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Reference interval for oxidative stress markers in young football and hockey players

SUROJIT SARKAR[1], SWAPAN KUMAR DEY[2], GOURIPROSAD DATTA[1], AMIT BANDYOPADHYAY[3]

Abstract

Introduction. Malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), and glutathione peroxidase (GPx) are widely accepted as biological markers for checking the redox balance and antioxidant status. Aim of Study. The purpose of the study was to frame the reference interval for antioxidant variables (MDA, SOD, GSH and GPx) in the young athletic population of various sports discipline. Material and Methods. 190 young male players [i.e., football (n = 89), and hockey (n = 101)] were recruited for the study (mean age = 18.3 ± 2.01 yrs). Assay of MDA, SOD, GAH and GPx was done by using the standard enzymatic protocol. 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. Results. Serum MDA range was from 23.75-36.19 µmoles/100ml serum with mean of 30.29 ± ± 3.24 µmoles/100 ml serum and median around 30.43. Serum SOD ranged from 0.05-0.14 U/min/mg protein with mean of 0.08 ± 0.01 U/min/mg protein and median around 0.08. The GSH was ranging from 43.21-55.55 mg/100 ml serum with mean of 46.43 ± 2.11 mg/100 ml serum and median around 46.10. The GPx was ranging from 9.04-14.33 µmol/min/mg protein with mean of 11.35 ± 1.38 µmol/min/mg protein and median around 11.05. Conclusions. Present study confers 24.55--35.58 µmoles/100 ml serum, 0.06-0.13 U/min/mg protein, 43.27--51.86 mg/100 ml serum, and 9.07-14.12 µmol/min/mg protein as the reference interval values for MDA, SOD, GSH, and GPx respectively. The present finding will guide the researchers to avoid misinterpretation of antioxidant biomarker values during any phase of competitive training of sports person.

KEYWORDS: lipid peroxidation, glutathione, reference interval, antioxidant biomarkers, endurance team-game.

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Corresponding author: bamit74@yahoo.co.in

[1] Rammohan College, Department of Physiology, Kolkata, India
[2] University of Calcutta, Department of Sports Science, Kolkata, India
[3] University of Calcutta, University Colleges of Science and Technology, Department of Physiology, Sports and Exercise Physiology Laboratory, Kolkata, India

Introduction

An exercise-induced oxidative stress condition following a high-intensity training session was (i.e., eccentric or reaped works) hypothesized to be metabolic, mechanical or both in nature during the temporary hypoxic condition that leads to excess reactive oxygen species (ROS) generation [17, 23]. The exercise-induced overproduction of ROS creates oxidative stress and challenge redox equilibrium, which further disrupts cellular homeostasis and leads to a rise in lipid peroxidation [13, 23]. The presently studied summary data of MDA, SOD, GSH, and GPx in reference to endurance team-games such as football and hockey have no game specific references in terms of antioxidant variables, which might due to the nature of energy requirements for the game and the high demand of recovery with a higher level of endurance capacity with a high burst of intense energy for short running sprints [1, 17]. However, a single high-intensity exercise and/or even a long duration moderate-high intensity training of endurance team-game such as football and hockey were observed to induce oxidative stress via

altering antioxidant biomarker enzymes and thus can lead to redox imbalance [1, 14, 17]. The present study can help to monitor the overreaching/overtraining condition of athletes by observing the resting data via comparing them with well-defined reference intervals of antioxidant biomarkers (i.e., MDA, SOD, GSH, and GPx). This can ultimately lead to ROS induced damage to muscle and create exercise-induced fatigue, which might limit sports performance.

Reference interval (RI) is a range of values for a certain parameter that is deemed to be normal or within the physiological limit for that particular population in that particular condition [10]. Preventing misinterpretation of any biochemical data can only be possible based on RI studies [16]. Interval values can be altered depending on the population variation such as e.g. sports palyers, healthy sedentary persons and patients, since all may have different reference intervals for a given parameter in the resting condition. Brancaccio et al. [2] reported an enzymatic alteration following adaptations to physical training, including the training volume and intensity. Standardized RI will help to assess the effect of training through blood biomarkers, which must be previously established in a specified group of the population [16].

