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Reference Interval of Muscle Damage Indices and Cortisol in Young Athletes of Various Sports Discipline

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Abstract: Creatine kinase (CK), lactate dehydrogenase (LDH) and cortisol are widely accepted as biological markers. The purpose of the study was to frame the reference interval for muscle damage indices (CK, LDH) and cortisol in the young athletic population of various sports disciplines. 260 young male players [i.e., football (n=62), hockey (n=60), gymnastics (n=36), swimming (n=28), table tennis (n=25), sprint-jump-throw (n=36) and middle-long distance running (n=13)] were recruited for the study (mean age = 15.6±1.59 yrs). Assay of LDH, CK and cortisol was done using the standard enzymatic protocol. The reference interval was calculated by following the Clinical and Laboratory Standard Institute (CLSI) C28-A3 guideline and "MedCalc" software (version 19) with a 90% confidence interval. Serum LDH range was from 148.00-324.00 IU/L with a mean of 233.2±34.74 and a median around 236.25. Serum CK ranged from 17.00-43.50 IU/L with a mean of 28.93±5.23 IU/L and a median around 28.00. Cortisol ranged from 4.99-15.78 µg/dl with a mean of 9.31±2.09 µg/dl and a median around 8.90. The present study confers 165.63 - 303.43 IU/L, 19.00 – 40.09 IU/L and 6.07-14.15 µg/dl as the reference interval values for LDH, CK and cortisol, respectively. The present finding will guide the researchers to avoid misinterpretation of muscle damage indices values during any phase of competitive training of sports person.

Keywords: Reference Interval, Creatine Kinase, Lactate Dehydrogenase, Cortisol, Sports Discipline.

About the Authors

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Dr. Gouriprosad Datta has obtained both B.Sc and M.Sc in Physiology and Human Physiology, respectively, from University of Calcutta. He has also completed his Ph.D. from the University of Calcutta. He has been actively engaged in both under-graduate and post-graduate teaching in the Department of Physiology, Rammohan College, since 1983 and 2006, respectively. He has served as HOD at the Department of Physiology, Rammohan College, India and is presently the Course Coordinator for postgraduate courses. Dr. Datta is also interested in research involving sports & exercise physiology and oxidative stress arising in athletes from eastern India due to over-training and the role of antioxidant vitamin supplementation and diet counselling in preventing oxidative damage. He was also involved in the prevalence of lifestyle induced non-communicable diseases in society, the risk factors associated with them and the role of nutraceuticals in preventing these diseases.

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1. Introduction

Exercise-induced muscle fibre damage following a high-intensity training session was (i.e., eccentric or reaped works) hypothesized to be metabolic, mechanical, or both in nature and that leads to leakage of intracellular muscle components into the blood due to the disruption in the sarcomere and rupture of sarcolemma [1,2]. Creatine kinase (CK), lactate dehydrogenase (LDH), aspartate aminotransferase (AST) and myoglobin are identified as potential markers of the functional status of skeletal muscle and an increase in their serum concentration serves as an indicator of muscle damage [3,4]. Bessa et al. [5] identified CK and LDH as the muscle cell damage markers to serve as an index of both intense training adaptations and overexertion of an athlete. On the other hand, Singh, Soodan, and Kumar [6] have reported an elevated stress marker (i.e., cortisol) level after an intense physical exercise bout, which correlates with increased serum CK level. LDH and CK were found to be elevated significantly even after 24 hours of various resistance and aerobic exercises [7]. The elevation in enzymatic level may be due to the membrane damage of muscle fibers, disorganization of the myofibrillar structure and a disruption of the Z line, extracellular matrix, basal lamina and sarcolemma [8].

Reference interval (RI) is a range of values for a specific parameter that is deemed to be normal or within the physiological limit for that particular population on that particular condition [9]. Preventing misinterpretation of any biochemical data can only be possible by RI studies [10]. Interval values can be altered as per the population variation. Sports individuals, healthy sedentary persons, and patients may have different reference intervals for a given parameter in resting conditions. Brancaccio et al. [3] have reported an enzymatic alteration following the adaptations to physical training, including the training volume and intensity. Standardized RI will help assess the effect of training through blood biomarkers that must be previously established in a specified population group [10].

