Impact of Calpain Activity Assay in Correlation to Human Sperm Parameters in Fertile and Infertile Men

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Abstract

Background: Sperm calpain is associated with the regulation and modification of physiological processes including cell fusion and motility that takes place during fertilization. However, the existence of a functional calcium- dependent protease, such as calpain, has not been focused in active mammalian spermatozoa.
Objective: The main aim of the study was to examine the certain sperm parameters and performed enzymatic analysis. In addition to assess the effects of calpain on the fertilization potential of fertile and infertile men.
Materials and Methods: This randomized study was carried out on 50 infertile asthenozoospermic men and apparently fertile men with total number of 50 were involved in this study. The two groups were subjected to evaluate the calpain activity assay in correlation to the certain sperm function parameters.
Results : The results of this study was revealed a highly significant($p<0.001$) differences in the calpain activity and sperm function parameters of infertile men compared with the fertile men. There was a high significance($p<0.001$) positive correlations in the calpain activity and sperm motility grade(a+b) between the two groups, the most high significant positive ones was in fertile men especially in sperm after activation line.
Conclusion: It was concluded that the high calpain activity assay was associated with high sperm motility grade (a+b) which indicate for the fertilizable sperms ability.
KEYWORDS: Calpain, sperm active motility, fertility, infertility

Introduction

For several years, interesting information about the role played by a calcium-specific protease, called calpain, in the functional activity of membranes has been gathered from a variety of somatic mammalian cells. Calpain is an intracellular, non-lysosomal protease that is isolated from the cytosolic fraction of tissues or cells (1). The catalytic site contains a cystein residue which places calpain in the cystein protease family (2). Its properties include an absolute dependence on Ca^{2+} for activity and an optimum pH of 7–8 (3).
In most mammalian tissues and cells, there are two forms of calpain with different sensitivities to Ca^{2+}, namely, calpain I and calpain II, which require low and high Ca^{2+} concentrations respectively. The two forms appear to have identical substrate specificity (1).

Calpain figures prominently in the intracellular regulatory activities mediated by calcium (4), including the calcium- mediated regulation of membrane fusion in somatic cells.
Several investigations reveal that some degree of proteolysis must occur for membrane fusion to occur and that a correlation exists between increased membrane fusibility and increased calcium-dependent proteolysis of membrane proteins (1,5). Indeed, there is evidence that calpain degrades membrane proteins and acts by limited proteolysis coupled to transient Ca^{2+}mobilization (6). Because mammalian spermatozoa have an absolute calcium requirement for certain specific functions such as sperm–egg interaction, it have been postulated that a calcium-activated protease functions in spermatozoa. According to the hypothesis, sperm calpain is associated with the cell fusion process that takes place during penetration of the oocyte. However, the existence of a functional calcium- dependent protease, such as calpain, has not been shown in mammalian spermatozoa.
For this purpose, the main aim of the study was designed to examine the sperm parameters and performed enzymatic analysis. In addition to assess the effects of calpain on the fertilization potential of fertile and infertile men and for sperms activated *in vitro* in relevance to morphologically normal sperm percentage .

Materials and Methods

The study was conducted in the High Institute of Infertility Diagnosis and ART at Al-Nahrain University through the period from March 2013 to February 2014.One hundred 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=50) who served as normal volunteers control and second one from infertile male partners (n=50 patients) with asthenozoospermia.
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 portion (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 (7, 8).
After complete liquefaction, the semen was analyzed by a macroscopic and microscopic examination using the standardization of WHO, (1999) (9).
-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 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 (10).

- Microscopic examination

1- Sperm concentration:

Assessment of sperm concentration is done by the estimation of the number of sperm per milliliter, the number was multiplied by a factor of one million (11, 12, 13).

A drop of 10µl spermatozoa suspension was placed on a microscopic slide and covered with a cover slip (12, 13). Certain sperm function parameters were recorded according to guidelines of WHO (1999) too (9).

2- Sperm motility:

The microscopic field was scanned systematically and the motility of each spermatozoon encountered was graded:-

• A - Rapid and linear progressive motility.
• B - Rapid non linear or linear non rapid progressive motility.
• C-Non progressive motility.
• D- Immotile.

