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STUDY OF CALPAIN ACTIVITY, SPERM PENETRATION ASSAY IN UNEXPLAINED INFERTILE MEN IN RELEVANCE TO ASSISTED REPRODUCTIVE TECHNOLOGIES

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ABSTRACT

Background: Sperm calpain is associated with the cell fusion and signal transduction processes that take place during penetration of the oocyte. However, the existence of a functional calcium-dependent protease, such as calpain, has not been focused in mammalian sperm-oocyte interaction. **Objective:** The main aim of the study was to examine the certain sperm function, sperm penetration assay parameters and performed enzymatic analysis. In addition to assess the effects of calpain on the fertilization capacity in relevance to assisted reproductive technologies of fertile and infertile men especially the unexplained ones. **Materials and Methods:** This study was carried out on infertile men especially the unexplained ones and apparently fertile men, the samples were well selected and prepared according to the long term procedures of the study in the laboratory which were reached for more than 10 hour. The study was carried out on 34 (16 infertile men and apparently 18 fertile men were involved in this study). The two groups were subjected to evaluate the effect of calpain activity assay with the certain sperm function, sperm penetration assay parameters and according to the certain accomplished assisted reproductive technologies like ICSI, IVF/Sperm penetration assay by using hamster oocytes as a model for human. **Results:** The results of this study were revealed a highly significant ($p<0.001$) difference in the calpain activity parameters in relevance to sperm function, sperm penetration assay parameters of fertile men when compared with the unexplained infertile men in each line of assisted reproductive technologies(SPA-ICSI and SPA-IVF). The levels of the studied calpain activity

parameters were higher in fertile in two lines of SPA especially in SPA-ICSI, the high levels of calpain activity parameters were in relevance to higher levels of sperm penetration assay parameters rather than unexplained infertile men. While the comparison Between the lines of SPA-ICSI and SPA-IVF within same group was not significant regardless the findings of the studied parameters were higher in SPA-ICSI rather than that of SPA-IVF in both of fertile and unexplained infertile groups. **Conclusion:** It was concluded that the high calpain activity assay was associated with high sperm function and high sperm penetration parameters which indicate for high potential of oocyte fertilization.

KEYWORDS: Calpain, sperm penetration assay, IVF, ICSI.

INTRODUCTION

Calpain is a cysteine protease that has absolute dependence on calcium (Ca^{2+}) for activity and stands as a unique receptor for Ca^{2+} signals.[1-3] Recently, it demonstrated by enzymatic and immunoblotting analysis that calpain plays main role in cellular and physiological pathways.[4] Calpain features prominently in Ca^{2+}-mediated regulation of membrane fusion during cell-to-cell interaction in somatic cells.[5] The enzyme degrades membrane proteins and acts by limited proteolysis coupled to transient Ca^{2+} mobilization.[6] Evidence that calpain mediates Ca^{2+}-regulated membrane fusibility in somatic cells prompted us to investigate the possible role of sperm calpain in the penetration of the oocyte by the human spermatozoa; this event is dependent on Ca^{2+} and involves membrane fusion between the two cells.[7, 8] For this purpose, the main aim of the present study was designed to assess the effects of a calpain activity parameters on the capacity of spermatozoa to fuse with and penetrate hamster oocytes by using assisted reproductive technologies (IVF/ICSI) as well as the examination of the certain sperm function, sperm penetration assay parameters and performed calpain enzymatic analysis of fertile and infertile men especially the unexplained ones.

MATERIALS AND METHODS

The study was conducted in the High Institute of Infertility Diagnosis and ART at Al-Nahrain University through the period from February 2014 to July 2014.Thirty four men were involved in this study who examined by a urologist. It can be divided into two groups first one from fertile volunteers with normozoospermia (n=18) who served as normal volunteers control and second one from infertile male partners ones (n=16 patients) with

unexplained infertility causes.

SPA-IVF was done for 10 fertile men, 8 unexplained infertile men, while SPA-ICSI was done for 8 fertile and 8 unexplained infertile men.

