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A novel diagnostic method for Duchenne Muscular Dystrophy using m-calpain as a quantitative marker: Biochemical marker for DMD diagnostics

Dr.Mohana Rao V[1], Dr.Sridevi K[2], Dr.G.Manoj Someswar[3], Dr.Anandraj MPJS[4]

1. Dept. of Biochemistry & Molecular Biology, Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad, A.P., India.
2. Associate Professor, Dept. of Pharmacology, Rajiv Gandhi Institute of Medical Sciences, Kadapa, A.P., India
3. Senior Scientist, Dept. of Molecular Biology and Genomics, SV Monco Biotech Pvt. Ltd., Hyderabad, A.P., India.
4. Former Director, Head-Dept. of Biochemistry & Molecular Biology, Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad, A.P., India and Department of Science and Technology, New Delhi, India.

***Abstract*:**

Calcium activated neutral protease-2 (m-Calpain) is a prominent catalytic protein found at the cellular level of human placenta. Earlier investigations stated its involvement in duchenne muscular dystrophy, which made us interested to isolate and purify m-Calpain with high yield to raise polyclonal antibodies. Here, we discussed indigenously developed reliable methodology by optimizing dependent parameters to isolate and purify m-Calpain with more yield. By currently developed methodology, significant yield of m-Calpain has been isolated and purified i.e 4-5 mg /1000g placenta tissue, which consists of 36.57% of total recovered protein. We have raised specific polyclonal antibodies against m-calpain in albino rabbits and serologically confirmed the purified enzyme by double diffusion and electrophoresis. Through development of this methodology, it enabled us to develop the cost-effective Quantitative ELISA (Q-ELISA) to identify Duchenne Muscular Dystrophy (DMD) patients and true carriers. Through Q-ELISA test we have found out that elevated levels of m-Calpain in platelets i.e 17.1±0.6 ng/ml in true carriers, 16.5±4.5 ng/ml in patients, 1.7±0.3 ng/ml in control males & 1.8±0.8 ng/ml in control females.

Keywords: *Calcium Activated Neutral Protease, Milli Calpain, Duchenne muscular dystrophy, Quantitative Enzyme-linked immunosorbent assay, Di Ethyl Amino Acetate, Phosphate buffered saline tween-20.*

Introduction

Calpains found to be associated with Z-disc of myofibrils and sarcolemma. Calpain is capable of proteolyzing a broad range of structural proteins found in muscles [1]. Z-disc proteins appear to be particularly susceptible to Calpains, which helps treating myoblasts through purified calpain [2] which made us interested to develop methodology to isolate and purify it with highest homogeneity. Two iso-enzymes [calcium-activated neutral protease (CANP1) or µ- calpain and CANP2 or m-calpain], with calcium requirements in micro molar and milli molar concentration respectively, have been identified at various tissue. Both are hetero-dimmers, composed of L-(large, catalytic, 80 kDa) and S-(small, regulatory, 30 kDa) subunits. The iso-enzymes differ in the L-subunit. The Calpain family comprises of several tissue-specific isoforms (n-Calpain) besides two ubiquitous iso-enzymes (µ- and m-calpain). Calpain in muscle cells & non-muscle cells contain both m- and µ-Calpain isoforms. Since placenta is a rich source of milli-calpain, we focused on developing a method to purify this enzyme from the source with high yield.