3- Sperm morphology:

The percentage of morphologically normal sperms was performed by using the same prepared slides for sperm motility. At least 100 spermatozoa were calculated by dividing the mean number of normal spermatozoa in four high power microscopic fields under magnification of (40x) (12, 13) on the number of sperm concentration (14).

- Sperm media preparation

The recommended sperm media in study is Ham's F-10 according to studies of (7, 8, 15).

- Wash and Spin method was done for all samples as described by (10).

- Homogenization by Liquid Nitrogen

One of the of homogenization methods that are available for enzymes extraction.

The washed spermatozoa were homogenized by nitrogen cavitation (1500 psi for 15 minutes) (3 shifts each one 5 minutes).

-Centrifugation and Determination of pellet weight

Then the homogenate was then centrifuged by Eppendorff centrifuge, at 32800 rpm (60minutes) or (100 000 G) for 60 minutes 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 (15). Then the pellet second weight was measured by balance after the last step of centrifuge.

-Sperm Protein Assay

The samples were determined by Bradford protein assay kit (16) which can be done by adding 100ul from the sample (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(17). The calpain activity was defined as the difference between the two readings (blank and the sample) read the samples by spectrophotometer at a

wave length 595nm after vortex for 10 minutes (17).

-Calculation of calpain activity

The calpain activity was expressed as certain units { 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 {units(IU)/mg } which equal (calpain activity value/ bradford assay value) it was calculated for each sample and for each line of study.

Results

In this study it has been carrying out a comparison for each line between the two groups), so the results of calpain activity, Specific calpain activity & sperm function parameters were with highly significant increase ($p \leq 0.001$) in the fertile group & in two lines , such as the results in the table (1,2) , except the sperm pellet weight were not significantly decrease in the fertile group & for each two lines as comparing with the infertile group.

Moreover, in this study it has been carrying out a comparison between the two lines within the same group.

In fertile group the parameters (calpain activity assay, specific calpain activity, Bradford protein assay) showed that there were an increase in all of them in the after activation with only a significant increase in calpain activity but the others were not significantly different in comparison to the before activation line in as interpreted in table(3).

The sperm pellet weight were decreased in after activation with no significant difference as compared to the before activation line. Same comparison done for the infertile group the increase in parameters(calpain activity, specific calpain activity) were highly significant between the two lines of study, while there were no significant increase in Bradford protein assay(Table 4).

As in fertile group the sperm pellet weight were decreased in after activation with no significant difference as compared to the before activation line.

The study of comparison were done also for the sperm function parameters like sperm concentration, motility and morphologically normal sperm for each line within the same group.

In fertile group the findings showed that there was highly significant increase in the parameters in after activation line except the sperm concentration which is decreased significantly in after activation line due to the screening principle of activation & reduction process of the non motile, non active and non fertilizable sperms numbers (Table3).

Same comparison and same findings were occurred in the infertile group with highly significant difference as shown in table4.

The study of the correlations between the calpain activity, specific calpain activity each for them with sperm motility grade for fertilizable sperms (a+b) in fertile group in each line (before and after activation).

In before activation line it shown there is a positive correlation because there were a high significant differences $p \leq 0.001, r=0.72$ $r=0.73$ respectively, the results of these correlations were obtained through statistical analysis of coefficients of these parameters are presented graphically as in (Figure a)

In after activation line it shown there is more positive correlation because of the high levels of calpain activity assay ,specific calpain activity with very high level of sperm motility grade (a+b) so the correlation with a high significance $p \leq 0.001$, & high coefficient of $r=0.9, r=0.89$ respectively, presented graphically as in Figure b).

Same correlations were done for the infertile group, in before activation it shown a little positive correlation than the fertile group but also with a high significance $p \leq 0.001, r=0.627, r=0.60$ respectively. Presented graphically as in figure(c).

In after activation line it shown also a little positive correlation than the fertile but higher than the before activation in the same group $r=0.634, r=0.69$ respectively the results of these correlations were also with a high significance are presented graphically as in figure(d).

Discussion

In this study, it has been trying to investigate the possibility to consider calpain activity assay measurement as predictor for fertilization potential of fertile men that complaining from unexplained infertility and for sperms activated *in vitro* with morphologically normal sperm percentage.