Freshly ejaculated samples of semen were collected by masturbation from fertile and infertile men directly into a clean, dry and sterile disposable plastic Petri–dish in an especially allocated room for this purpose in the Institute. For each subject with acquaintance in the abstinence period from 3-5 day, the sample was transported to the semen analysis laboratory immediately. After liquefaction time each sample was divided into two equal portions (each 1ml) for two lines first line: 1ml for before activation and second: 1ml for after activation. After 30-60 minutes each semen sample was allowed to liquefy according to methods described previously.[9, 10] After complete liquefaction, the semen was analyzed by a macroscopic and microscopic examination using the standardization of WHO, (1999).[11]

Sperm calpain assay

Two ml of the liquefied semen sample were taken for the assay and were divided into two equal portions for two lines, in the first line was centrifuged at 2000 rpm (400g) by centrifuge (for 10 minutes (Before activation line)), and the same step of centrifugation was done of the second 1ml after their mixing with 1ml of Ham's-F10 medium in the second line (after activation line).

The pellet were formed after the centrifugation, the first weight of the pellet was measured by electric sensitive balance for the two lines. The supernatant was discarded and the sperm cells were washed twice at 1500 rpm(200g) for 5 min with 1 ml Ham's F10 medium (Before activation line),and among the wash and spin technique, it was added 0.5 ml of the Ham's F10 medium in the final step after incubation time for 30 minutes in after activation line.[12] The sperm function parameters were the microscopic examination of the seminal fluid for each subject which includes: sperm concentration, sperm motility grades and morphologically normal sperms. These certain sperm function parameters were recorded according to guidelines of WHO (1999). *In vitro* sperm activation was the wash and Spin method that done for all samples as described by Mahadevan *et al.* in 1984. [12]. The washed spermatozoa were homogenized by nitrogen cavitation (1500 psi for 15 minutes) (3 shifts each one 5 minutes, this step must be done for all the samples and for each line of study.

Centrifugation and Determination of pellet weight

Then the homogenate was then centrifuged by Eppendorff centrifuge, at 32800 rpm (60minutes) and the supernatant and pellet were recovered to obtain the pellet by the eppendorff centrifuge after the adding process of the calpain extraction materials respectively According to the methods of Rojas *et al*.,1999.[13]

Then the pellet second weight was measured by balance after the last step of centrifuge, also this step must be done for all the samples and for each line of study.

Sperm Protein Assay

The samples were determined by Bradford protein assay kit[14] which can be done by adding 100ul from the sample and for each line (recovered pellet and supernatant) & mixing with 5ml dye reagent of Bradford protein assay and were incubated in incubation for 5 minute at 37C°, then measured the absorbance at wave length 595nm by the spectrophotometer with a quartz and to assay the value it was made a fall in the standard curve of the bradford protein assay.

Determination of calpain

The determination of calpain was done by Calpain assay kit according to Buroker-Kilgore and Wang method.[15] The calpain activity was defined as the difference between the two readings (blank and the sample) read the samples and for each by spectrophotometer at a wave length 595nm after vortex for 10 minutes.[15]

Calculation of calpain activity

The calpain activity was expressed as { units(IU)/ml } multiplied by dilution factor 40 for before activation samples & 50 for after activation samples according to the added volumes of media within the original 1 ml volume of the seminal fluid. Each experiment was run in triplicate and was repeated at least three times.

One unit is the amount of enzyme that catalyses the reaction of 1µmol of substrate per one minute, so we divided the values by 10 according to our determination. The activity units(IU)/ml is given by the determination the differences between the two readings (blank and the sample) read by spectrophotometer and then multiplied by the appropriate dilution factor 40 or 50 accordingly to the preparation method of the seminal fluid.

Determination of specific calpain activity: the specific calpain activity was expressed as certain units {units(IU)/mg } which equal (calpain activity value/ bradford assay value)[16] it was calculated for each sample and for each line of study.

Methods of Oocyte –Sperm interaction assay

Superovulation induction

Mature female Golden Syrian Hamsters superovulation was induced by intra peritoneal injection (IP) of pregnant mare serum gonadotropin (PMSG)(Folligon®,Intervet Company,Holland). PMSG was used to mimic the oocyte maturation effect of the endogenous FSH. While human chorionic gonadotropin (hCG) (Pregnyl ®Oss,Holland) was used to mimic the ovulation induction effect of LH.