An earlier study suggests Calpain has shown to be significantly elevated in dystrophic muscle, implicating their role in muscle necrosis as in Duchenne Muscular Dystrophy (DMD) in `mdx' mice [3]. Studies have demonstrated an increase in the activity and concentration of calpain in DMD and mdx muscle, suggesting an mRNA dependent de novo synthesis of enzyme under dystrophic conditions [4]. Platelets contain both high and low $Ca2^{+}$ requiring calpain forms (m and µ) [5]. Calpain is located throughout the interior of human platelets, and its activation in platelets involves translocation to membrane and $Ca2^{+}$ dependent auto-proteolytic activation at the membrane [6-7]. It is suggested that the loss of membrane phospholipids asymmetry in activated platelets is due to the degradation of cytoskeletal proteins by calpain [8]. Pp60 Src, a major tyrosine kinase, is known to be easily cleaved by calpain and has been demonstrated as an endogenous substrate for calpain in human platelets [9,10]. Platelet Calpains may be involved in modulation of protein-tyrosine phosphorylation through activation of protein tyrosine phosphatase [11]. Calpain was demonstrated to play a role in $Ca2^{+}$ homeostasis through $Ca2^{+}$ ATPase activation by limited proteolysis [12]

Studies have demonstrated a significant increase in calpain activity [13-14] and concentration in the platelets from DMD patients and carriers. Such an increase in the concentration of calpain has been monitored to test its usefulness for the identification of disease and carrier states and found to be useful and reliable for disease and carrier detection in DMD [15]. Other findings corroborate the phenomenon of significant increase in the calpain activity in the non-muscle cells (platelets, lymphocytes and erythrocytes) in DMD patients and the carriers. In fact such an increase in calpain concentration has been monitored in platelets and lymphocytes for the identification of disease and carrier states of DMD. Calpain is believed to play a major role in muscle necrosis, especially during the early stages of pathogenesis of DMD [16]. The platelets,

which possess contractile protein apparatus and calcium fluxes regulations identical to that of muscle, showed elevated calpain [17].

This research article describes a simple, indigenously developed method by optimizing physical parameters such as conditions of precipitation, chromatographic separation specifications which includes pH, temperature and salt concentrations used to obtain m-calpain in high yields. Later on separation, specific polyclonal antibodies against this purified m-calpain have been raised in albino rabbits, and the purified enzyme has been serologically confirmed by double diffusion and electrophoresis. The development of this method enabled us to develop a cost-effective Q-ELISA method to identify Duchenne Muscular Dystrophy (DMD) patients and true carriers, as the function of calpains in muscle has received increased interest because of the discoveries that the activation and concentration of the ubiquitous calpains increase in the mouse model of DMD. Calpain concentration has been found to be raised in the platelets and lymphocytes of patients and carriers of DMD by dot blot immunoassay, while other myopathies did not show a significant increase in calpain levels, and this was proposed to be used as a pathological index for DMD [18]. However, isolating and purifying these enzymes to use in an assay has been difficult. Therefore, the cost of testing has been high.

Materials and methods

This study was performed after obtaining approval from the ethics committee (Institutional Ethical Committee For Biomedical Research) at Institute of Genetics and Hospital for Genetic Diseases, Osmania University, Hyderabad, Andhra Pradesh, India. Furthermore, informed consent was obtained from parents of all participants involved in the study. The informed consent was written and specified in the research consent.

Purification of m-Calpain

The placental tissue (500 gms), free of membranous material, was washed several times with normal saline and homogenized with buffer containing 20mM NaHCO3 and 5mM EDTA. Homogenate of placental tissue was centrifuged at 10, 000xg for 20 minutes. The pH of the supernatant was adjusted to 4.5 with 0.1N acetic acid. After 30 minutes the precipitate was pelleted by centrifugation at 20, 000xg for 20 minutes. The pellet was extracted in a buffer-A containing 20mM Tris-HC1, 5mM EDTA and l0mM 2-mercaptoethanol (pH 7.4). The pH of the extract was adjusted to 7.5 with 1N NaOH and left overnight with stirring. The extract was centrifuged at 20, 000xg for 20 minutes and supernatant, which contains the enzyme, was collected.

The clear supernatant was loaded on to a DEAE-cellulose column (35cm x 3.5cm) previously equilibrated with buffer-A. The column loaded with placental m-calpain was washed with buffer-A containing 250 mM NaCl, before elution of the enzyme with 340 mM NaCl. Active fractions determined by activity assay (described below) were pooled and were added with ammonium sulphate up to 60% saturation to precipitate the protein. The precipitate was collected by centrifugation at 200, for 10 minutes. The precipitate was dissolved in a buffer and loaded with a Sephadex G-150 column (100 cm x 1.5 cm) previously equilibrated with buffer-A (Figure-1).