The findings of the present study are in a good agreement with findings reported by the study of Rojas *et al* (15), demonstrating that calpain is mostly localized in the membrane fraction of spermatozoa & could be determined by enzymatic analysis.

In contrast to somatic calpain, sperm calpain requires signicantly higher pH for optimal activity and is located in the membrane, not in the cytosolic fraction (15). These observations raise the possibility that sperm calpain can be specially targeted for fertility control.

The present results show that the swim-up method for recovery of motile sperm is reliable. The washing procedure is necessary to remove prostaglandins, infectious agents and leukocytes (18)·

One advantage of the method is the limited number of technical steps that besides being more practical & important in the study aim avoids damage to the spermatozoal cytoplasmic membrane (19). Furthermore, it was reported that common laboratory factors like centrifugation, washing, temperature fluctuation, and processing delay harmfully affect semen quality both positively and negatively due to direct influence of laboratory interventions on the cytoskeletal assemblies of sperm (20).Regarding the calpain activity assay in each group & especially in post activation line, the calpain enzyme that is a cystein protease which has absolute dependence on calcium (Ca^{2+}) for activity and stands as a unique receptor for Ca^{2+}signals, the increased calpain activity level & its strong positive correlations to the high fertilizable sperms percentage grade(a+b) might be ascribed to the biochemical events of sperm activation & the role of intracellular free Ca^{2+} for calpain activity which is exhibited more in normal &

good motile sperms, these findings were conducted by the studies(21,5,1). So the biochemical events that occurred through post activation leads to calpain activation contributes to sperm motility as well as to the acrosome reaction. These results suggest the possibility that activation of calpain in human sperm plays an important role in fertilization. These findings were similar to the findings reported by Ozaki *et al* study(22). Regarding the not significant lower levels of sperm pellet weight in post activation in both groups of study & in fertile subjects in comparison to infertile ones, these findings could be ascribed to whether or not spermatozoa are found in the pellet depends on the centrifugation time and speed (23,24) and on how much of the pellet is centrifuged; for e.g. the centrifugation at 3000*g* for 15 minutes does not pellet all spermatozoa from a sample (25); and after centrifugation & due to the high weak sperms number, the motility can be lost (26) and concentration will be underestimated, these findings similar to findings reported by Cooper TG *et al* study(27).

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Table (1): Comparison of some studied parameters before activation line between fertile and infertile group.

Parameters	Fertile	Infertile	Significances
	M±SE	M±SE	
Calpain activity assay (IU/ml)	0.643±0.031	0.323±0.0171	HS
Bradford protein assay(mg/ml)	0.095±0.0038	0.078±0.0025	HS
Specific calpain activity(IU/mg)	6.983±0.289	4.366±0.257	HS
Sperm pellet weight(g)	0.070±0.0049	0.074±0.0053	NS
Sperm conc.(m/ml)	58.300±2.471	39.340±1.926	HS
Sperm grades (a+b+c)(%)	72.100±0.991	42.800±2.051	HS
Sperm grades (a+b)(%)	61.200±1.139	21.160±1.956	HS
Morphologically normal sperm(%)	39.000±0.833	27.000±0.589	HS

n=50, M±SE=mean± standard error, HS=P<0.001, NS=Not significant

Table (2): Comparison of some studied parameters after activation line between fertile and infertile group.

Parameters	Fertile	Infertile	Significances
	M±SE	M±SE	
Calpain activity assay(IU/ml)	0.712±0.037	0.429±0.011	HS
Bradford protein assay(mg/ml)	0.098±0.0033	0.082±0.0031	HS
Specific Calpain activity(IU/mg)	7.515±0.386	5.402±0.162	HS
Sperm Pellet weight(g)	0.060±0.0049	0.070±0.0036	NS
Sperm conc.(m/ml)	24.52±1.138	9.44±0.793	HS
Sperm grades (a+b+c)(%)	87.00±0.989	62.40±2.191	HS
Sperm grades (a+b)(%)	71.1±1.342	40.20±2.166	HS
Morphologically normal sperm (%)	62.200±0.731	43.50±1.013	HS

n=50, M±SE=mean± standard error, HS=P<0.001, NS=Not significant

Table (3): Comparison of some studied parameters in fertile group between before and after activation.

