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DETERMINATION OF OXIDATIVE STRESS FACTORS IN PATIENTS WITH HEREDITARY MUSCLE DISEASES: ONE POSSIBLE DIAGNOSTIC AND OPTIONAL MANAGEMENT OF THE PATIENTS

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ABSTRACT

Superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LP) are familiar parameters for investigating the oxidative stress induced pathogenesis of hereditary muscle diseases. Results stated that oxidative stress may be hosting a role in the pathogenesis of hereditary muscle diseases. Evaluated defensive parameters (SOD, GPx & CAT) and indicative parameter (LP) are collectively considered as oxidative stress factors and represented the oxidative stress status of the patients. Alterations in the levels of these factors follow the uniform pattern in patients with muscular dystrophies and mitochondrial myopathies. These factors also provide the possibility for the development of alternate diagnostic method. On the basis of such informations, clinicians would be able to manage the patients with addition of appropriate antioxidants in the medication or diet. There is no permanent treatment for patients with hereditary muscle diseases and management of disease is a solitary option of delaying the deterioration progress.

KEYWORDS: Regression analysis, antioxidants, muscular dystrophy, mitochondrial myopathy, pathogenesis.

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INTRODUCTION

Hereditary muscle diseases share a common end-point represented by muscle wasting.[1]Common of the hereditary muscle wasting diseases among Indian population are muscular dystrophies (27.4%) and metabolic myopathies (4.66%).[2] The muscular dystrophies are caused by mutations in genes encoding the extracellular matrix, transmembrane and membrane-associated proteins, cytoplasmic enzymes and nuclear proteins.[1]Duchenne Muscular Dystrophy (DMD), Becker Muscular Dystrophy (BMD), Limb Girdle Muscular Dystrophy (LGMD) and Facioscapulohumeral dystrophy (FSHD) are most prevalent types of muscular dystrophies in Indian population.[2] Insufficient supply and irregular regeneration of the ATP molecules, are responsible for anomalous muscle function in the patients with metabolic myopathies. The major clinical entities of such types are mitochondrial myopathies, GSD (glycogen storage disease), carnitine deficiency etc.[3-4] Genetic basis of several varieties of muscular dystrophies has been characterized but the mechanism behind the pathophysiology is still ambiguous.[1] In literature, there are several studies, indicating the central role of oxidative stress and abnormal production of reactive oxygen species (ROS) in the pathogenesis of muscular dystrophies.[1] Dystrophin-deficient (mdx) mice are more susceptible to damage induced by free radical species.[5] Primary genetic defects and abnormal ROS production are responsible for the pathophysiology in patient with DMD and mdx mouse model.[6] Possibility of myofiber necrosis is observed in these diseases due to oxidative stress.[5] Oxidation induced damage is not only responsible, for the damage of skeletal muscle but it may also be involved in the pathogenesis of the heart failure in DMD and mdx mice.[7] The functional impairment of muscle force generating capacity in DMD patients happens due to, the oxidative stress dependable events. These events are the extensive disruption of the dystroglycan complex and the upregulation of inflammatory transcription factor NF-B.[8] Skeletal muscle immobility is associated with loss of muscle protein and reduced force generation capacity in the muscular dystrophies. Oxidative stress is also contributing to the muscle protein loss, in calpainopathy. [9] Lattanzi et al (2012) documented the altered expression of extracellular matrix components due to the accumulation of oxidative stress induced damaged DNA in the muscular dystrophies [10], which is further responsible for the muscle cell death. The role for oxidative stress, in DMD is supported by a wealth of pre-clinical studies in mdx mice, that report benefits such as improved muscle pathology and decreased necrosis, for example various kinds of antioxidant drugs and interventions such as resveratrol, green tea extracts, catalase, ubiquinol-9, biquinol-10 and coenzyme Q10.[11-14] Dysferlin deficiency is also associated with excessive inflammation and it has been hypothetically put forward, that this leads to an increased level of reactive oxygen species (ROS).[15] DUX4 protein initiates a large transcription deregulation cascade leading to muscle degeneration and oxidative stress in patients with FSHD .[16] Catalase (CAT) levels are found to increase in muscle samples of DMD patients, as well as in dystrophic mouse models mdx (pre-necrotic), mdx (4-5 mo) and dy/dx.[17-20] CAT activity is also enhanced in the dysferlin deficient muscle cell. Superoxide dismutase (SOD)-1 and SOD-2 level are also found to be enhanced in DMD patients.[17] Level of these two enzymes was also increased in dystrophic mouse models mdx (pre-necrotic) and mdx (4-5 mo).[18-19] Only the level of SOD-2 is increased in dy/dx dystrophic mouse model.[20] Glutathione peroxidase (GPx) level were found to increase in patients with DMD and BMD as well as in dystrophic mouse models mdx (pre-necrotic), mdx (4-5 mo) and dy/dx.[17-21] Lipid peroxidation is also enhanced in muscle samples from patient with DMD, LGMD and FSHD as compared to control.[17] Asayama et al (1981) concluded that the muscle protein breakdown, occurs independent of the lipid peroxidation, despite the presence of tissue-specific abnormality, of redox metabolism in dystrophic muscles of mice.[18] Enhanced rate of lipid peroxidation is also observed in the dysferlinopathy, as well as in DMD and sarcoglycanopathy.[15] Oxidative stress is not only responsible for muscular dystrophy but it also plays a major role in mitochondrial myopathies. In mitochondrial myopathies, a varying degree of dysfunctions of the mitochondrial respiratory chain occur, which leads to the generation of reactive oxygen species (ROS), followed by oxidative stress and cellular damage. Over expression of antioxidant enzymes, in the muscle tissue has been documented, probably as an attempt to counteract the free radical generation.[22] Mitochondrial DNA mutations are also involved in oxidative stress, oxidative damage and altered gene expression as well as in pathogenesis and progression of MERRF syndrome.[23] Enhanced ROS production is leading to

apoptosis and over expression of antioxidant enzymes, which may play an important role in the pathophysiology of COX deficient mitochondrial encephalomyopathies.[24]The pre-clinical studies with CoQ10 supplementation in MEREF patient showed the reduction of oxidative stress with the recovery of fibroblasts and cybrid pathophysiology.[25]There are several reports related to therapeutic trials also, which have proved that the oxidative stress induces damage in mitochondrial myopathies.[26-28] Measurement of oxidative stress is performed via the estimation of SOD, GPx, CAT & LP in various diseases such as in chronic renal failure,[29] heart disease[30] and breast carcinoma[31] as well as in muscular dystrophies.[5-7]In literature, there is a lack of complete and systematic analysis of antioxidant enzymes and lipid peroxidation in both muscle as well as serum samples of the hereditary muscle diseases (muscular dystrophies and metabolic myopathies). None of the study has reported correlation analysis and the possible diagnostic role for all these parameters. In this regard, our this study is designed to perform, the appropriate analysis of SOD, CAT, GPx and LP in muscle and serum samples from patients with muscular dystrophies and metabolic myopathies along with the normal/ control individuals. Correlation analysis of enzyme activities and lipid peroxidation in muscle as well as in serum samples was performed. Regression analysis was performed for obtaining the linear equations between the parameters. DFA (Discriminant function analysis) was also performed for the discrimination of patients and healthy individuals.

MATERIALS AND METHODS

Approval from ethical committee of All India Institute of Medical Sciences, New Delhi, was obtained before collecting the samples. Blood and muscle sampless of fourty seven (N= 47; N= number) (age, mean$\pm$SD; 22.9$\pm$7.2 year) clinically, electrophysiologically and histopathologically proven patients with muscle diseases were collected from outpatient clinics (OPD) and inpatient wards of the Neurology Department of All India Institute of Medical Sciences, New Delhi, India. In all these patients, thirty four (N=34) were male and thirteen (N =13) were female. Written consent from the relatives of the patients was obtained before taking the blood and muscle samples. Normal muscles samples of thirty (N=30) (age, mean$\pm$SD; 25.9$\pm$12.0 year) control individuals were obtained from Neurosurgery Department of All India Institute of Medical Sciences, New Delhi, India. In all these control subjects, twenty three (N=23) were male and seven (N =7) were female. It was ensured that control individuals had no evidence of neuromuscular disease. Muscle tissue was obtained from those control individuals whose para spinal muscles had to be sacrificed during separation from underlying bone. A written consent was obtained from all the control individuals before removing the tissue. The muscle tissue sample was collected directly in a sterilized tube and stored in the liquid nitrogen (-196°C). Blood samples were collected from thirty healthy volunteers (N=30) (age, means; 24.6$\pm$3.3). These samples were collected during their routine health examination. In all the healthy individuals, seventeen (N=17) were male and thirteen (N =13) were female. All these patients and healthy individuals were of North India origin. All of the samples were collected directly into sterilized tubes. Blood samples were collected for the separation of serum. All the serum samples were stored in the liquid nitrogen (-196°C).