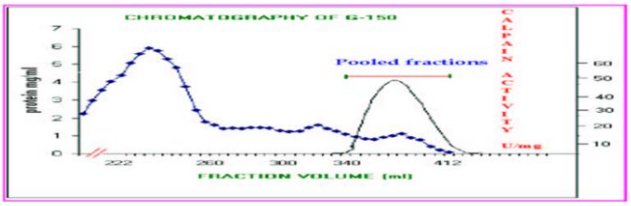

Fig.1: Chromatogram of m-Calpain with Sephadex G-150

The elution was done with buffer-A. The active fractions were pooled and cchromatography on DEAE-Sephadex A50 column (10 cm x 1.2 cm) (Figure-2). A linear gradient of NaCl (0.2-0.4M) was used for this purpose. The active fractions were pooled and subjected to denaturing polyacrylamide gel electrophoresis (PAGE) for the confirmation of molecular weight and homogeneity of subunits of purified protein. The 80 kDa subunit of m-

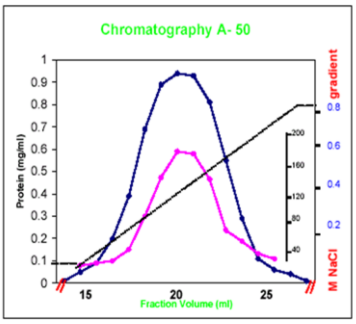

Fig.2 Chromatogram of m-Calpain with DEAE-Sephadex-A50

A linear gradient of NaCl (0.2-0.4M) was used for this purpose. The active fractions were pooled and subjected to denaturing polyacrylamide gel electrophoresis (PAGE) for the confirmation of molecular

weight and homogeneity of subunits of purified protein. The 80 kDa subunit of m-Calpain was separated by preparative gel, electroeluted and was used for raising polyclonal antibodies.(Figures - 3 & 4)

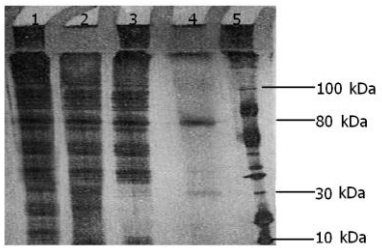

Fig.3: Placental m-calpain (80kDa) protein purified from 10% SDS PAGE, Lane 1: Crude extract of placenta, Lane 2: Chromatogram of DEAE cellulose, Lane 3:Chromatogram of Sephadex -G150, Lane 4:Chromatogram of affinity Lane 5:Marker kDa from 100 –1 0

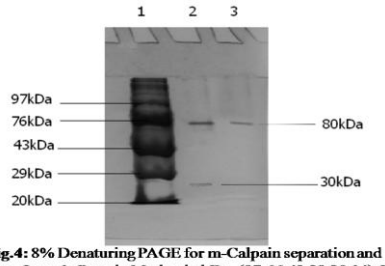

Fig.4: 8% Denaturing PAGE for m-Calpain separation and purification, Lane 1: Protein Marker in kDa (97,66,43,29,20,14), Lane 2: Separated subunits m-Calpain of 30 kDa and 80kDa, Lane 3: Purified m-Calpain 80 kDa

Enzymatic Assay for m-Calpain

The proteolytic activity of m-Calpain was monitored using alkali- casein as a substrate. The assay mixture consisted of enzyme in a total volume of 0.5mL, containing 0.25% alkali- casein in 20mM Tris-HCl buffer (pH7.4), 10mM 2-mercaptoethanol and 5mM CaCl2. Controls had 5mM EDTA instead of CaCl2. The reaction was carried out at 30°C for 90 minutes and was terminated by adding 10% cold Tri-chloro acetic acid (TCA). TCA-soluble peptides were measured at 280 nm in the spectrophotometer (Shimadzu, Japan). A unit of enzyme activity is defined as the amount of enzymes that catalyzes an increase of 0.01 absorbance at 280nm at 30°C in 90 minutes.