According to estrus cycle detection procedure, superovulation was induced by IP injection of 40 IU of PMSG, and then followed by IP injection of 40 IU of hCG after 42-47hours.[17]

Oocytes collection

Sterile surgical instruments and gloves were used for removal of uterine horns and uterine tubes from each animal.After 15-18 hours post –hCG injection, the females hamsters were sacrificed by Ether inhalation for few minutes, so the oviducts were isolated.

For flushing the oocytes, the ampulla was tearing to release the cumulus masses. Then all the cumulus masses were transferred to a single fertilization dish by using a mouth—controlled pipette under aseptic conditions.

Pipettes were used to pick up the oocytes , then they were transferred to a central well-culture dish containing 1 ml of culture medium (pH=7.4-7.6) and kept at 37° C with 5 %CO2 and 95% humidity within CO2 incubator.

The mature oocytes were subjected to insemination (human sperms) by either IVF or ICSI after stripping process to evaluate the sperm penetration assay and the calpain activity parameters assay were done for each sample as a third line.

SPA-IVF

The insemination by IVF procedure as described by Edwards *et al.*,1984[18] was done by

1- Two out of four IVF wells filled with equal mixture of IVF medium. The other two wells loaded with IVF medium alone. All wells covered with paraffin oil.

2- Insemination of mature oocytes were done by adding 1-2 x 10^5 of the incubation sperm to the IVF well containing 5 oocytes .

SPA-ICSI

Female hamsters receive the same ovarian stimulation as those undergoing a routine IVF treatment. Then the sperms collection at the time of the oocytes collection or shortly thereafter. The human sperms were prepared for injection using *In vitro* Sperms Activation Technique (wash and spin) and kept at 37ºC in an incubator until the moment of injection. IVF medium and polyvinylpyrrolidone (PVP) solution were used to prepare the ICSI dish at least two hours before ICSI procedure[19] and kept at 37ºC in the incubator. The assessment of the oocytes was done similar to the standard IVF procedure. The cells covering the oocytes were gently removed, using a mild hyaluronidase solution(40-80IU/ml medium) and treated consecutively with trypsin (to remove the cumulus cells attached to the zona pellucida and then remove the zona pellucida itself), and zona free eggs are then combined with (capacitated) sperm, and hand drawn pipettes. The oocytes were subsequently rinsed several times in phosphate buffer solution and carefully examined for oocyte maturity.

Then Intracytoplasmic sperm injection was carried out on mature oocytes. Prior to injection, the sperm was prepared and immobilized using an extremely viscous solution of (PVP), enabling to aspirate a single sperm for injection directly into the cytoplasm of the oocyte. After ICSI which done via micromanipulators ,the oocytes were transferred into IVF medium alone for culture in the incubator.

So the sperm penetrating criteria were assessed as what's known by Sperm penetration assay (SPA) in relevance to calpain level for each sample for each subject, in addition to the sperm-oocyte assay protein assay.

Sperm Penetration Assay

Sperm penetration assay is an *in vitro* technique that evaluates the ability of human spermatozoa to penetrate zona free hamster oocytes. The test was initially developed by[20] who used the hamster oocyte as a substitute for human ova in the assessment for the fertilizing capacity of human spermatozoa. The hamster oocyte is unique in that in its zona free state which can be penetrated by every species of spermatozoa. This assay evaluate the ability of sperm to successfully undergo capacitaion, acrosome reaction, membrane fusion with oocytes and chromatin decondensation. SPA does not evaluate sperm binding and zona

pellucida penetration. This test is also used as screening procedure before IVF.

So mixing of sperms either fresh or processed, then the concentration is adjusted.

The zona-free hamster oocytes were placed in 50µl sperm droplets(concentration adjusted to 3-5x 10^6 sperm/ml added for 3 hours before determining the percentage of oocytes penetrated and number of spermatozoa penetrated per oocyte. The penetration are noted by detecting swollen sperm heads within the oocytes cytoplasm. Sperms that have penetrated the zona free hamster eggs have "decondensed" heads, which are much larger than the other surrounding sperm heads.

$$\text{Sperm penetration Index} = \frac{\text{No. of sperm swollen head / oocytes}}{\text{Total No. penetrated oocytes}}$$

Normal Value ≥ 5

$$\text{SPA} = \frac{\text{No. of Penetrated oocytes}}{\text{Total oocytes}} \times 100$$

The passing level for the SPA is 10% or more of oocytes are penetrated.