Chemicals

All the reagents for the enzyme assay and lipid peroxidation were obtained from Sigma- Aldrich, UK.

Methods

Clinical examination

All suspected patients with muscle diseases were examined by a neurologist, in the outpatient clinic of AIIMS, New Delhi. All the patients complained about the general problems, such as difficulty in walking and standing, difficulty in climbing the stairs, frequent fall and muscular wasting and weakness. The patients were examined for the well-known signs and symptoms. Gower's and valley sign, along with calf and other muscle hyper trophy [32], which are the clinical signs to diagnose DMD (Duchenne Muscular Dystrophy) and BMD (Becker's Muscular Dystrophy). Polly- hill sign and calf head on trophy sign are helpful to diagnose the FSHD (Facioscalpulo humeral muscular dystrophy) and LGMD-2B (Limb Girdle Muscular Dystrophy type-2B or Dysferlinopathy).[32] Patients with mitochondrial myopathies were also examined for well known symptoms and signs.[32]

Electromyographical (EMG) examination

EMG examination was performed for confirming the diagnosis of myopathy by using concentric bipolar needle. Myopathy was established after the expression of the myopathic EMG pattern.

Histopathological and immuno histopathological examination

Muscle samples for biopsy from all the suspected muscle disease patients were performed for the histopathological and immunohistochemical examinations. Immunoblotting was also performed for further confirming the diagnosis, wherever, felt necessary.

Biochemical estimations from serum and muscle tissue

Blood was collected in plain vials followed by centrifugation at 2000 rpm for 10 minutes. Clear serum was aliquoted and stored in liquid nitrogen (at -196°C) for biochemical studies. To prepare muscle tissue homogenate, the muscle tissue was crushed in mortar and pestle in presence of liquid nitrogen for making fine powder. After this, the muscle tissue was homogenized in 0.05M Tris-HCl buffer (pH= 7.4) with protease inhibitor cocktail (3µl per 10ml buffer) to yield a 10% (w/v) homogenate. The homogenate was centrifuged at 1000g for 10 minutes. The resultant supernatant was transferred into pre-cooled centrifugation tubes and centrifuged at 12,000 g for 30minutes. The supernatant (having cytosolic fraction), after discarding any floating lipid layer, was used for the estimation of LP, SOD, CAT and GP_x activities. Homogenates were kept at -80ºC until the experiments were performed.

(a) Estimation of lipid peroxidation

Thiobarbituric acid-reactive substance (TBARS), an index of lipid peroxidation was estimated by the method of Ohkawa et al 1979.[33] The amount of TBARS was determined spectrophotometrically at 532nm and expressed as nanomoles of malondialdehyde (MDA)/mg protein. Tetramethoxy propane (TMP) was used as a standard.

(b) Determination of superoxide dismutase, catalase and glutathione peroxidase activities

The activity of SOD, CAT and GPx was determined spectrophotometrically at 37º C utilizing the technique of Singh et al.[34] Enzyme activity was expressed as units/mg protein.

Statistical analysis

Mean levels of SOD, GPx, CAT and LP in serum and muscle samples of the patients with muscle disease and control/ healthy individuals were compared by independent sample t-test for independent groups. Mean levels of all the above described enzymes and lipid peroxidation in serum and muscle samples of the patients with muscle diseases were compared by paired t-test for paired groups. The p-values, less than 0.05 were considered significant. Correlation between the levels of the enzymes (SOD, GPx and CAT) and LP in serum and muscle samples of the patients with muscle diseases were performed by Pearson correlation analysis. Correlation between the ratio of the levels of the enzymes (SOD, GPx and CAT) and LP in serum and muscle samples of the patients with muscle diseases were performed by Spearman correlation analysis. The regression analysis was also performed for these values. DFA (discriminant function analysis) was also performed for the discrimination of the patients and control/ healthy individuals. The ratios of antioxidant enzymes & LP in serum and muscle samples of the patients with muscle diseases were compared by Mann-Whitney U test. The data management and analyses were performed by using the statistical software SPSS version 15.0.

Sample size determination

The parameters under study are quantative. Therefore, on the basis of some pilot experiments, the mean level of SOD, GPx, CAT and LP are taken under consideration. Highest variation in parameter CAT is considered for covering the sample size for other parameters. Each group (disease and control/healthy individuals) is required twenty nine samples for a two-tailed “t” test on considering the clinical difference (CD) = 15, standard deviation (SD) = 20, significant level (p) =0.05 and 80% power level. The mean level of SOD, GPx, CAT and LP will be compared by student “t” test. The p value less than or equal to 0.05 will be considered significant. The data management and analysis will be carried out using statistical software SPSS version 13.0.

RESULTS

Clinical examination

All the 47 cases of muscle diseases could be suspected for having DMD, BMD, FSHD and LGMD, unclassified muscular dystrophies and mitochondrial myopathies on the basis of clinical examination, including symptoms, signs and family history

EMG examination

All the 47 cases of muscle diseases showed

myopathic EMG pattern in the form of almost complete interference pattern and low amplitude (<500 µv) MUAP were of short duration (5-8 ms), and were most of polyphasia (Figure 1).

Histopathological and immuno-histochemical examination

Based on the histopathological and immuno-histochemical examination of the muscle biopsy samples, following types of muscle diseases were confirmed, the four cases (N=4) of DMD & BMD, ten cases of (N =10) LGMD-2B, eight cases of (N =8) LGMD-2A, six cases of (N =6) mitochondrial myopathies, four cases (N =4) of FSHD and fifteen cases (N=15) of unclassified muscular dystrophies (Figure-2).

Biochemical estimations in serum and muscle tissue

(a) Estimation of lipid peroxidation

Level of LP in the serum of 47 cases of patients with muscle diseases (mean ±SD; 9.94± 5.16 mmol TBARS/mg protein) is significantly higher ($p< 0.05$) as compared to 30 normal individuals (mean ±SD; 7.58±2.23 TBARS/mg protein). In the muscle samples of the patients with muscle diseases, value of lipid peroxidation of 47 cases (mean ±SD; 5.39±5.11 TBARS/mg protein) is significantly higher ($p< 0.05$) as compared to 30 control individuals (mean ±SD; 3.22±2.73 TBARS/mg protein) (Figure-3 &4).

(b) Determination of superoxide dismutase, catalase and glutathione peroxidase

Enzymatic activities of SOD, CAT and GPx in serum of the 47 cases of patients with muscle diseases are 1.45 ± 0.78, 42.77±19.36 and 68.50±45.92, units/mg protein, respectively. Levels of SOD, CAT and GPx in serum of 30 cases of normal individuals are 1.08±0.63, 30.84±16.96 and 49.12± 23.15, units/mg protein, respectively. SOD, CAT and GPx activities in serum of the patients with muscle diseases are significantly higher ($p< 0.05$) as compared to normal individuals (Figure-3). SOD activity (2.53±4.09 units/mg protein) in the muscle samples of the 47 cases of the muscle diseases is significantly higher ($p< 0.007$) as compared to the 30 normal individuals (0.82±0.64 units/mg protein). Similarly, GPx and CAT activity in the muscle samples of the 47 patients with muscle disease are 221.29±195.83 and 326.13±171.49 units/mg protein, respectively. Levels of GPx and CAT in the muscle sample of the 30 control individuals are 81.47±52.09 and 192.35±134.54 units/mg protein, respectively. Activities of GPx and CAT in muscle samples of the patients with muscle diseases are significantly higher ($p< 0.0001$ & $p< 0.001$) as compared to control individuals (Figure-4).

(c) Comparison of LP, SOD, CAT ,GPx in muscle and serum samples of the patients

Enzymatic activities (SOD, CAT & GPx) and LP level in muscle and serum samples of the patients with muscle diseases were compared by "paired t test". Level of SOD, GPx & CAT was found significantly higher ($p<0.0001$) in muscle as compared to serum samples. LP level was significantly reduced ($p<0.0001$) in muscle as compared to serum samples (Figure-5).