Parameters	Before activation	After activation	Significances
	M±SE	M±SE	
Calpain activity assay (IU/ml)	0.643±0.031	0.712±0.037	HS
Bradfordprotein assay (mg/ml)	0.095±0.0038	0.098±0.0034	NS
Specific Calpain activity (IU/mg)	6.983±0.289	7.515±0.386	NS
Sperm Pellet weight(g)	0.070±0.0049	0.060±0.0049	NS
Sperm conc. (m/ml)	58.30±2.470	24.52±1.138	HS
Sperm grades (a+b+c) (%)	72.10±0.991	87.00±0.989	HS
Sperm grades (a+b) (%)	61.20±1.139	71.1±1.342	HS
Morphologically normal sperm (%)	39.00±0.833	62.20±0.731	HS

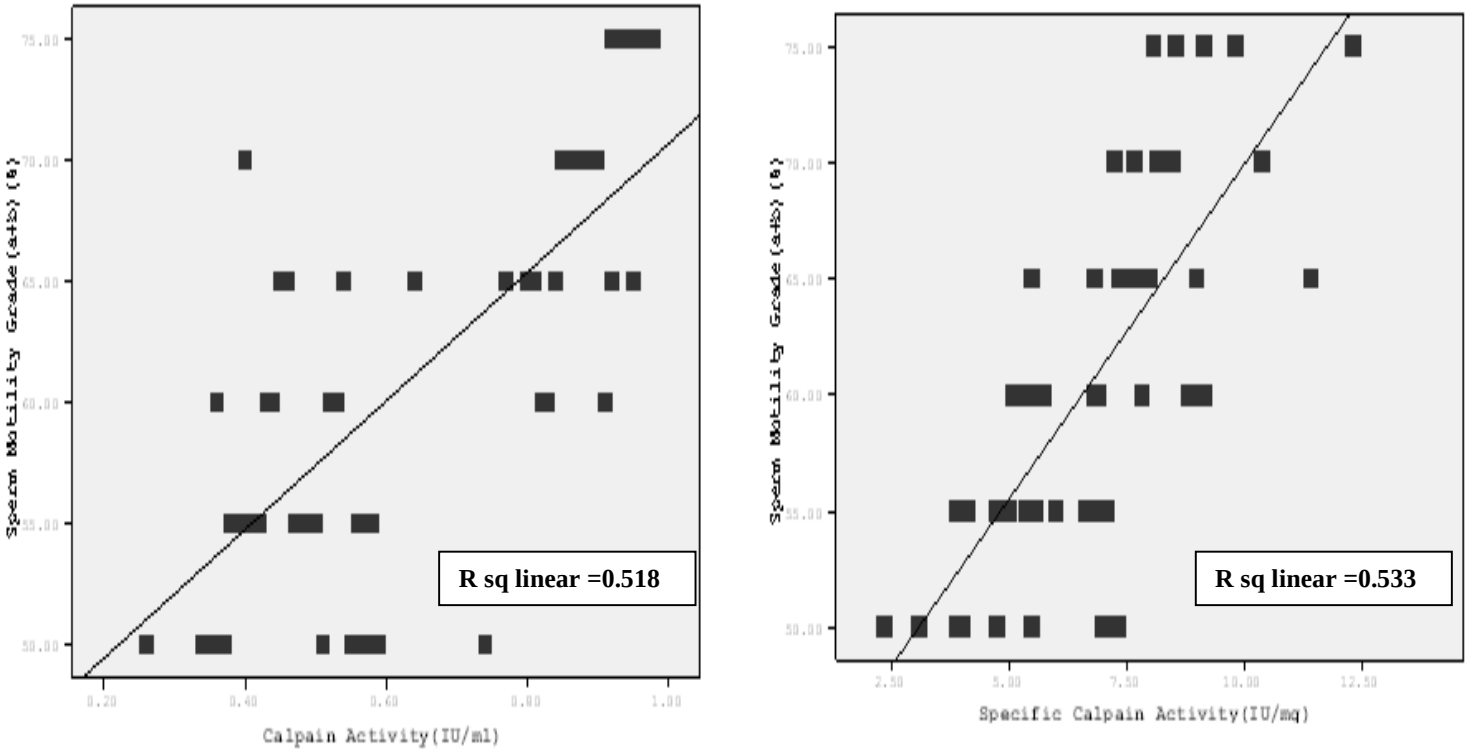
n=50, M±SE=mean± standard error, HS=P<0.001, NS=Not significant

Table (4): Comparison of some studied parameters in infertile group between before and after activation.