A few studies predicted RI values for LDH in healthy populations [11,12]. But for an athletic population, the study number was even less and was reported for CK [13,14] and LDH [15]. Studies like Mahmutyazicioglu et al. [13] have differentiated the reference values for the normal population from the athletic population. There is a huge gap among the athletic population in terms of the reference range of values for important variables like CK, LDH and cortisol, which can give insight into muscle damage and stress responses against exercising/ training. So, the present study aimed to frame the reference interval for muscle damage indices (i.e., CK, LDH) and stress markers (i.e., cortisol) in the young athletic population of various sports disciplines.

2. Materials and Methods

2.1 Subjects

260 young male state level athletes (mean age = 18.3±2.01 yrs) of 7 different sports discipline i.e., football (FB, n=62), hockey (HOC, n=60), gymnastics (GYM, n=36), swimming (SWIM, n=28), table tennis (TT, n=25), sprint-jump-throw (SJT, n=36) and middle-long distance running (ML, n=13) were recruited as subjects for the present cross-sectional study. The sample size has been calculated using G*Power software (version 3.1.9.7) over the statistical test of one-way ANOVA. Where effect size f=0.25(medium), α error probability=0.05, Power (1-β error probability) = 0.8, number of groups=7 and critical F=2.19991 were used to complete the sample size calculation. Therefore, the calculated total sample size will be 231 subjects. However, considering 10% dropouts, minimum of 254 subjects need to be recruited. However, to be safe sided, 260 subjects will be recruited in the present investigation [14,15]. ML group includes the particular events of 100m, 400m, 8000m and 1500m race. All participants had a minimum of 4 years of formal training history and had played at least state-level competition. Players were considered to be homogeneous in terms of the similar socio-economic group, dietary habits and identical environmental conditions during training sessions. Subjects were clinically examined before commencing the study and only the fit players were chosen as the study subjects. During the accomplishment of the present study, all the players were in a pre-competitive training phase. The training regimen of the players includes practice interval of 2-3 hours/ day, excluding Sundays, which comes around 30 hours in a week. Daily practice was equally divided into morning and evening sessions which comprised of physical training for 1hour and skill training for about 2 hours. The ethical guidelines of Helsinki's Declaration (1975) were maintained throughout the study protocol and written informed consent was also obtained from each subject. Proper ethical clearance (Ref No. IHEC/AB/P82/2019) was obtained from the Institutional Human Ethical Committee (IHEC), Department of Physiology, University of Calcutta.

2.2 Biochemical Analysis

2.2.1 Process of Blood Collection and Plasma Sample Preparation

Venous blood samples were collected from the antecubital vein into centrifuge tubes for serum preparation (without anticoagulant) between 6:00 AM – 8:00 AM in the pre-prandial state (after 8-10 hrs of fasting) to avoid possible differences due to diurnal variation. Each blood supernatant was centrifuged at 3000 rpm for 15 minutes to ensure complete serum separation. The samples were then transferred into cryo-vials and stored and preserved at –20ºC for later biochemical analyses [16]. All laboratory tests were performed at a room temperature varying from 23º C to 25º C with 50-60% relative humidity.

2.2.2 Determination of Creatine Kinase (CK)

Creatine kinase (CK) helps turn phosphocreatine into creatine and subsequently forms ATP. CK assay was based on the dephosphorylation of creatine phosphate, the liberated ATP linked to the glucose/hexokinase/glucose-6-phosphate dehydrogenase reactions. N-acetyl cysteine was used to reactivate the enzyme, which was rapidly inactivated due to the oxidation of the sulphydryl groups in the enzyme's active site. The rate of NADPH formation is proportional to the catalytic concentration of CK in the blood sample and was measured photo-metrically at 340 nm [17].

2.2.3 Determination of Lactate Dehydrogenase (LDH)

This method was based on converting pyruvate to lactate at a temperature of 37°C and a total reaction time of 120s. A measured amount of threefold diluted serum was added to the strip, which contained pyruvate and NADH. LDH activity was measured using reflectance spectroscopy by the rate of disappearance of NADH at 340 nm [18].

LDH activity (U/I) = (change in OD/min) X 8095

2.2.4 Determination of Cortisol

10% of total cortisol is the unbound or free plasma cortisol which is mainly the active form to react and the rest is the bounded cortisol to corticosteroid-binding globulin (CBG) and albumin. Plasma cortisol was measured by a commercial immunoassay (Modular Analytics E170, Roche Diagnostics, Mannheim, Germany) and the analytical sensitivity was 8.5 nmol/L. The intra-assay and inter-assay coefficient of variation for cortisol were 1.7% (at 129 nmol/L), 4.7% (at 102 nmol/L) and 2% (at 940 nmol/L) respectively [19]. Plasma free cortisol was calculated by using Coolens' equation [20].