(d) Correlation between the antioxidant enzymes (SOD, GPx & CAT) and LP levels in muscle tissue & serum of the patients with muscle diseases, respectively

Correlation analysis was performed for SOD, GPx, CAT and LP levels in muscle and serum of the patients with muscle diseases, respectively. There is no correlation observed for SOD, GPx, CAT & LP in between muscle and serum of the patients. Weak positive correlation was observed in between the following pairs of analysis, muscle (SOD) vs. muscle (LP), muscle (GPx) vs. muscle (LP), muscle (CAT) vs. muscle (LP), muscle (SOD) vs. muscle (CAT) & muscle (SOD) vs. muscle (GPx). A moderate correlation is observed for muscle (SOD) vs. muscle (GPx+CAT). There is no correlation observed for muscle (GPx) vs. muscle (CAT) (Table-1). No correlation is observed for serum of the patients. Weak negative correlation is observed for serum (GPx) vs. serum (CAT).

(e) Correlation between the ratios of the antioxidant enzymes (SOD, GPx & CAT) and LP levels in muscle tissue & serum of the patients with muscle diseases, respectively

Correlation analysis was performed for the ratios of SOD, GPx & CAT and LP levels in muscle and serum of the patients with muscle diseases, respectively. There is no correlation observed for the ratios of SOD, GPx, CAT & LP in between muscle and serum of the patients. Strong positive correlation is observed for muscle (SOD/CAT) vs. muscle (SOD/LP), muscle (SOD/GPx) vs. muscle (SOD/LP) and muscle (SOD/GPx+CAT) vs. muscle (SOD/LP). Moderate correlation is observed for

muscle (SOD/GPx) vs. muscle (SOD/CAT) and muscle (SOD/GPx/CAT) vs. muscle (SOD/LP). Weak positive correlation is observed for serum (SOD/CAT) vs. serum (SOD/LP), serum (SOD/GPx) vs. serum (SOD/LP), serum (SOD/GPx+CAT) vs. serum (SOD/LP), serum (SOD/GPx) vs. serum (SOD/CAT) and serum (SOD/GPx/CAT) vs. serum (SOD/LP) (Table-2).

(f) *Comparison of the ratios of antioxidant enzymes (SOD, GPx & CAT) levels and the ratios of SOD to LP, GPx to LP and CAT to LP levels in muscle tissue & serum of the patients with muscle diseases and control/healthy individuals*

Ratios of SOD/GPX/CAT, SOD/LP, GPx/LP, CAT/LP in muscle tissue of patients as compared to the control individuals showed no significant difference ($p>0.05$). Similarly, these ratios showed no significant difference in the serum of the patients as compared to the healthy individuals ($p>0.05$). Loverro's coefficient (P/A ratio; LP: SOD+GPx+CAT) [32] is also insignificant for muscle and serum of patients as compared to normal individuals, respectively (Table-3).

Derivation of Linear Equations for Parameters Estimation

Regression analysis was performed for the parameters of antioxidant enzymes and lipid peroxidation in the muscle and serum samples of the patients with muscle diseases. Estimation of the parameters is performed by the linear equations presented in the figure-6 & 7.

Diagnostic possibility of parameters under consideration

DFA analysis was performed with the parameters muscle (SOD), muscle (GPx), muscle (CAT), muscle (SOD/LP) and muscle (LP) for 47 patients and 30 control individuals. This analysis correctly classified the 73% of original grouped cases and 71% of cross-validated grouped cases correctly classified. DFA analysis was also performed with the serum (SOD), serum (GPx), serum (CAT), serum (SOD/LP) and serum (LP) for 47 patients and 30 control individuals. This analysis correctly classified the 78% of original grouped cases and 74.0% of cross-validated grouped cases correctly classified.

Table 1

Comparison of the antioxidant enzymes (SOD, GPx & CAT) and LP levels in muscle tissue & serum of the patients with muscle diseases and control/healthy subjects

Subjects/ Statical analysis	ΔSOD (units/mg) (mean ± SD*)	GPx (units/mg) (mean ± SD)	CAT (units/mg) (mean ± SD)	LP (nmol TBARS/ mg protein) (mean ± SD)
Muscle tissue				
#Muscle disease patients (n = 47)	2.53±4.09	221.29±195.83	326.13±171.49	5.39±5.11
Control subjects (n= 30)	0.82±0.64	81.47± 52.09	192.35± 134.54	3.22±2.73
Independent sample 't' test	P< 0.007, Significant	P< 0.0001, Significant	P< 0.001, Significant	P< 0.035, Significant
Serum				
Subjects/ Statical analysis	SOD (units/mg) (mean ± SD)	GPx (units/mg) (mean ± SD)	CAT (units/mg) (mean ± SD)	LP (nmol TBARS/ mg protein) (mean ± SD)
#Muscle disease patients (n = 47)	1.45 ± 0.78	42.77±19.36	68.50±45.92	9.94±5.16
Control subjects (n =30)	1.08±0.63	30.84±16.96	49.12± 23.15	7.58±2.23
Independent sample 't' test	P< 0.032, Significant	P< 0.007, Significant	P< 0.017, Significant	P< 0.007, Significant

Note: # including the 41 cases of muscular dystrophies and 6 cases of mitochondrial myopathies;* SD; Standard Deviation; Δ SOD; Superoxide dismutase, GPx; Glutathione peroxidase, CAT; Catalase & LP; Lipid peroxidation.

Table 2

Comparison of the antioxidant enzymes (SOD, GPx & CAT) and LP levels in between muscle tissue & serum of the patients with muscle diseases, respectively

Specimens/ Statical analysis	[a]SOD (units/mg) (mean ± SD*)	GPx (units/mg) (mean ± SD)	CAT (units/mg) (mean ± SD)	LP (mmol TBARS/ mg protein) (mean ± SD)
# Muscle disease patients				
Muscle specimens	2.53±4.09	221.29±195.83	326.13±171.49	5.39±5.11
Serum specimens	1.45 ± 0.78	42.77±19.36	68.50±45.92	9.94±5.16
Paired sample 't' test	P< 0.0001, Significant	P< 0.0001, Significant	P< 0.0001, Significant	P< 0.0001, Significant

Table 3

Examine the correlation between the antioxidant enzymes (SOD, GPx & CAT) and LP levels in muscle tissue & serum of the patients with muscle diseases, respectively

Muscle		
Subjects of correlation	Pearson's correlation coefficient	Inferences
Correlation between Muscle SOD & Muscle LP	0.23	Weak (+) correlation
Correlation between Muscle GPx & Muscle LP	0.22	Weak (+) correlation
Correlation between Muscle CAT & Muscle LP	0.30	Weak (+) correlation
Correlation between Muscle SOD & Muscle CAT	0.22	Weak (+) correlation
Correlation between Muscle SOD & Muscle GPx	0.25	Weak (+) correlation
Correlation between Muscle GPx & Muscle CAT	-0.14	No correlation
Correlation between Muscle SOD & Muscle GPx+ Muscle CAT	0.41	Moderate (+) correlation
Serum		
Subject of correlation	Pearson's correlation coefficient	Inferences
Correlation between Serum SOD & Serum LP	-0.08	No correlation
Correlation between Serum GPx & Serum LP	-0.14	No correlation
Correlation between Serum CAT & Serum LP	-0.14	No correlation
Correlation between Serum SOD & Serum CAT	-0.00	No correlation
Correlation between Serum SOD & Serum GPx	-0.01	No correlation
Correlation between Serum GPx & Serum CAT	-0.21	Weak (-) correlation
Correlation between Serum SOD & Serum GPx+ Serum CAT	-0.04	No correlation

Table 4

Comparison of the ratios of antioxidant enzymes (SOD, GPx & CAT) levels and the ratios of SOD to LP, GPx to LP and CAT to LP levels in muscle tissue & serum of the patients with muscle diseases and control/healthy subjects

Subjects/ Statical analysis	¹SOD:GPx:CAT (mean ± SD*)	SOD:LP (mean ± SD)	GPx:LP (mean ± SD)	CAT:LP (mean ± SD)	LP:SOD+GPx+CAT (mean ± SD) (P/A ratio)
Muscle tissue					
#Muscle disease patients (n = 47)	7.21±13.25	0.69±0.93	71.47±90.43	108.36±112.14	0.01±0.01
Control subjects (n= 30)	3.83±8.96	0.68± 1.32	53.34± 49.92	133.17±141.69	0.02±0.03
Nonparametric test (Mann-Whitney U test)	P>0.05, Insignificant	P>0.05, Insignificant	P>0.05, Insignificant	P>0.05, Insignificant	P>0.05, Insignificant
Serum					
Subjects/ Statical analysis	¹SOD:GPx:CAT (mean ± SD*)	SOD:LP (mean ± SD)	GPx:LP (mean ± SD)	CAT:LP (mean ± SD)	LP/SOD+GPx+CAT (mean ± SD) (P/A ratio)
#Muscle disease patients (n = 47)	2.97 ± 3.67	0.20±0.18	6.16±5.28	9.67±9.06	0.12±0.09
Control subjects (n =30)	2.71±4.72	0.16±0.11	4.61± 3.01	7.23±4.41	0.11±0.06
Nonparametric test (Mann-Whitney U test)	P>0.05, Insignificant	P>0.05, Insignificant	P>0.05, Insignificant	P>0.05, Insignificant	P>0.05, Insignificant

Note: P = prooxidant & A = antioxidant; LP/ SOD+GPx+CAT is also known as prooxidant/antioxidant ratio or Loverro ratio.