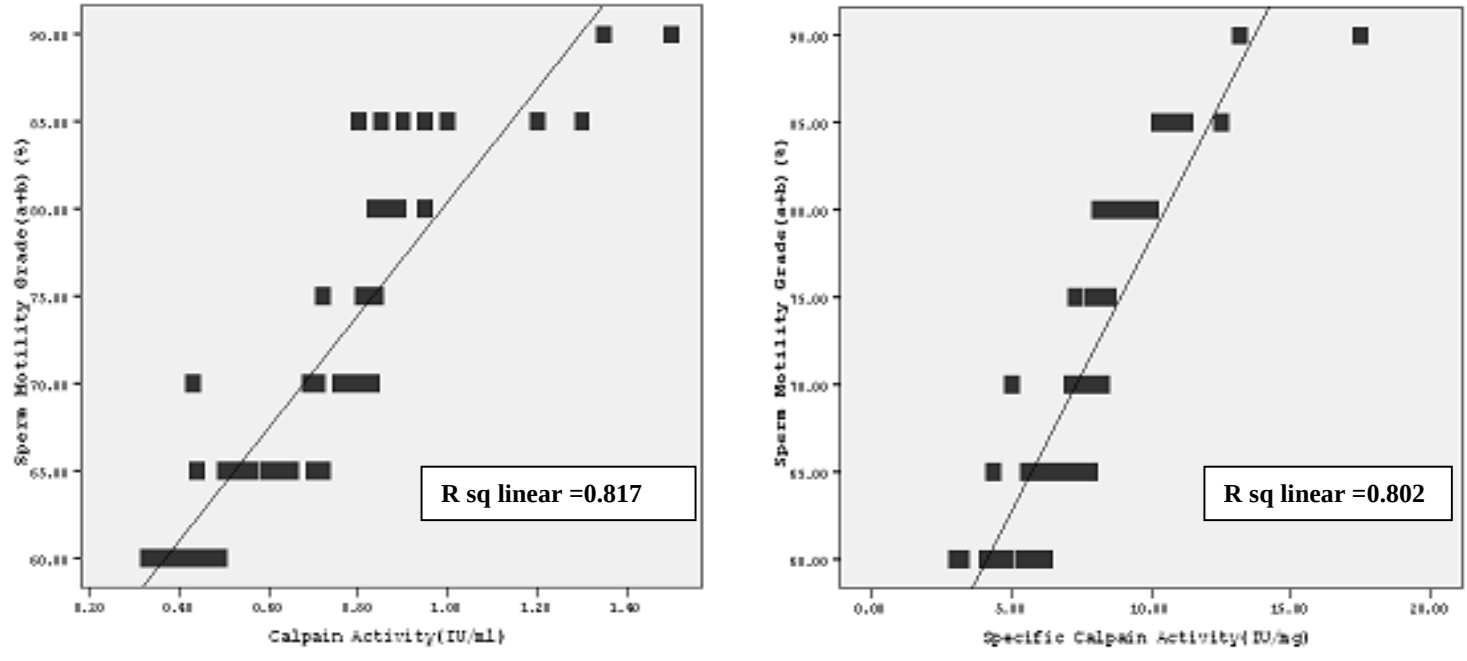
Parameters	Before activation	After activation	Significances
	M±SE	M±SE	
Calpain activity assay (IU/ml)	0.323±0.017	0.429±0.0117	HS
Bradford protein assay (mg/ml)	0.078±0.0025	0.082±0.0031	NS
Specific Calpain activity (IU/mg)	4.366±0.257	5.402±0.162	HS
Sperm Pellet weight(g)	0.074±0.0054	0.070±0.0037	NS
Sperm conc. (m/ml)	39.34±1.926	9.44±0.793	HS
Sperm grades (a+b+c) (%)	42.80±2.051	62.40±2.191	HS
Sperm grades (a+b) (%)	21.16±1.956	40.20±2.166	HS
Morphologically normal sperm (%)	27.00±0.589	43.50±1.013	HS

