C b	8 34.5% C a	7 30.5% A a
Total number of 5-8 cell stage of embryo	Ham's F-12 medium	38	5 13% A a	11 29% A b	11 29% A b	11 29% A b
	pentoxifylline medium	31	6 19.5% B b	10 32% B a	10 32% B a	5 16.5% B c
	L-carnitine medium	0	---	---	---	---
	pentoxifylline and L-carnitine medium	20	3 15% C c	7 35% C a	3 15% C c	7 35% C a
Different capital letters mean a significant difference at p<0.05 between the embryo grades.						
Different small letters mean a significant difference at p<0.05 between different media at each embryo grades.						

Discussion:

-Effect of PX ,LC and PX+LC media on Epididymal Sperms In vitro Activation after Cryopreservation: The significant improvement in forward progressive motility may be due to the effect of PX on energy supplementation by cAMP. It is well known that PX is a phosphodiesterase inhibitor of the methylxantine group(16) . 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. 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(17) L-carnitine increases sperm motility by affecting the metabolism of fatty acids (18) . Fatty acid metabolism occurs in the mitochondria of sperm middle-piece. It has been demonstrated that LC regulates the amount of acetyl coenzyme A(19). Acetyl-CoA is necessary for tricarboxylic acid cycle and energy production(20). Therefore, the increased motility of sperm by LC in this study might be due to the effects of LC on oxidative phosphorylation and energy production.It has been shown that LC can increase ejaculatory sperm motility of men with asthenozoospermia(21). In another study it has been demonstrated that testicular sperm motility improved after exposure to LC in vitro(22). Similarly, PX has been reported to increase testicular sperm motility (23) . L-carnitine has a key role in sperm metabolism by supplying the required energy and has a positive effect on sperm production, maturation and motility (24) .

Aliabadi et al.(25)showed that mice testicular sperm motility can be improved after exposure to LC and PX in vitro (26). It has been believes that sperm motility is dependent on the amount of its energy production. Sperm stimulators, such as PX and LC, are antioxidants and ROS scavengers (27). Extensive studies have been done in vivo and in vitro on different compounds, including LC and PX, found their affecting on sperm motility in Assisted reproductive technology(28) . Oxidiative Stress (OS) can influence cell membrane structures such as proteins (29) . Therefore, ROS scavengers such as LC and PX may affect sperm metabolism (27) , motility (25) and its plasma membrane (30).

-Effect of Cryopreservation on Certain Sperm Parameters:Long-term storage of sperm into the epididymis seems related to a loss of sperm quality(31). Cell metabolic changes, as well as plasmatic membrane alterations, due to the decreasing temperature through freezing steps and due to the increasing in temperature during thawing, are considered as responsible factors for each abnormal changes (32) . In the case of epididymal sperm, the fact that the cells have not been in contact with seminal plasma might further decrease their resistance to these procedures(33) .

The morphologic structure of the spermatozoon is extremely important for the processes of fertilization and embryo development. There was no significant change in the morphologic abnormalities of head, acrosome cap and mid piece in general. The sperm parameter among tail abnormalities which showed tail significant changes, was revealed in tail coiling after thawing and increase in immotile spermatozoa and the decrease in viability after thawing had strong correlation, it is likely that the main reason for the decrease in motility is the loss of vitality(34) .Tail defects after cryopreservation have been previously reported, and plasma membrane destruction in this region has been suggested as the probable reason for these defects. It has been examined an increase in coiled tails, which usually occur after osmotic changes. Media such as cryoprotactent agents, addition-removal of them and also changes occurring in water content during freezing of the compounds may lead to coiling of the tails (35). -

Sperm Parameter and Fertilization Rate: This finding is in agreement with Battin(36) who recognized that the sperm motility after swim-up method was associated with the rate of fertilization. Sperm progressive motility is essential for the vigorous sperm to penetrate the cumulus cells ,corona radiate, ZP both in vivo and in vitro. Therefore; this factor has been considered one of the most important factor in determining the FR (37). In this study, the result of FR in treated groups media by(PX alone or with LC) was shown a highly significant improvement compared to control group (Hams F-12 medium).The medium which containing PX was revealed a high selected motile sperm populations by incubation through IVF procedure (38) , that may increase intra cellular cAMP concentration often cause an increase in the agonist-induced acrosome reaction(39)and FR (40) .

-Embryonic Development: The current study found that IVF results by a medium containing PX alone or with LC improved significantly the number of ED in all grades at difference cell stages .Pentoxifylline has been shown to scavenge hydroxyl as well as superoxide radicals and inhibit their release (41) . It has been reported that small doses of PX have proven to be effective in their ability to scavenge ROS in cryopreserved sperm (42) .Therefore ,the addition of PX result in normal fertilization and ED. Human embryos generated from IVF also exhibit varying degrees of cytoplasmic fragmentation (43). Thus , it seemed that in the present study the PX showed an beneficial effect in reducing ROS induced embryo damage and hence improves IVF outcomes (44).

Moreover, LC medium have a positive effects on embryonic development rate. LC is able to stabilize mitochondrial membranes and increase the supply of energy to the organelle and protect the cell from apoptotic death. Reduction of apoptosis through the mitochondrial pathway by the administration of LC to mouse fibroblasts in culture medium has been demonstrated(45).

It has been shown that embryo quality is one of the most important prognostic factors for pregnancy chance in IVF (46) . LC medium may significantly reduced ROS levels in 2-cell stage embryos but did not reduce ROS levels at later stages ,this finding is compatible with the study of Takahashi et al (47) . L-carnitine significantly increased ATP levels in 2-cell embryos but not at the 8-cell or blastocyst stages.

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