IVF patients may require a 20% or more oocyte penetration criterion.

After the Sperm Penetration Assay which be either SPA-IVF or SPA-ICSI type and according to their accomplishment within study that compare the SPA-IVF between the two different groups and within the same group, same comparison were done in SPA-ICSI, so after the SPA line of study and after the 3 hours of incubation it were measured the calpain parameters and sperm-oocyte protein assay to evaluate the role of calpain through the sperm-oocyte interaction.

RESULTS

In this study it has been carrying out a comparison for each line of assisted reproductive technologies (SPA-ICSI or SPA-IVF) in both fertile and infertile groups. The result of SPA-ICSI showed that sperm concentration m/ml of 8 fertile men was (31.875±1.244), while of 8 unexplained infertile men was (20.625±2.457).There was a high significant ($p<0.001$) difference between them. A significant ($p<0.001$) increase in sperm motility grade (a+b+c) % of fertile men was (94. 375±1.320) compared to unexplained infertile men (71.875±2.599).

Active sperm motility grade (a+b) % of fertile men (88.125±1.314) was significantly ($p<0.001$) higher than that of unexplained infertile men was (50.625±2.481).

The morphologically normal sperm % of fertile men was (71.250±1.249), while of unexplained infertile men was (52.5±1.888).There was a high significant ($p<0.001$) difference between them.

Also it showed that the SPA (%) of fertile men (70±3.777)was significantly($p<0.001$) higher than that of unexplained infertile men (55±3.269) and the SPI (%) of fertile men was (9.500±1.710), while for unexplained infertile men was (4.75±0.860).There was a significant ($p\leq 0.05$) difference between them.

To study the main events after (Sperm-oocyte interaction) in this study it has been carrying out a comparison between before and after SPA-ICSI/SPA-IVF results including percent of change. The results of calpain activity were with highly significant increase ($p\leq0.001$) in the fertile group after the SPA- ICSI, and were with significant increase ($p\leq0.05$) in both Bradford protein and specific calpain activity as compared with the before of SPA-ICSI (table 1). In infertile group within the same SPA-ICSI assay, after SPA-ICSI the increase were highly significant($p\leq0.001$) in calpain & speicifc calpain activity, while the increase were not significantly different in Bradford protein assay as compared with the before of SPA-ICSI (table 2). The increase in the sperm pellet/oocyte weight were with no significant difference in both groups within this assay of SPA-ICSI. (table 1,2). Correlations were done between calpain activity parameters and SPA-ICSI results for fertile and unexplained infertile groups (table (3).

In fertile group it showed that calpain activity assay was significantly positive correlated ($p <0.050$) with SPA, SPI ($r=0.798$, 0.778 respectively), while Bradford protein assay showed no significant ($p >0.050$) positive correlation with SPA, SPI($r=0.261$, 0.311 respectively).The specific calpain activity had a significant($p <0.05$) positive correlation with SPA, SPI($r=0.796$, 0.774 respectively)(table 3).

In contrast, in unexplained infertile group it showed no significant ($p >0.050$) positive correlations between all calpain activity parameters with SPA,SPI, the correlation coefficients of calpain activity assay with SPA,SPI were ($r=0.417$,0.259 respectively),Bradford protein assay with SPA,SPI($r=0.183$,0.253, respectively),and specific calpain activity with SPA,SPI were($r=0.466$,0.303, respectively) as shown in (table 3).

Regarding SPA-IVF line ,the Sperm concentration m/ml of 10 fertile men was (34.5±2.337), while of 8 unexplained infertile men was (13±1.508) .There was a high significant (p<0.001) difference between them.A significant (p<0.001) increase in sperm motility grade (a+b+c) % for the fertile men was (92.5±1.344) compared to unexplained infertile men (68.125±1.651).Active sperm motility grade (a+b) % of fertile men (84±2.081) was significantly (p<0.001) higher than that of unexplained infertile men (41.875±2.126).Morphologically normal sperm % of the fertile men was (69.5±0.500), while of the unexplained infertile men was (51.25±2.058).There was a high significant (p<0.001) difference between them. There was a significant (p<0.001) increase in SPA of fertile men was (68±3.264)compared to unexplained infertile men (47.5±3.640).A significant (p<0.05) increase in the SPI of fertile men (8.70±1.271) was observed compared with unexplained infertile men (4.50±0.377).