Standard troubleshooting rules for general protein purification have been applied during development of this purification method (i.e., use of protease inhibitors when a necessary, addition of required co-factors for a given target protein to promote stability, proper buffer salts, pH, time and conditions, etc.). Here, we have achieved time taken for completion of purification in 36hrs as against 72hrs taken in earlier cases.

Raising Antisera (Polyclonal antibody)

Immunization of rabbits

Young and healthy New Zealand rabbits were used for immunization. 100µL (150µg) of purified 80-kDa m-calpain was emulsified with an equal volume of complete Freund's adjuvant and injected intra-dermally at multiple sites of the rabbits with three booster doses (i.e. with interval of 20days). After 2nd booster dose

with incomplete Freund's adjuvant, after 10 days of 2nd booster dose total 60ml of blood (i.e each draw of 20ml) is collected with frequent interval of 10 days.

Antisera collection and preservation

The immunized rabbits were bled after 10 days of the last injection, by making a cut on the ear vein with a sharp blade. Blood was collected into sterile tubes, kept at room temperature for clotting, and subsequently at 4ºC for serum release. The serum was collected into sterile centrifuge tubes and centrifuged at 6000xg for 15min. The antiserum was aseptically aliquoted in 1.5 mL volumes and stored at –20ºC. The rabbits were further bled at 10 days intervals to repeat the above sera separation procedure.

Purification of IgG from antisera with protein A-Sepharose separation

Serum, diluted with an equal volume of PBS, was passed through the Protein-A column in the IgG Purification kit (Invitrogen, India) at a rate of about 0.2mL/min. The purified immunoglobulin of m-calpain was scanned from 200–300nm. The immunoglobulin has maximum and minimum absorptions at 278.4 nm and 250.6 nm, respectively. Purified IgG was quantified using the Molar Extinction Co-efficient value of 1.4 OD=1mg/mL of purified m-Calpain. The Purified IgG has been tested by immunoelectrophoresis, double immuno-diffusion and ELISA methods for the quantitation of m-Calpain.

Serological Detection

Double immuno-diffusion test was performed to know the Antibody titer in antiserum as described by Purcifull and Batchelor (1977). The gel was prepared to use 1% molten agarose in 0.01M phosphate buffer pH 7.2, 0.5% SDS and 0.1% sodium azide. The molten agarose at 500C was poured on to a 5x5 cm clean glass plate with the help of pipette and allowed to solidify. The wells (4 mm diameter) were cut in the solidified medium by using a template and cork borer (six peripheral wells at a distance of 3mm from the edge of the central well). The agarose plugs were taken out with the help of a needle. The bottom of the each well was sealed with molten agarose to prevent seepage of the samples.

To determine the antiserum titer the purified m-calpain was used in the central well (20µL) and the peripheral wells were filled with 20µL of different dilutions (1/2, 1/4, 1/8, 1/16, 1/32) of antiserum. The antigen- and antibody- loaded plate was incubated in a moist chamber at room temperature for 36 hours. The mono-specificity of the raised polyclonal antibodies were confirmed by double diffusion and immunoelectrophoresis. In the immunoelectrophoresis 80µg antigen was electrophoresed.

Enzyme linked immunosorbent assay (ELISA) for the Quantitation of platelet m-calpain

Isolation of platelets

Peripheral blood platelets were isolated from heparinized venous blood from DMD, patients and carriers (obligate & probable) as described earlier[19]. Briefly, platelets from the blood samples were isolated on Ficoll-Hypaque (Sigma) gradient centrifugation and the washed cells were sonicated by eight cycles for 15 sec bursts with intermittent cooling on ice using Imeco sonicator.