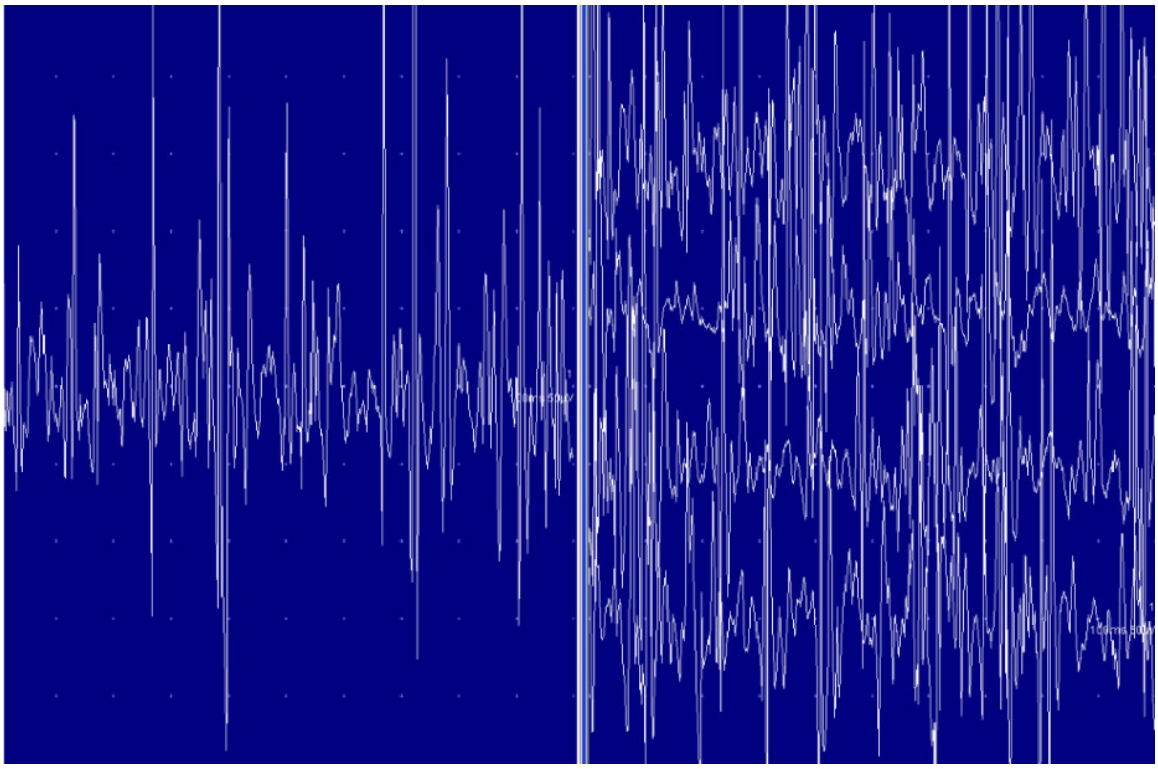

Figure 1

Electromyography measurement showed the myopathic pattern in the patients with muscle disease (Pattern in the form of almost complete interference pattern and low amplitude and short duration).

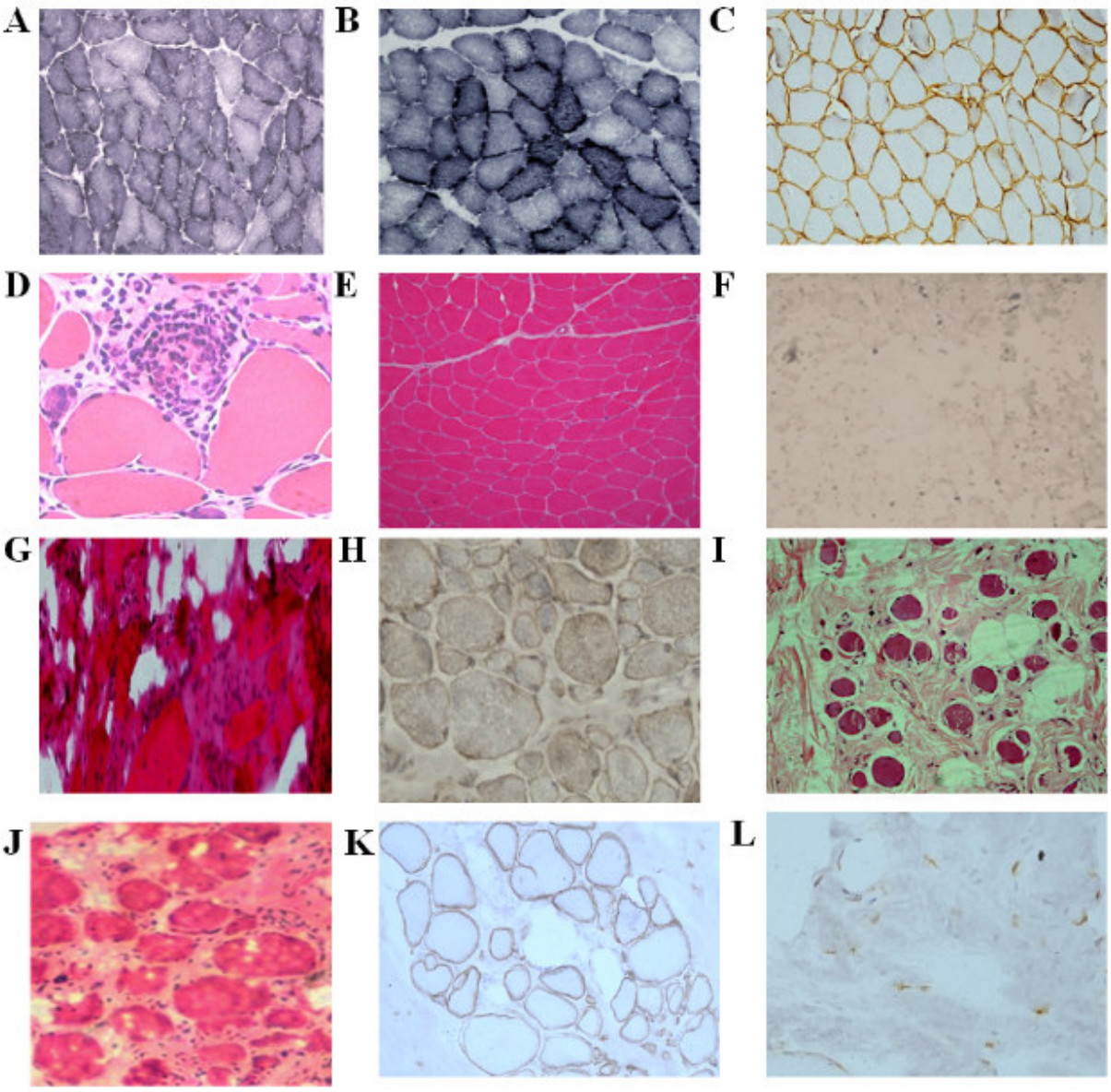

Figure 2

Histopathological and immuno histopathological examination of muscle samples confirmation the (A) mitochondrial myopathy (NADH-TR staining), (B) mitochondrial myopathy (Succinic dehydrogenase staining), (C) FSHD (Beta staining), (D) FSHD (H & E staining), (E) LGMD-2B (H & E stain), (F) LGMD-2B (negative dysferlin staining), (G) LGMD-2A (H & E stain), (H) LGMD-2A (showing partial reduction of dysferlin, on Immunoblotting, there was complete loss of calpain, but dysferlin was normal), (I) DMD (H & E stain), (J) BMD (H & E stain), (K) BMD (reduce and discontinuous dystrophin staining), (L) DMD (complete loss of dystrophin in dystrophin staining).