2.3 Statistical Analysis

Statistical analysis was done using Statistical Program for Social Sciences (SPSS) version 18.0 for Windows (SPSS Inc., Chicago, IL, USA). All values were expressed as means ± standard deviation (SD). ANOVA was separately performed along with Bonferroni's post hoc test to assess the group-specific differences among CK, LDH and cortisol. Descriptive statistics were also done which included the mean, median, mode, standard deviation, standard error of the mean, range, percentile, etc. One sample Kolmogorov Smirnov test and histogram were performed to check the frequency distribution of the data set. The approved guideline of the Clinical and Laboratory Standard Institute (CLSI, C28-A3) and the International Federation of Clinical Chemistry (IFCC) was followed to frame the reference interval at a 95% confidence interval (CI). "G*Power" software (version 3.1.9.7) was used for sample size calculation over the one-way fixed effects ANOVA statistical test at 95% CI. "MedCalc" software (version 19, MedCalc Software bvba) was used to calculate the reference intervals in the present study [21-23].

3. Results

Table 1 depicts the age, body height and body mass of young male athletes from various sports disciplines. Height and body mass were significantly ($p<0.05$) higher among the middle-long distance runners when compared to others. Whereas mean age was found to be altered among the groups in a statistically insignificant manner.

Table 2 shows the mean values of muscle damage indices (i.e., CK, LDH and cortisol) in young male athletes of different sports disciplines.

Table 1. Mean and standard deviation of age, body height and body mass of young male athletes of various sports discipline

Sports discipline	Age (yrs)	Body height (cm)	Body weight (kg)
Football (n=62)	15.6±2.09	167.1±6.66	55.7±6.81
Hockey (n=60)	15.8±1.90	169.0±6.21	59.5±6.81
Gymnastics (n=36)	16.2±2.61	164.1±8.67	55.8±7.51
Swimming (n=28)	15.6±2.07	166.6±6.90	55.9±7.90
Table Tennis (n=25)	15.6±1.46	164.9±7.82	55.5±9.02
Sprint-jump-throw (n=36)	16.5±1.62	167.3±10.91	58.8±11.35
Middle-long (n=13)	16.3±1.70	170.4±5.49	61.2±6.56
F value	1.295(NS)	2.309*	2.491*
P value	P=0.260	P=0.035	P=0.023
Bonferroni's post hoc	-	ML vs GYM, TT	ML vs FB, GYM, SWIM, TT

Values are expressed as mean ± SD, *= $p<0.05$, NS-not significant, ML= middle long-distance running, FB= football, GYM= gymnastics, SWIM= swimming, TT= table tennis.

Table 2. Mean and standard deviation of CK, LDH and cortisol level of young male athletes of various sports discipline

Sports discipline	CK (IU/L)	LDH (IU/L)	Cortisol (µg/dl)
Football (n=62)	30.8±4.99	239.1±29.15	9.5±1.98
Hockey (n=60)	29.3±5.08	232.2±39.99	9.3±1.99
Gymnastics (n=36)	27.7±4.94	228.1±31.83	9.0±2.28
Swimming (n=28)	28.5±4.23	227.8±46.03	9.1±2.34
Table Tennis (n=25)	26.4±4.61	226.5±31.73	9.0±1.81
Sprint-jump-throw (n=36)	30.1±5.97	245.2±25.21	9.8±2.25
Middle-long (n=13)	24.5±4.12	214.7±32.27	9.1±2.11
F value	4.914***	2.084(NS)	0.603(NS)
P value	P<0.001	P=0.056	P=0.728
Bonferroni's post hoc	ML vs FB, HOC, SJT	-	-

Values are expressed as mean ± SD, ***= $p<0.001$, NS-not significant, CK= creatine kinase, LDH= lactate dehydrogenase, ML= middle long-distance running, FB= football, HOC= hockey, SJT= sprint-jump-throw.

CK value was significantly ($p<0.001$) lowest in the middle-long group and highest in the sprint-jump-throw and football groups. Whereas LDH and cortisol were found to differ among the groups insignificantly with lowest value in middle-long and gymnastic, table tennis respectively and highest value in sprint-jump-throw for both variables.