Optimization of m-calpain ELISA

The method described is an indirect non-competitive ELISA with antigen immobilized on the solid phase microtitre plate (Nunc-Denmark). Microtitre plate was coated with 100 µl serially diluted purified m-calpain (50 ng-0.097 ng/ml) or platelet cytosolic proteins from DMD patients, carrier mothers, (10-0.009 µg/ml) platelet cytosolic proteins from DMD patients, carrier mothers in carbonate buffer (14mM Na2CO3, 46 mM NaHCO3) and the plate was incubated at 40C overnight. The plate was washed thrice with PBS-T at 5 min interval. Non-specific binding sites were blocked with 3% BSA/PBS for 2 hours at room temperature. After washing the plate three times with PBS-T, primary antibody (1:1500)(Figure 6) diluted in 1% BSA/PBS was added and the plate was incubated for three hours at room temperature. After rinsing three times with PBS-T at 5 minutes interval 100µ1 of horse radish peroxidase (HRP) conjugated secondary antibody (1:10000) diluted in 1% BSA/PBS-T was added. The plate was further incubated for two hours at room temperature. The bound peroxidase was detected by the addition of Tetramethyl benzidine(TMB) substrate solution and the reaction was carried out for 20 minutes at room temperature, and was stopped by adding 5% HCL stop solution and the absorbance was measured at 450 nm.

The standard curve obtained using purified m- calpain was used for the quantitation of m-calpain in the cytosol of platelets from DMD patients, carriers and normal controls. To determine the absolute amounts of m-calpain in the cytosol of platelets from DMD patients, carriers(Figures7-8) and controls the midpoint from the linear part of the respective exponential curves was compared with the standard curve.

Calpain assessment in DMD and carriers

In order to develop an ELISA, the standard dose response curves were generated using antibody dilutions of 1:500, 1:1000, 1:1500 and 1:2000 with serial dilution of antigen (m-calpain) ranging from 0.09-50 ng/ml. Dose response curve obtained with antibody dilution of 1:1500 (Figure-4) was selected as standard curve, which maintained the linearity between 0.75-6.25ng/ml of antigen concentration. Similarly, dose response curves using antigen (cytosolic protein of platelets from DMD patients, carriers and controls) from DMD

patients and obligate carriers showed the linearity between 78-625 ng/ml, while the antigen from normal control showed the linearity of the curve between 625-5000 ng/ml. In order to measure the absolute amount of m-calpain present in the cytosol of platelets from DMD patients, (Figure-5) carriers (Figure-6) and controls, respective dose response curves were compared with the standard curve.

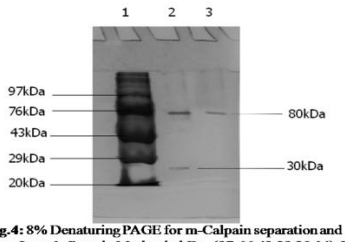

Fig.4: 8% Denaturing PAGE for m-Calpain separation and purification, Lane 1: Protein Marker in kDa (97,66,43,29,20,14), Lane 2: Separated subunits m-Calpain of 30 kDa and 80kDa, Lane 3: Purified m-Calpain 80 kDa

The amount of m-calpain present in 60 DMD patients was found to be 16.5 ± 1.9 ng/µg(mean±SD) (Table-1) of platelet protein in 45 risk females; it was 14.7±4.1 ng/µg(man±SD) in 18 obligate carriers. The m-calpain value present in 24&3 probable carriers with high and low values was observed to be 12.5 ± 5.4 and 2.4±0.6 ng/µg (mean±SD) (Table 1).Therefore, compared to control a 10-fold increase in the amount of m-calpain was registered in all the DMD patients, obligate and probable carriers tested in the present study.