Table (1) : Calpain activity parameters in fertile group between before and after SPA-ICSI.

Parameters	SPA-ICSI for unexplained infertile men		Percent of change (%)	Significances
	Before	After		
	M±SE	M±SE		
Calpain activity assay (IU/ml)	0.407±0.0126	0.890±0.0373	+118.67%	HS
Bradford protein assay (mg/ml)	0.0613±0.0023	0.0619±0.001	+0.97%	NS
Specific Calpain activity (IU/mg)	6.660±0.119	14.410±0.702	+116.366%	HS
Sperm Pellet/Sperm-oocyte weight(g)	0.0087±0.0023	0.0147±0.004	+69.94%	NS

n=8

M±SE=mean± standard error

HS= P<0.001

S= P<0.005

NS=Not significant.

Table (2) : Calpain activity parameters in unexplained infertile group between before and after SPA-ICSI.

Parameters	SPA-ICSI for fertile men		Percent of change (%)	Significances
	Before	After		
	M±SE	M±SE		
Calpain activity assay (IU/ml)	0.723±0.087	1.509 ±0.176	+108.71%	HS
Bradford protein assay (mg/ml)	0.0676±0.0026	0.0811±0.0027	+19.97%	S
Specific Calpain activity (IU/mg)	10.533±1.039	18.58±2.087	+76.39%	S
Sperm Pellet/Sperm-oocyte weight(g)	0.083±0.0060	0.0914±0.0063	+10.12%	NS

n=8

M±SE=mean± standard error

HS=P<0.001

NS=Not significant

Table (3) : The correlation coefficient of calpain activity parameters with SPA–ICSI results in fertile and unexplained infertile groups.

Parameter	Calpain Activity Assay (IU/ml)		Bradford Protein Assay (mg/ml)		Specific Calpain Activity (IU/mg)	
	Fertile	Unexplained Infertile	Fertile	Unexplained Infertile	Fertile	Unexplained Infertile
SPA (%)	r=0.798* p=0.018	r=0.417 p=0.304	r=0.261 p=0.533	r=0.183 p=0.665	r=0.796* p=0.018	r=0.466 p=0.244
SPI (%)	r=0.778* p=0.023	r=0.259 p=0.536	r=0.311 p=0.454	r=0.253 p=0.545	r=0.774* p=0.024	r=0.303 p=0.465

**** Correlation is significant at the 0.05 level (2-tailed)***

To study the changes before & after SPA-IVF, the results of calpain activity,speicifc calpain activity were with highly significant increase ($p \leq 0.001$) in the fertile group after the SPA-IVF , and were with significant increase ($p \leq 0.05$) in Bradford protein (table 4)as compared with the before of SPA-IVF assay. In infertile group within the same SPA-IVF assay, after this assay the increase were significant ($p \leq 0.05$) in calpain & with a highly significant difference in specific calpain activity, while the increase were not significantly different in Bradford protein assay as compared to the before of this assay (table 5). The increase in the sperm pellet/oocyte weight were with no significant difference in both groups within this assay of SPA-IVF. (table 4,5).

Regarding the correlations between calpain activity parameters and SPA-IVF results**,** in fertile group it showed that calpain activity assay had significantly positive correlation ($p < 0.050$) with SPA, SPI ($r=0.648$, 0.611 respectively), while Bradford protein assay had no significant ($p > 0.050$) positive correlation with SPA, SPI($r=0.204$, 0.179 respectively) as shown in table 6. Also it showed that specific calpain activity had a significant ($p < 0.050$) positive correlation with SPA, SPI($r=0.644$, 0.689 respectively)(table 6).

In unexplained infertile group it showed no significant ($p > 0.050$) positive correlations between all calpain activity parameters with SPA,SPI, the correlation coefficients of calpain activity assay with SPA,SPI were ($r=0.227$,0.127 respectively) ,Bradford protein assay with SPA,SPI($r=0.040$,0.135, respectively),and specific calpain activity with SPA,SPI were($r=0.089$,0.069, respectively) as summarized in table 6.