There were very few studies which predicted RI values for CK [12, 18] and LDH [18, 22] for an athletic population. Studies such as e.g. Mahmutyazicioglu et al. [12] differentiated reference values for the normal population from the athletic population. However, to date, there have been no studies of reference intervals for antioxidant biomarkers and in terms of the athletic population there is a huge gap. Only an unclear prediction can be made based on some of the studies, where initial data of antioxidant variables was present, although all of them originate either from the study of training intervention [17, 24], nutritional intake profiling [6, 7], or a generalized profile study [5]. More importantly, none of those studies were aimed for reference intervals of antioxidant variables. Additionally, all those previous studies only included very small athlete numbers which restricts their effectiveness and as a result they may not be a source of reference ranges. The reference range values for important biomarkers such as MDA, SOD, GSH and GPx will provide an insight into oxidative stress responses against the exercising/training induced damaging condition of muscles, which might be accompanied by hampering inflammatory responses. All together the antioxidant enzymatic biomarker alteration and redox equilibrium imbalance could help to predict the indirect fatigue limit of physical functioning and the overtraining/overreaching condition. Thus, the present study was aimed at identifying reference intervals for antioxidant biomarkers (i.e., MDA, SOD, GSH and GPx) among young athletes practicing football and hockey.

Material and Methods

Subjects

A total of 190 young male state level athletes (mean age = 18.3 ± 2.01 yrs) of 2 endurance-based team--games, i.e., football (FB, n = 89), and hockey (HOC, n = 101), were recruited as subjects for the present cross--sectional study. All participants had a minimum 4 years of formal training history and had played at minimum state-level competition. Players were considered to be homogeneous in terms of the socio-economic group, dietary habits and identical environmental conditions during training sessions. Subjects were clinically examined before commencing the study and only fit players were chosen as the study subjects. During the present study, all the players were in the pre--competitive training phase. The ethical guidelines of Helsinki's Declaration 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 also obtained from the Institutional Human Ethical Committee (IHEC), Department of Physiology, University of Calcutta.

Training regimen

Players of both the games were trained for 5 hours/day, which was further divided into 2:30 hours in equal halves of morning and evening sessions daily excluding Sundays. Thus, the weekly training volume amount to approx. 30 hours/week. The players were at the early pre-competitive phase where maintenance was conducted with a training load of work : rest = = 1 : 1. Strength training sessions were performed twice a week and endurance training sessions were conducted once a week, although all the other sessions included 70-80% of game specific skill trainings supplemented with the physical training part where various interval running, shuttle runs, plyometrics, etc. were carried out. Game specific skill training includes ball bridling, goal practice, long/short pass practice, and training match play, etc.

Biochemical analysis

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 and 8:00 AM in the pre-prandial state (after 8-10 hours of fasting) to avoid possible differences due to diurnal variation. Each blood supernatant was centrifuged at 3000 rpm for 15 minutes to ensure complete separation of serum. The samples were then transferred into cryo-vials and stored and preserved at –20°C for later biochemical analyses [19]. All laboratory tests were performed at room temperature varying from 23ºC to 25ºC with the relative humidity of 50-60%.