Table 3 represents the descriptive statistics of muscle damage indices (i.e., CK, LDH and cortisol) for combined young male athletes of different sports disciplines. Where percentiles were only done at 3 points 25%, 50% (median value) and 75%.

Table 4 depicts the reference interval for CK, LDH and cortisol in the combined group (n=260) and individual game-specific population.

Reference intervals were calculated with a 90% confidence interval limit for all the sports disciplines. The reference interval of the total athletic population/combined group was calculated by using the non-parametric percentile method and individual sports-specific groups by using the robust method (CLSI guideline, C28-A3). Reed's method was applied for the outlier measurement, but no outlier was found in the following three tables.

Following figures [1(a), 2(a), 3(a)] represent the histogram of CK, LDH and cortisol respectively. On the other hand, figures [1(b), 2(b), 3(b)] show the Box-and-Whisker plot of CK, LDH and cortisol respectively for the median, lower quartile, upper quartile, lower extreme and upper extreme values.

Table 3. Descriptive statistics of CK, LDH and cortisol for combined group of young male athletes

		CK (n=260)	LDH (n=260)	Cortisol (n=260)
Mean		28.93	233.17	9.31
Median		28.00	236.25	8.90
Mode		26.00	231.00	7.68
Std. Deviation		5.23	34.74	2.09
Std. Error of Mean		0.32414	2.15421	0.12941
Variance		27.317	1206.564	4.354
Range		26.50	176.00	10.79
Minimum		17.00	148.00	4.99
Maximum		43.50	324.00	15.78
Percentiles	25%	26.00	208.00	7.82
	50%	28.00	236.25	8.90
	75%	32.00	255.75	10.48

CK= creatine kinase, LDH= lactate dehydrogenase, Std. Deviation= standard deviation, Std. Error of Mean= standard error of mean.

Table 4. Reference interval for CK, LDH and cortisol of young male athletes of various sports discipline

Sports discipline	Reference Interval		
	CK (IU/L)	LDH (IU/L)	Cortisol (µg/dl)
Combine (n=260)	19.00-40.09	165.63-303.43	6.07-14.15
Football	20.14-40.66	180.94-298.62	5.21-13.20
Hockey	17.56-38.64	148.52-310.61	4.92-13.00
Gymnastics	17.80-38.43	162.32-294.63	3.69-13.45
Swimming	19.65-37.45	130.80-326.45	3.79-14.25
Table tennis	16.71-36.31	159.88-294.65	5.05-12.72
Sprint-jump-throw	17.19-41.94	193.89-297.78	4.90-14.32
Middle-long	14.59-34.59	135.08-292.07	3.93-14.09

CK= creatine kinase, LDH= lactate dehydrogenase.

Figure 1: Histogram (a) and Box-and-Whisker plot (b) of creatine kinase (CK):

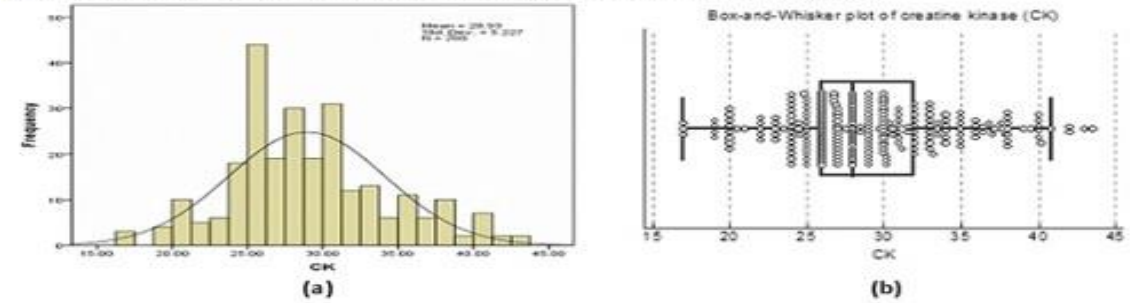

Figure 2: Histogram (a) and Box-and-Whisker plot (b) of lactate dehydrogenase (LDH):