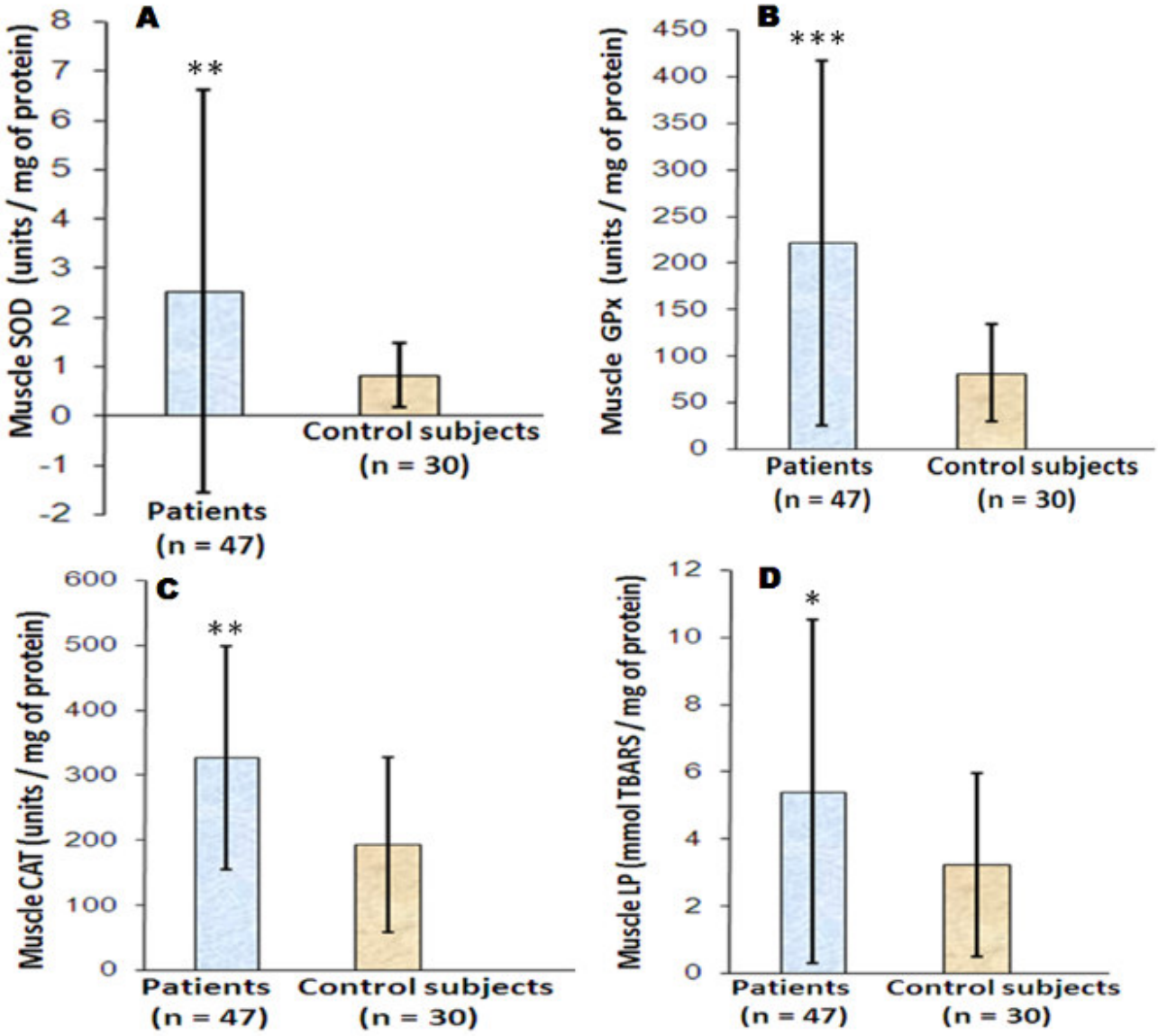

Figure 3

Comparison of (A) SOD, (B) GPx, (C) CAT and (D) LP in muscle samples of patients with muscle diseases (N=47) and control/ healthy individuals (N=30) [values are expressed as mean ± SD of unit/mg of protein for SOD, GPx and CAT but mmol TBARS/mg of protein for LP. P values: *< 0.05, **< 0.01 and ***< 0.0001].

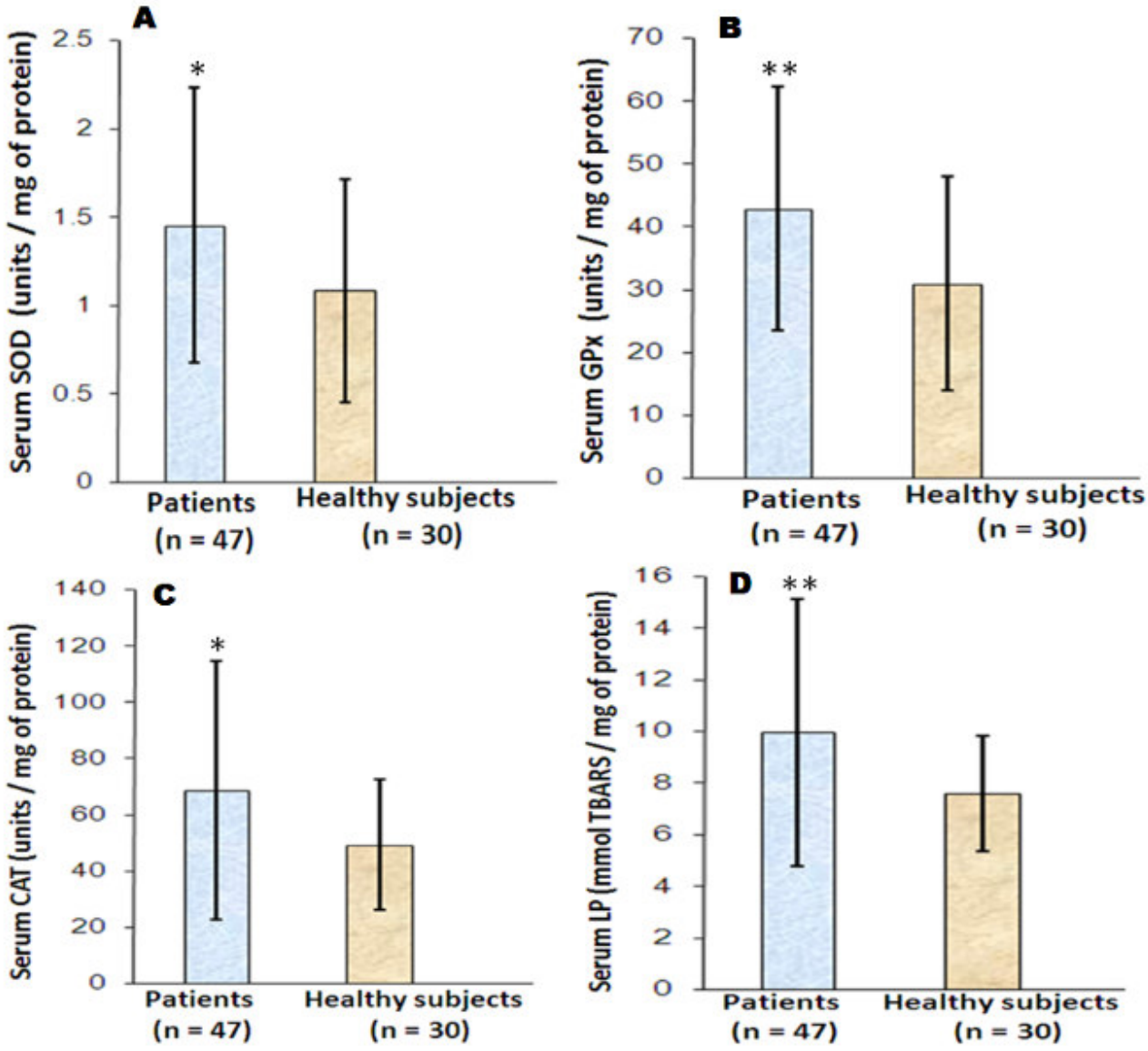

Figure 4

Comparison of (A) SOD, (B) GPx, (C) CAT and (D) LP in serum samples of patients with muscle diseases (N=47) and control/ healthy individuals (N=30) [values are expressed as mean ± SD of unit/mg of protein for SOD, GPx and CAT but mmol TBARS/mg of protein for LP. P values: *< 0.05 and **< 0.01].