Moreover, in this study it has been carrying out a comparison between the two lines within the same group. So all the mentioned parameters had been exhibited no significant difference in the two lines for each group, regarding the not significant increase of the calpain activity, specific calpain activity, Bradford protein assay , sperm penetration assay and Sperm penetration Index.

In addition to the no significant difference in sperm function parameters in SPA-ICSI line for each group as compared to SPA-IVF line results (table 7,8).

Table (4) : Calpain activity parameters in fertile group between before and after SPA-IVF.

Parameters	SPA-IVF for fertile men		Percent of change (%)	Significances
	Before	After		
	M±SE	M±SE		
Calpain activity assay (IU/ml)	0.786±0.0686	1.386±0.0576	+76.28%	HS
Bradford protein assay (mg/ml)	0.070±0.003	0.080±0.0022	+14.14%	S
Specific Calpain activity (IU/mg)	11.211±0.918	17.30±0.424	+54.31%	HS
Sperm Pellet/Sperm-oocyte weight(g)	0. 106±0.011	0.120±0.010	+13.20%	NS

n=10

M±SE=mean± standard error

HS= P<0.001

S= P<0.005

NS= Not significant

Table (5) : Calpain activity parameters in unexplained infertile group between before and after SPA-IVF.

Parameters	SPA-IVF for unexplained infertile men		Percent of change (%)	Significances
	Before	After		
	M±SE	M±SE		
Calpain activity assay (IU/ml)	0.468±0.0282	0.817±0.0893	+74.57%	S
Bradford protein assay (mg/ml)	0.0604±0.002	0.0610±0.0057	+0.99%	NS
Specific Calpain activity (IU/mg)	7.74±0.391	13.33±0.654	+72.2%	HS
Sperm Pellet/Sperm-oocyte weight(g)	0.0230±0.0062	0.032±0.0074	+39.13%	NS

n=8

M±SE=mean± standard error

S= P<0.005

NS=Not significant

HS= P<0.001

Table (6) : showed the correlation coefficient of calpain activity parameters with SPA – IVF results in fertile and unexplained infertile groups

Parameter	Calpain Activity Assay (IU/ml)		Bradford Protein Assay (mg/ml)		Specific Calpain Activity (IU/mg)	
	Fertile	Unexplained Infertile	Fertile	Unexplained Infertile	Fertile	Unexplained Infertile
SPA (%)	r=0.648* p=0.043	r=0.227 p=0.589	r=0.204 p=0.572	r=0.040 p=0.925	r=0.644* p=0.044	r=0.089 p=0.835
SPI (%)	r=0.611* p=0.004	r=0.127 p=0.764	r=0.179 p=0.620	r=0.135 p=0.750	r=0.689* p=0.027	r=0.069 p=0.870

**** Correlation is significant at the 0.05 level (2-tailed)***

Table (7) : Comparison of some studied parameters in fertile group

Parameters	Fertile		Significances
	(SPA-ICSI)	(SPA-IVF)	
	M±SE	M±SE	
Calpain activity assay (IU/ml)	1.509 ±0.176	1.386 ±0.0576	NS
Bradford protein assay mg/ml)	0.0811±0.0026	0.080±0.0022	NS
Specific Calpain activity (IU/mg)	18.58±2.087	17.30±0.424	NS
SPA (%)	70±3.777	68±3.264	NS
SPI (%)	9.500±1.710	8.7±1.271	NS
Sperm conc. (m/ml)	31.875±1.244	34.5±2.337	NS
Sperm motility grade (a+b+c) (%)	94.375±1.320	92.5±1.344	NS
Active sperm motility grade (a+b) (%)	88.125±1.314	84±2.081	NS
Morphologically normal sperm(%)	71.250±1.249	69.5±0.500	NS

n of SPA-ICSI=8

n of SPA-IVF=10

M±SE=mean± standard error

NS= Not significant

Table (8) : Comparison of some studied parameters in infertile group

Parameters	Unexplained Infertile		Significances
	(SPA-ICSI)	(SPA-IVF)	
	M±SE	M±SE	
Calpain activity assay (IU/ml)	0.890±0.0373	0.817±0.089	#NS
Bradford protein assay (mg/ml)	0.0619±0.001	0.0610±0.0057	NS
Specific Calpain activity (IU/mg)	14.410±0.702	13.33±0.654	NS
SPA (%)	55±3.269	47.5±3.640	NS
SPI (%)	4.75±0.860	4.5±0.377	NS
Sperm conc. (m/ml)	20.625±2.457	13±1.508	NS
Sperm motility grade (a+b+c)(%)	71.875±2.599	68.125±1.651	NS
Active sperm motility grade (a+b)(%)	50.625±2.481	41.875±2.126	NS
Morphologically normal sperm(%)	52.5±1.888	51.25±2.058	NS