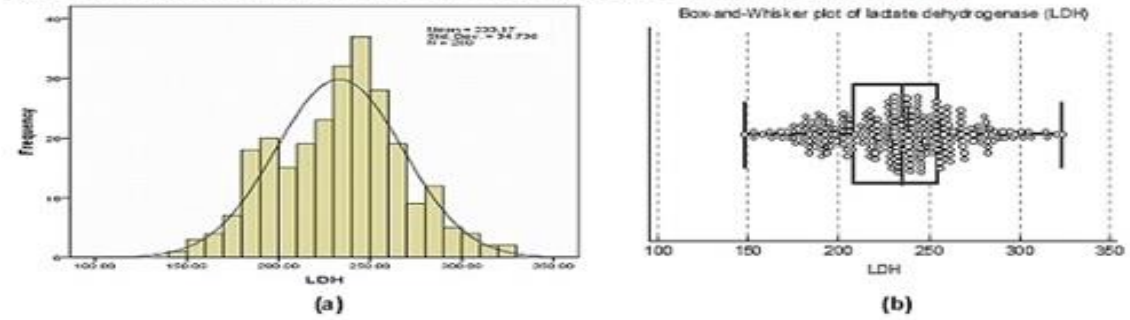

Figure 3: Histogram (a) and Box-and-Whisker plot (b) of cortisol:

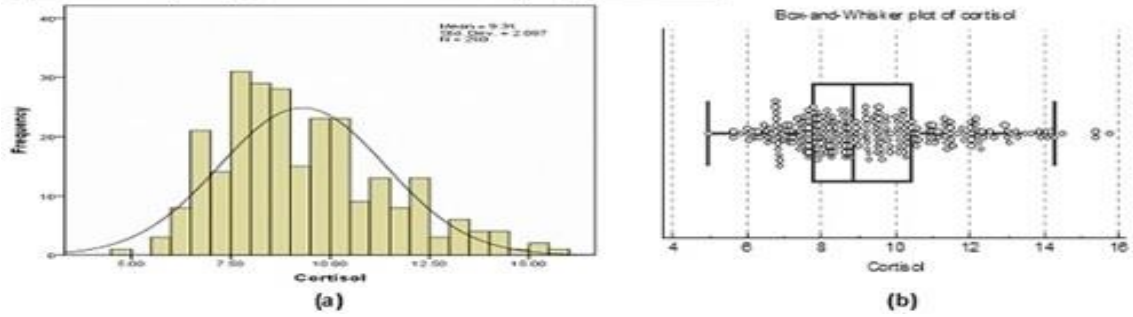

Figure. [1] Representing the histogram (a) and Box-and-Whisker plot (b) of CK; **Figure [2].** Representing the histogram (a) and Box-and-Whisker plot (b) of LDH; **Figure [3].** Representing the histogram (a) and Box-and-Whisker plot (b) of cortisol.

4. Discussion

Functional and non-functional overreaching may result from physical training. Physical activity alters the enzymatic alterations in an athlete's body and LDH, CK was subjected to serve as indices for monitoring the athlete's training status [24,25,26]. Elevated CK and cortisol activities were assumed to indicate excessive training load and training intensity resulted in exercise-induced muscle injury [26]. Increased LDH, CK and cortisol level during muscular damage / non-functional overreaching state was reported to be associated with a reduced physical performance [26,3,24].

In the present study, the 95% reference interval range for muscle damaged indices (i.e., LDH, CK) and stress marker parameter (i.e., cortisol) of all athletes (n=260) were calculated by following the guideline of IFCC and CLSI (C28-A3) of non-parametric percentile method (CLSI standard C28-A3) using reference limits at 0.025 fractile (2.5th percentile) for the lower reference limit and 0.975 fractile (97.5th percentile) as the upper reference limit. Data were transformed to the Gaussian distribution using Box-Cox transformation and then Outliers were detected and removed using Dixon's method [15]. But for individual groups (i.e., football, hockey, gymnastics, etc.), the interval was calculated according to the 'robust method' as those groups had a smaller sample size (<120) [21,27]. One sample Kolmogorov Smirnov test clarifies the distribution of the data set and depicts statistical significance value (2 tailed) for both CK (p=0.013) and cortisol (p=0.047) which can conclude the frequency distribution of CK and cortisol were skewed. Positive

skewness (0.416 and 0.688) and kurtosis (0.074 and 0.103) were found in CK and cortisol. Whereas the Kolmogorov Smirnov test, histogram and Box-and-Whisker plot depict that LDH has a normal frequency distribution.