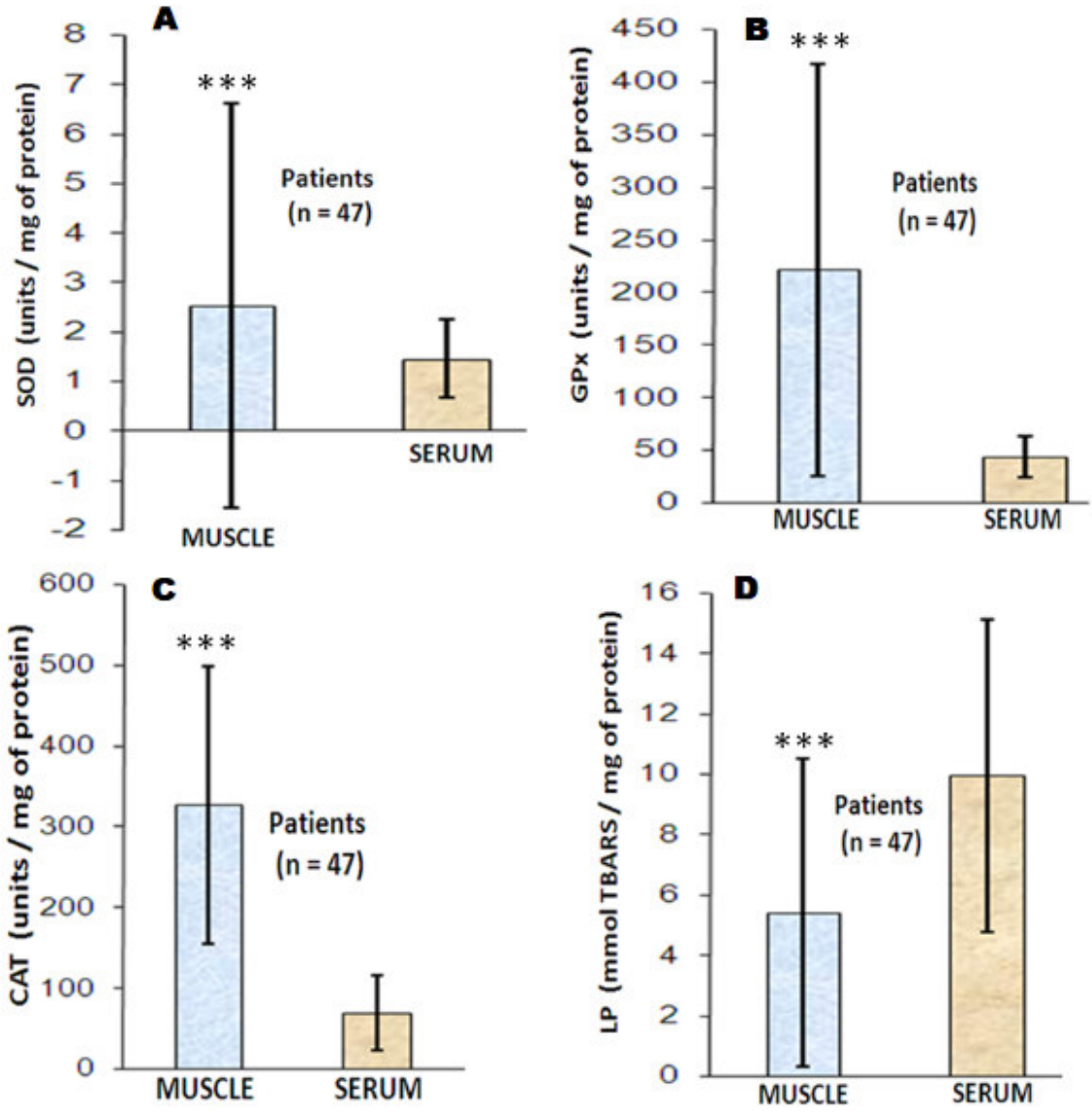

Figure 5

Comparison of (A) SOD, (B) GPx, (C) CAT and (D) LP in muscle and serum samples of patients with muscle diseases (N=47) [values are expressed as mean ± SD of unit/mg of protein for SOD, GPx and CAT but mmol TBARS/mg of protein for LP. P values: ***< 0.0001].

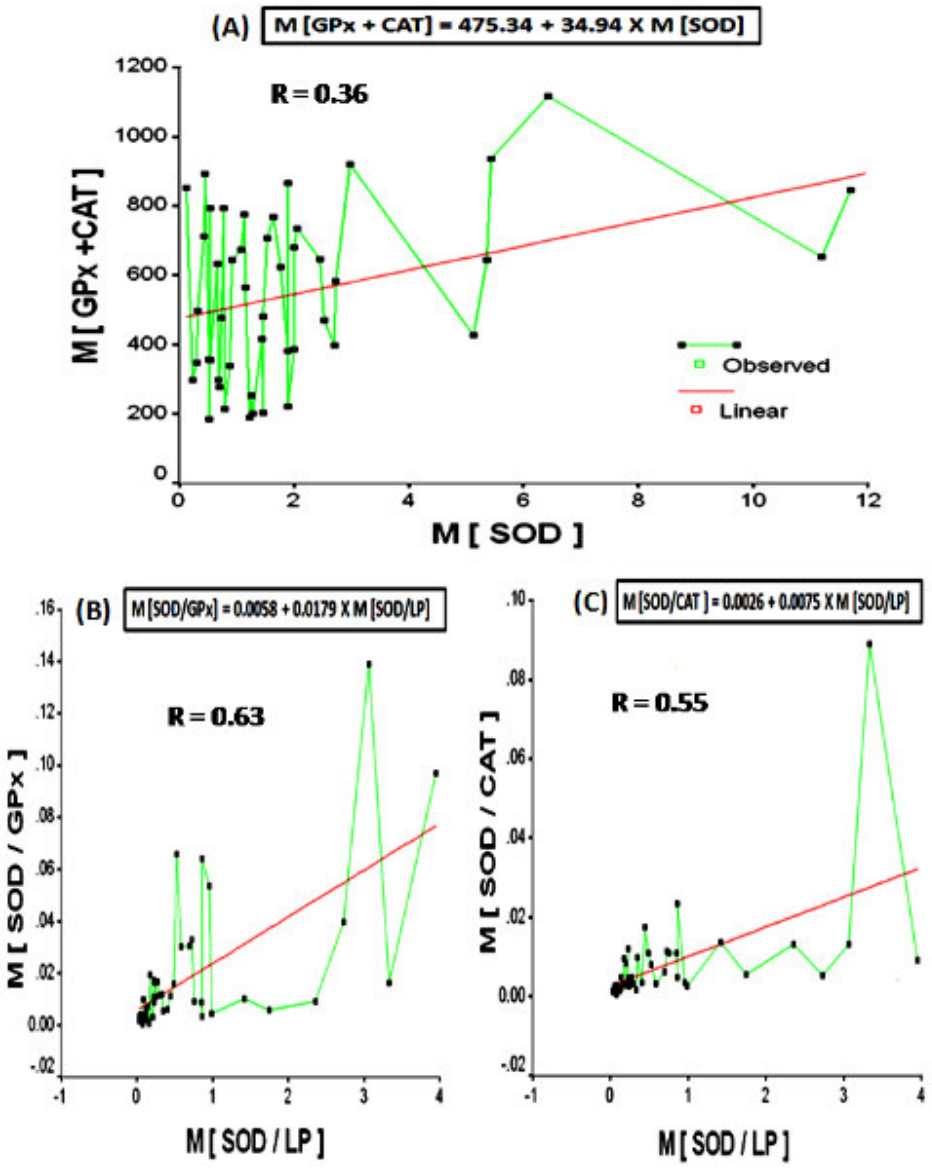

Figure 6

Regression analysis derived the linear equations (A) M [GPx + CAT] = 475.34 + 34.94 X M [SOD], (B) M [SOD / GPx] = 0.0058+ 0.0179 X M [SOD/LP] and (C) M [SOD/CAT] = 0.0026 + 0.0075 X M [SOD/LP] (M = muscle).