S.No	Purification Steps of m-calpain from Placenta	Total Protein (mg)	Specific activitiy (u/mg)	Total activity (folds)	Purification (fold)	Recovery percentage of m-Calpain
1	Homogenate	24,600	-	-	-	-
2	Isoelectric precipitate	6,150	-	-	-	-
3	DEAE – cellulose Chromatography	96.5	54.2	10650.3	1	100
4	Ammonium sulphate precepitation	32.5	124.66	6170.67	2.3	74.75

5	Sephadex-G 15O gel filtration	12.37	261.78	3238.29	4.82	59.62
6	DEAE–Sephadex-A50chromatography	3.03	654.29	1982.49	12.07	36.57

Table.1: Purification fold and percent recovery during various stages of purification of m-calpain from Human Placenta

Total number of patients screened	**DMD Patients with m-calpain levels**	**True Carriers with m-calpain levels**	**Control Boys**	**Control Females**
m-Calpain ng/µg	16.5±4.5 (mean±SD) P<0.001	17.1±0.6 (mean±SD) P<0.002	1.7±0.3 (mean±SD)	1.8±0.8 (mean±SD)

Table-2: m-Calpain levels in the studied groups. m-Calpain was expressed as nanogram of m-calpain per microgram of platelet cytosolic protein. Statistical significance values (P) are given, P: statistical significance values

Results & Discussion

Typical results for the purification of m-Calpain from human placental tissue, such as fold-purification and percent recovery, are tabulated (Table-1). The results suggest that human placenta extracts contain m-Calpain in significant quantities, of around 4-5 mg m-Calpain/1000g placenta tissue constituting 36.57% of total recovered protein, obtained through the method used to isolate and purify the protease. The concentration of the enzyme was estimated as 62.7mg/mL in the final sample. Presence of proteolytic

activity was seen from a purified protein sample while performing qualitatively and quantitatively estimation through enzymatic assay. After affinity column purification, protein was identified & characterized (Figure-3) by SDS PAGE (Figure-4).

The highly purified, homogeneous m-calpain obtained was used in raising the polyclonal antibodies in rabbits. From the total collected blood of 60ml, after separation 18ml of antisera of m-Calpain is collected, and further it is serologically tested and confirmed by Immuno electrophoresis and Double Diffusion tests. In double diffusion, the arc was identified between 1/16 and 1/32 Ab dilutions (Figure-5), thereby confirming the antigen (80 kDa) and antisera relationship. The precipitation band was observed 1/32 dilution at when 50µL of antisera (IgG) was used (Figure-6). No precipitation line was observed when pre-immune serum was used. The sera has been preserved to develop a quantitative enzyme-linked immunosorbent assay (Q- ELISA) method for detection of DMD patients and true carriers.

As observed in erythrocytes, platelets and lymphocytes of DMD patients, calpain activity and concentration were also found to be raised in DMD carriers(Figure-7). In order to diagnose DMD patient & true carriers(Figure-8), m-Calpain levels in platelets(Figure-9) and lymphocytes are tested with CPK assay as preliminary diagnostic tool. Since values of CPK and other

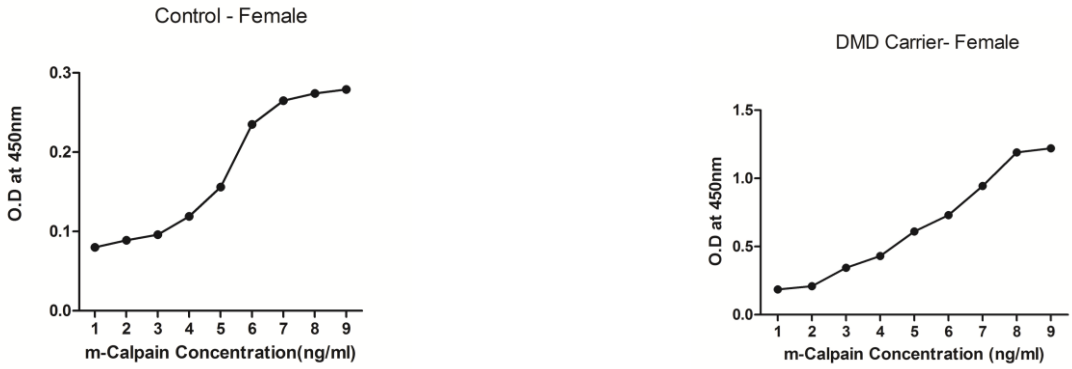

Figure-7 : Dose response curve using antibody dilution 1:1500with serial dilution of m– Calpain 2.2ng/ml in control female & 8.5ng/ml in Carrier