n=50, M±SE=mean± standard error, HS=P<0.001, NS=Not significant

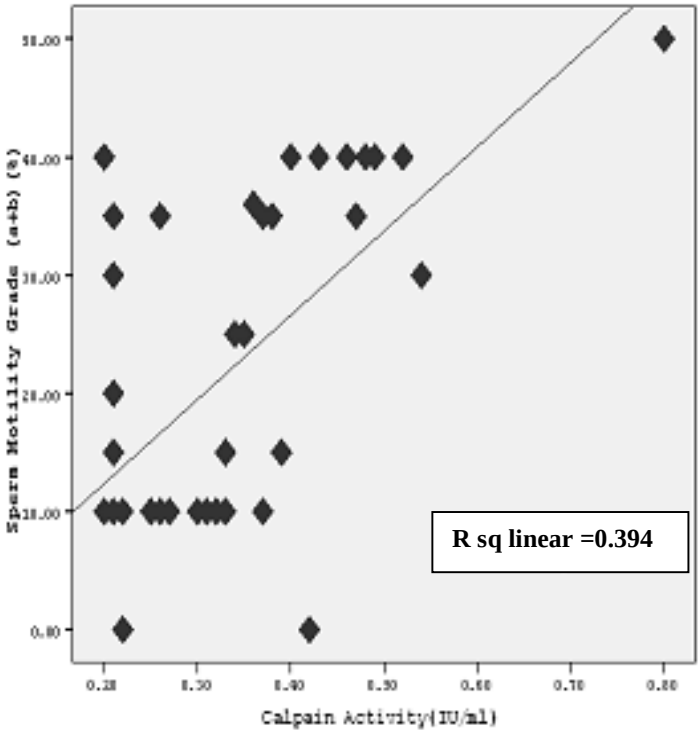
Figure(a): Correlations of calpain activity, specific calpain activity with sperm motility grade (a+b) in Fertile -Before activation

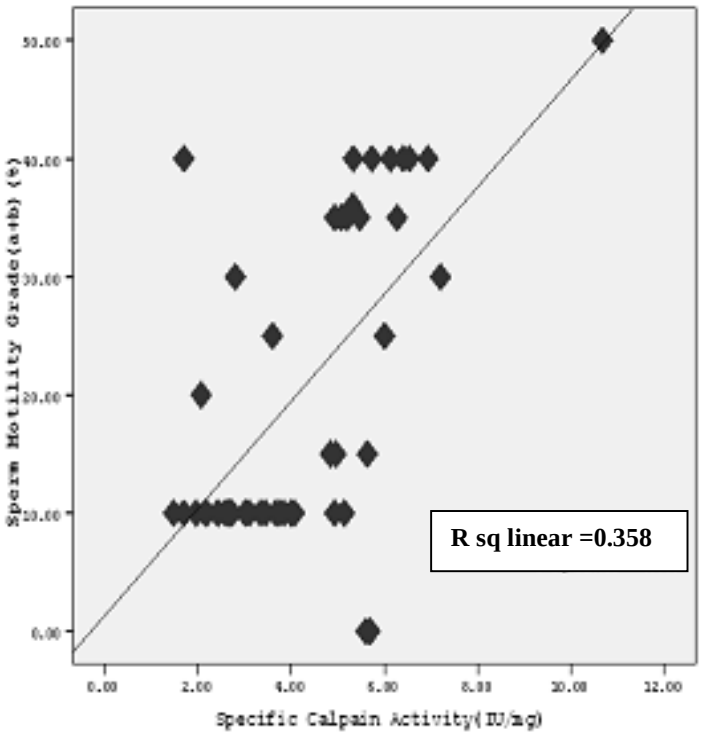


Figure(b): Correlations of calpain activity, specific calpain activity with sperm motility grade (a+b) in fertile -After activation



Figure(c): Correlations of calpain activity, specific calpain activity with sperm motility grade (a+b) in infertile -Before activation





Figure(d): Correlation of calpain activity, specific calpain activity with sperm motility grade (a+b) in infertile -After activation