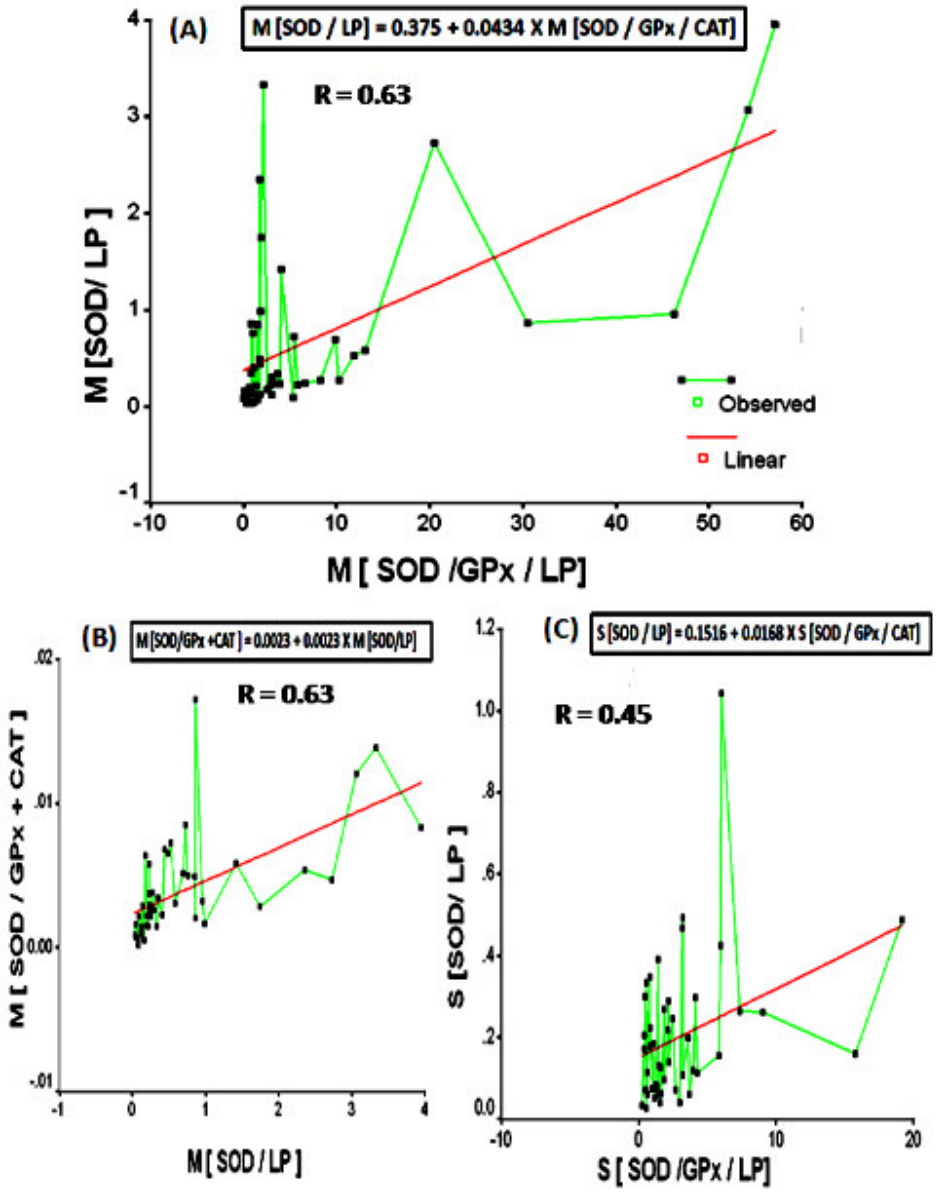

Figure 7

Regression analysis derived the linear equations (A) M [SOD/LP] = 0.375 + 0.0434 X M [SOD/GPx/CAT], (B) M [SOD/ GPx+CAT] = 0.0023 + 0.0023 X M [SOD/LP] and (C) S [SOD/LP] = 0.1516 + 0.0168 X S [SOD/GPx/CAT] (M = muscle; S = serum).

DISCUSSION

Present study contributed to the complete and systematic analysis of LP and antioxidant enzymes (SOD, CAT & GPx) in patients with major types and unclassified forms of muscular dystrophies as well as in mitochondrial myopathies. Similar pattern of enhanced lipid peroxidation and level of antioxidant enzymes was observed in serum and muscle samples of the patients with muscular dystrophies and mitochondrial myopathies as compared to normal/control individuals. Here, SOD, GPx and CAT are considered as defensive parameters because these enzymes are acting against the oxidative stress and having the protective function.[5-9] LP is considered as indicative parameter because free radicals induced oxidative stress first oxidized the lipid molecule of the membrane. [5, 15, 17] Evaluated defensive

parameters (SOD, GPx & CAT) and indicative parameter (LP) are collectively considered as oxidative stress factors and represented the oxidative stress status of the patients. The first part of results for increased LP and levels of SOD, GPx & CAT in muscle samples of the patients with muscular dystrophies and mitochondrial myopathies as compared to normal/control individuals are supported by various studies. Kar et al (1979) also reported about the enhanced LP and increased level of SOD, CAT & GPx in the muscle samples of the DMD patients.[17] Levels of CAT, SOD and GPx increased in muscle specimens of mdx (pre-necrotic), mdx (4-5 mo) and dy/dy mouse model of muscular dystrophy.[18-20] Higher level of GPx has been reported in muscle of DMD and BMD.[22] SOD was found to increase in complex I (Mitochondrial NADH Dehydrogenase) deficient patients.[36] Increased levels of SOD, GPx and CAT have also been observed in ragged-red fibers of skeletal muscle from patients with mitochondrial encephalomyopathies.[37]In all these studies and from the results obtained, it is evident that the membrane integrity in muscle diseases (muscular dystrophies/mitochondrial myopathies) decreases and becomes weak. Muscle membrane behaves abnormally in the muscular dystrophies [17-18] as well as in mitochondrial myopathies. Numerous evidences suggest that oxidative stress induced damage to membrane may contribute to the loss of membrane integrity in muscular dystrophies[38] as well as in mitochondrial myopathies. There are similarities between the pathologic changes that occur in muscular dystrophies and changes that occur in muscle under different conditions of oxidative stress such as ischemia, exhaustive exercise, and vitamin E deficiency.[18, 38-40]Various reports have documented biochemical changes in dystrophic muscle that are characteristic of oxidative damage.[40-43] Oxidation induced muscle damage is also the major feature of mitochondrial myopathies because the results obtained revealed, that the pattern of antioxidant enzymes and lipid peroxidation is similar in the muscular dystrophies and mitochondrial myopathies. Oxidative stress induced damage, of dystrophin deficient muscle cells is specifically more sensitive as compared to normal muscle cells.[5, 18] Enhanced oxidative stress in muscle of mouse with muscular dystrophy happens due to genetic alterations of free radical metabolism. [5]Now, it is very clear that the oxidative stress is responsible for weakening of integrity and abnormal behaviour of muscle membrane. Abnormal membranes are more susceptible to oxidative stress induced lipid peroxidation and thus lipid peroxides levels are very high in the muscle of the patients with muscular dystrophies, as well as in mitochondrial myopathies. These lipid peroxides can suddenly disrupt the lysosomal membranes and thereby release the hydrolytic enzymes in the cytoplasm, which are further responsible for damage of muscle cells in muscular dystrophies[15, 17]and mitochondrial myopathies. SOD which catalyzes the dismutation of the super-oxide anion radicals is found in all aerobic cells and is believed to exert an important protective function against the toxicity of oxygen.[17] Oxidative stress induced over expression of SOD enzyme, in murine muscular dystrophy, which is further responsible for activity enhancement.[44] Over expression of SOD, GPx and CAT are also observed in the ragged-red fibers of skeletal muscle from patients with mitochondrial encephalomyopathies.[37]SOD enzyme performs the elimination of superoxide ions through its conversion into hydrogen peroxide. Hydrogen peroxide is eliminated by GPx and CAT.[45-46] Normally the GPx and catalase activities are sufficient to remove the hydrogen peroxide,[47] but enhanced ROS production induces the amplification of gene expression of GPx and CAT, respectively.[48-49] These events are responsible for enhancing the activities of GPx and CAT in dystrophic muscle. The second part of results for increased lipid peroxidation and levels of SOD, GPx & CAT in serum of the patients with muscular dystrophies and mitochondrial myopathies as compared to normal/control individuals are also supported by various studies. Elevated level of SOD, CAT and LP were observed in the red blood cells (RBC) of DMD patients.[50]Significant elevation of serum SOD, GPx and LP was found in