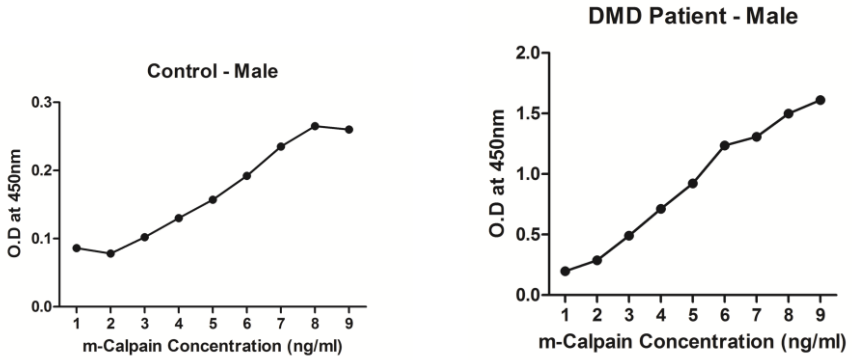

Figure-8: Dose response curve using antibody dilution 1:1500with serial dilution of m– Calpain- 13.4ng/ml in control male and 11.5 ng/ml in patient male

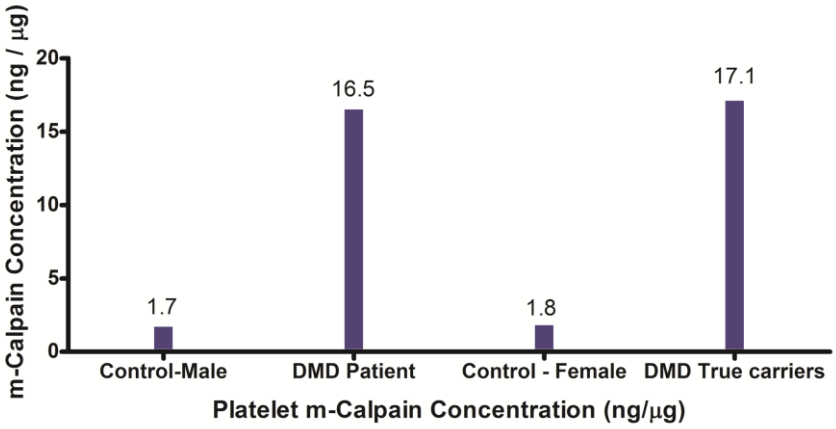

Figure-9: Bar Graph representation of m-Calpain concentration(ng/microgram) in platelets of Control-Male, Patient-Male, Control-female & DMD True Carrier

phenotypic indices shown considerable overlap with that of controls, these tests are considered unreliable for true carrier detection. In order to achieve better diagnostic results, Q-ELSA methodology was developed to cater the needs. The earlier method used for quantitating the m-calpain was semi quantitative, the development of a method such as quantitative ELISA would enable facile, accurate and cost effective quantitation of m-calpain. Incidentally, monitoring the calpain levels in patients and 'carriers' of DMD would provide a handle for detection of disease and true carrier states. The enhanced level of m-calpain as measured by ELISA and the presence of deletions or the mutant chromosome tracked by inherited STR polymorphisms while validating the status of m-calpain as marker for diagnosis of disease/true carrier state would form part of a strategy for definitive diagnosis of true carriers and prenatal diagnosis. Such a marker would also help in population screening.

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