n=8

M±SE=mean± standard error

NS= Not significant

DISCUSSION

The development of relatively simple assays that can assess the functional activity of spermatozoa should be of use in the diagnosis of the infertile men especially the unexplained ones. Present day semen analyses primarily determine sperm function parameters such as concentration, motility, morphology. Although these are representative of the characteristics of spermatozoa, they give inadequate information about the fertilizing capacity of the spermatozoa, i.e., their ability to undergo penetration through the oocyte's investments.

Current knowledge indicates that calcium is a signal transducer that specifically mediates membrane fusion following sperm–oocyte interaction.[8] In this study regarding the evidence that calpain mediates Ca^{2+}regulated membrane fusibility in somatic cells, this prompted us to investigate the possible role of sperm calpain in the penetration of the oocyte by the human spermatozoa; this event is dependent on Ca^{2+} and involves membrane fusion between the two cells.[7,8] In present study in regarding the calpain activity assay with relevance to sperm penetration assay and certain assisted reproductive technologies (ICSI/IVF), the fertile group exhibited the high levels of sperm function and sperm penetration (SPA, SPI) parameters rather than unexplained infertile group and in both assisted reproductive technologies (SPA-ICSI, SPA-IVF), this could be ascribed to the higher calpain activity assay parameters that were cascaded to the high level sperm function, penetration parameters which were exhibited more in fertile group. These findings are in a good agreement with the findings reported by the studies of (Ozaki *et al.*,2001; Ayoama *et al.*,2001) [21,22]. that showed the main role of calpain which appears to be involved in the regulation of sperm motility.

The findings of sperm function, penetration parameters in relevance to higher levels of calpain activity parameters within SPA-ICSI were higher than findings of SPA-IVF and in both fertile and unexplained infertile groups, this could be ascribed to the main role of calpain activity which were revealed in the SPA-ICSI criteria by the cell fusion process that takes more place during penetration of the oocyte as reported by the study of Redpath *et al.*,2014 [4]. in addition to the recognition, signal transduction and binding process between the extracellular coat of the oocyte with components of the sperm plasma membrane, it is widely accepted that the binding process is mediated by complex carbohydrates of the oocyte coat that are decoded by complementary sites of carbohydrate-binding proteins located on the

sperm surface.[23] Also It has been observed that injection of sperm cytoplasmic extracts into oocytes triggers Ca^{2+} activity similar to those observed during fertilization.[24-27]

In this study and within SPA-ICSI,SPA-IVF assays the fertile and unexplained infertile groups showed that calpain activity parameters were increased and in high percent of change after the assays in comparison to before state, this could explain the main role of increased calpain in the sperm-oocyte interaction in cascading to intracellular Ca^{2+}, these findings are so close to what reported by the studies of (Rojas *et al*.,1999;Yangimachi *et al*.,1988)[13,8]. that showed the binding criteria of Ca^{2+}dependent calpain system and the critical membrane fusion events between the spermatozoa and oocyte which are likely modulated by this system. Although some argument is still present in regard to the human sperm - hamster oocyte test, most investigators agree that this test or assay generally not absolutely reflects the fertilizing potential of human spermatozoa.[28-31]

That's could reveal the role of other predictors for fertilization potential like the calpain activity assay, so this study exhibited the no significant difference in the calpain activity parameters which were in accordance to the no significant different sperm function ,and penetration parameters between the SPA-ICSI ,SPA-IVF findings within each group of study.Regarding the sperm-oocyte pellet weight within SPA-ICSI,SPA-IVF assays within each group of study, it showed that were with no significant difference in comparison , this could be relevant to standard conditions within ICSI procedure,[32] same findings were showed within IVF procedure[33] in addition to the standard conditions of the mature hamster oocytes-human sperm interaction within the study.

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