patients with myotonic dystrophy type-1 (DM-1).[51] Enhanced lipid peroxidation and CAT level are also observed in the plasma of broiler chickens with Muscular Dystrophy. [52] Increased level of lipid peroxidation is also observed in the serum of DMD patients as compared to controls.[53]All these supported studies and obtained results clearly raised the point of elevated level of antioxidant enzymes and increased lipid peroxidation rate in serum/plasma of patients with muscular dystrophies as well as in mitochondrial myopathies. Similar pattern of antioxidant enzymes and lipid peroxidation in serum/plasma is also observed in muscular dystrophies and in mitochondrial myopathies, and in this regard, the explanation of their cause is same. Non-plasma/ serum-specific enzymes are intracellular enzymes normally present in plasma at minimal levels or at concentrations well below those in tissue or cells. Their presence in plasma/ serum is normally due to turnover of tissue or cells, but they are released into the body fluids in excessive concentrations as a result of cellular damage or abnormality of membrane functions. Membrane permeability changes and cell destruction affects the release of intracellular enzymes.[54-56] Here due to abnormality of muscle membrane in muscular dystrophies and mitochondrial myopathies, the antioxidant enzymes are leaked into the blood or plasma/serum. Muscle cell or tissue destruction is also responsible for excessive concentrations of antioxidant enzymes in plasma/ serum.[56-57] Such events are responsible for the elevated levels of antioxidant enzymes in serum of the patients with muscular dystrophies and mitochondrial myopathies. Oxidative-stress induced LP is also higher in the muscle tissue[15, 17] of the patients with muscular dystrophies as well as in mitochondrial myopathies,[58-59]which further releases the lipid peroxide products into plasma/ serum and in this regard, the LP value in plasma/ serum goes up. The third part of results showed the comparison of the levels of LP, SOD, GPx and CAT in between the serum and muscle specimen of the patients with muscular dystrophies and mitochondrial myopathies. Significantly higher level of SOD, GPx and CAT was found in muscle as compared to serum. Oxidative stress is responsible for the elevated levels of LP and SOD, GPx & CAT in muscle. According to established facts, the non- plasma specific enzymes are present in plasma because of normal turnover of tissues, leakage through cell membranes, tissue necrosis and increased enzyme synthesis. Removal of these non-plasma specific enzymes is depending on the factors such as intravascular inactivation (dilution, lack of substrates and coenzymes, inhibitors and proteinases etc.), uptake by tissue with subsequent inactivation and removal by the reticuloendothelial system.[53, 60] In this regard, the alterations of the level of enzymes in tissue are not reflected in the similar way in blood. This is the major cause of this event is responsible for higher levels of antioxidant enzymes in muscle as compared to serum of the patients with muscular dystrophies and mitochondrial myopathies. LP was significantly reduced in the muscle as compared to serum. The lipid peroxide products i.e. malondialdehyde (MDA) is released from muscle to blood through leakage, necrosis and degeneration.[56-57] Malondialdehyde is a stable and degraded product of lipids, and not easily removed from the blood.[62-63] In this regard, higher concentration of malondialdehyde in blood is responsible for the higher the value of LP in serum as compared to muscle of the patients with muscular dystrophies and mitochondrial myopathies. The fourth part of results showed the correlation analysis. No correlation was found for levels of LP, SOD, GPx & CAT in muscle and serum samples of the patients with muscular dystrophies and mitochondrial myopathies, due to the entry and exit factors of the antioxidant enzymes & lipid peroxidation in blood.[60] Weak positive correlation was found in between the muscle antioxidant enzymes & lipid peroxidation because oxidative stress induces the lipid peroxidation first and further lipid peroxides induces the elevation of antioxidant enzymes.[63-64] The fifth part of results showed the correlation of the ratios of individual antioxidant enzymes to lipid peroxidation in muscle as well as in serum specimen of the patients with

muscular dystrophies and mitochondrial myopathies. Strong positive correlation is observed for the ratios of muscle SOD/CAT, SOD/GPx and SOD/GPx+CAT to the muscle SOD/LP because of the weak positive correlation of the antioxidant enzymes to the lipid peroxidation. This is, one of the results, of the presented study. Weak positive correlation is observed, for all these parameters in serum clearly stating that the disturbances in the muscle, is also fully reflected in the blood. The sixth part of results showed the regression analysis. This is based on the fourth & fifth parts of results and established the linear equations. These equations are helpful for the calculation of many other parameters, by introducing one parameter value. In this regard, only lipid peroxidation and any one value of the antioxidant enzymes assay are sufficient to obtain the values of others. There is no need to perform the assays of all the antioxidant enzymes, which is time consuming and not feasible in clinical set up. The seventh part of results showed the comparison of the ratios of individual antioxidant enzymes, to lipid peroxidation & their mutual ratios in muscle as well as in serum specimen of the patients with muscular dystrophies and mitochondrial myopathies, as compared to control/ healthy individuals. No significant difference is observed for all these ratios in muscle and serum specimens which shows the characteristics feature that in muscle diseases, the levels of lipid peroxidation and antioxidant enzymes are altered but the proportion of ratios of the altered levels of enzymes are always constant. Oxidative stress induced damage depends on the generation of free radicals.[6-10] These free radicals perform the lipid peroxidation of the membrane. Lipid peroxide products again stimulate the expression of antioxidant enzymes (SOD, GPx & CAT).[64-73] In this regard, levels of antioxidant enzymes is approximately proportionate to the lipid peroxidation. Such type of proportionality is responsible for the constant values of the ratios of antioxidant enzymes to the lipid peroxidation. Mutual ratios of antioxidant enzymes are also constant because the constant values of their ratios to lipid peroxidation as well as their association in the removal of free radicals. The eight parts of results shows the Discriminant Function Analysis (DFA) and stated the diagnostic possibility of the antioxidant enzymes and lipid peroxidation in muscle and serum specimens of the patients with muscular dystrophies and mitochondrial myopathies. In serum, the diagnostic differentiation is more prominent as compared to the diagnostic differentiation in muscle because LP value is higher in serum as compared to muscle in patients. There are various reports about the diagnostic use of antioxidant enzymes in the diseases.[73-74] Such reports showed and provided the track for possible diagnostic use of antioxidant enzymes in the case of patients with muscular dystrophies and mitochondrial myopathies.

CONCLUSION

Evaluated defensive parameters (SOD, GPx & CAT) and indicative parameter (LP) are collectively considered as oxidative stress factors and represented the oxidative stress status of the patients. Antioxidant enzymes and lipid peroxidation are collectively considered as oxidative stress factors and they present the oxidative-stress status of the patients. Alteration in the levels of antioxidant enzymes and lipid peroxidation follow the uniform pattern in muscular dystrophies and mitochondrial myopathies. This uniformity is same in muscle as well as in serum samples. Lipid peroxidation is higher in the serum as compared to the muscle of the patients. The proportionate ratio of all these factors is constant in disease state. This is the characteristics of the muscular dystrophies and mitochondrial myopathies. Linear equations are helpful for the calculation of the values of parameters only performing the assays of any two enzymes. These factors also provide the possibility of development of alternate diagnostic methods. On the basis of such information, clinicians would be able to manage the patients with appropriate addition of antioxidants in the medication or diet. There is no permanent treatment for patients with hereditary muscle diseases and management of disease is an option for delaying the degenerative process.

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ABBREVIATIONS

SOD: Superoxide dismutase; GPx: Glutathione peroxidase; CAT: Catalase; LP: Lipid peroxidation; DMD: Duchenne Muscular Dystrophy; BMD: Becker Muscular Dystrophy; LGMD: Limb Girdle Muscular Dystrophy; FSHD: Facioscapulohumeral dystrophy; ROS: Reactive oxygen species; TBRAS: Thiobarbituric acid-reactive substance.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Niraj Kumar Srivastava, Achal Kumar Srivastava, Somanath Mukherjee and Rohan Sharma performed all the experimental works and data collection. All the authors contributed in preparing the manuscript. Ashok Kumar Mahapatra and Deepak Sharma have done overall supervision. All authors read and approved the final manuscript.

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