In the present study, the CK value was found to be lowest among the middle-long distance runners (24.5±4.12) and highest among footballers and sprinters-jumpers-throwers (30.8±4.99 and 30.1±5.97 respectively), which coincide with the study report of Debnath *et al.* [28] where footballers were also found to have the highest CK value in comparison to others. Presently reported, the reference interval of resting serum LDH was 165.63 - 303.43 IU/L with a mean of 233.2±34.74 and a median around 236.25 coincides with the LDH interval range of sedentary population (105 - 333 IU/L) and athletic population (138.4-746.0 IU/L) [15,29]. In the present study, the reference interval of resting serum CK was 19.00-40.09 IU/L with a mean of 28.93±5.23 IU/L and a median of around 28.00, which also corroborates with the interval range of professional footballers (64.9 – 1971.7 U/L), male athletes (82-1083 U/L) [13,14]. Presently reported reference interval of resting cortisol was 6.07-14.15 µg/dl with a mean of 9.31±2.09 µg/dl and a median of around 8.90, which was in agreement with the cortisol range of the athletic population 5.3-15.7 nmol/l [30].

According to Debnath et al. [28], the elevated level of CK and LDH may be due to the detrimental consequence of high-intensity rapid exercise like eccentric muscle contractions, required for adaptive remodeling, which can result in residual muscle overstretching and damage which finally causes leakage of CK and LDH from damaged muscle bed and induces a muscular fatigue condition. Fatigue caused by the exercises characterized by a substantial fraction of eccentric force production and higher muscular tension development, causing rapid use of the stretch-shortening cycle and resulting in muscle tear and damage [28,15]. Srividhya, Majumdar and Subramanian [15] have also depicted that training may result in functional overreaching (improved performance) or non-functional overreaching (decreased performance) depending on the training workload, which CK and LDH can easily monitor. On the other hand, Cevada et al. [30] have reported the direct linear relation between lactate and cortisol, directly correlating cortisol to exercise-induced muscular fatigue and counted positive for the elevated cortisol level for oxidative stress generation.

The present studied summative data of CK, LDH and cortisol refer that SJT group athletes may have the highest level of muscle damage which might be due to their intense training protocols, which include eccentric exercises to adapt to the fast production of explosive strength [15,28,30]. However, the footballers were prone to muscle damage only in terms of the highest CK level, which might identify their overreaching condition [28]. Thus, the present study can help monitor athletes' overreaching conditions by observing the well-defined reference interval of skeletal muscle-damaging enzymes (i.e., CK and LDH), which can finally limit the muscle damage and exercise-induced fatigue to optimize the sports performance.

4.1 Study Strengths and Limitations

This is one of the pioneer studies to access the reference interval of muscle damage indices and cortisol in the sports/ athletic population of the Indian subcontinent/ origin. The study result will help set a guideline for the athletes and coaches but also helps to maintain the balance between increasing training load and muscle damage/ injury within the physiologic fatigue limit to reach optimal performance. The major limitation of the present study was the moderate sample size (n=260). However, bigger sample size will be more accurate for standardizing the reference interval value in any population. However, the study clarifies the sample size calculation to ensure its acceptability. Another limitation of the study is the ethnicity of the study sample, which may only be bound to the south-east Asian part.

5. Conclusion

The present study concluded the reference interval range for LDH (165.63 - 303.43 IU/L), CK (165.63 - 303.43 IU/L) and cortisol (6.07-14.15 µg/dl) with precise limits. Sprint-jump-throw athletes were identified to have the highest muscle damage due to high-intensity eccentric exercise protocols that allow them to adapt faster for explosive strength production. Further, footballers were prone to muscle damage only in terms of the highest CK level, which might identify their overreaching condition. Thus, the present study will help the athletes and coaches avoid overreaching or overtraining and maintain/ maximize their performance within the physiologic fatigue limit. The study will also help clarify the misinterpretation of muscle damage indices and stress marker parameters during any phase of competitive training. Lastly, the

study will help standardize and enrich the population data set of reference values that can be used for future research.

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All participants gave signed consent for this study.

Conflict of interest

All authors have agreed to publish the present article and declared that no competing interest exists.

Does this article screened for similarity?

Yes

Author's contribution

Surojit Sarkar[ABCDEF], Swapan Kr Dey[ABDE], Gouriprosad Datt[BDEF], Amit Bandyopadhyay[ACDEF] **[A:** Study design, **B:** Data collection, **C:** Statistical analysis, **D:** Data interpretation, **E:** Manuscript preparation, **F:** Literature search]