Assays for antioxidant status variables (MDA, SOD, GSH and GPx):
Malondialdehyde (MDA) was measured by reacting with thiobarbituric acid (TBA) to form TBA-MDA under acidic conditions at an elevated temperature at 532 nm and expressed as moles of MDA/100 ml serum. Superoxide dismutase (SOD) was estimated by inhibiting the auto-oxidation of pyrogallol at 420 nm. The SOD activity was expressed as U/min/mg protein and 1 U of the enzyme is defined as the enzyme activity that inhibits auto-oxidation of pyragallol by 50%. Reduced glutathione (GSH) content was estimated from a yellow coloured complex after reacting to DTNB with an absorbance maximum at 412 nm expressed as mg/100 ml serum. The glutathione peroxidase (GPx) enzyme degrades H_2O_2 in the presence of GSH. The remaining GSH was measured via its reaction with DTNB. GPx activity was expressed as µmoles of GSH consumed/min/mg protein. All oxidative stress markers (MDA, SOD, GSH, GPx) were measured following the standard procedure [11, 17]. Protein levels were estimated following the Lowry method where the Folin–Ciocalteu reagent was used to produce a blue-purple coloured complex, with maximum absorption at 660 nm.

Statistical analysis
Statistical analysis was performed using the 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 to assess the group-specific differences among MDA, SOD, GSH, and GPx. Descriptive statistics were also calculated, 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. Approved guidelines of the Clinical and Laboratory Standard Institute (CLSI, C28-A3) and the International Federation of Clinical Chemistry (IFCC) were followed to identify the RI at the 95% confidence interval (CI). The G*Power software (version 3.1.9.7) was used for sample size calculation over the statistical test of one-way fixed effects ANOVA at 95% CI. The MedCalc software (version 19, MedCalc Software bvba) was used to calculate the reference intervals in the present study [3, 15, 20].

Results

Table 1 presents general anthropometry and antioxidant variables of young male athletes from various sports disciplines. All variables inducing anthropometry and antioxidant biomarker variables were found to show no game-specific significant differences when compared between football and hockey.

Table 1. Mean and standard deviation of general anthropometry and antioxidant variables of young male athletes of football and hockey sports discipline

Variables	Football (n = 89)	Hockey (n = 101)	t value	P value
Body height (cm)	167.77 ± 6.04	168.70 ± 4.97	–1.158	0.248 NS
Body weight (kg)	57.05 ± 6.98	59.42 ± 5.58	–2.591	0.010 NS
BMI	20.23 ± 1.86	20.85 ± 1.37	–2.650	0.009 NS
Fat%	14.36 ± 4.60	14.16 ± 5.53	0.261	0.794 NS
MDA (µmoles/100 ml serum)	30.00 ± 3.00	30.55 ± 3.44	–1.167	0.245 NS
SOD (U/min/mg protein)	0.08 ± 0.01	0.09 ± 0.02	–1.725	0.086 NS
GSH (mg/100 ml serum)	46.13 ± 1.36	46.70 ± 2.58	–1.852	0.066 NS
GPx (µmol/min/mg protein)	11.54 ± 1.36	11.19 ± 1.39	1.711	0.089 NS

Note: Values are expressed as mean ± SD; NS – non-significant, MDA – malondialdehyde, SOD – superoxide dismutase, GSH – glutathione, GPx – glutathione reductase

Table 2 represents descriptive statistics of antioxidant variables (i.e., MDA, SOD, GSH, and GPx) for the combined population of young male athletes. Here percentiles were only given at three points: 25%, 50% (median value) and 75%. MDA ranges from 23.75 to 36.19 µmoles/100 ml serum with a range of 12.44, for SOD it is 0.05-0.14 U/min/mg protein with a range of 0.09, GSH ranges from 43.21 to 55.55 mg/100 ml serum with a range of 12.34, while for GPx it is 9.04--14.33 µmol/min/mg protein with a range of 5.29, respectively.

Table 2. Descriptive statistics of oxidative stress variables for the combined group of young male endurance team-game athletes

		MDA	SOD	GSH	GPx
Mean		30.29	0.08	46.43	11.35
Median		30.43	0.08	46.10	11.05
Mode		30.55	0.07	45.20	11.05
Std. Deviation		3.246	0.016	2.113	1.385
Std. Error of Mean		0.235	0.001	0.153	0.100
Variance		10.53	0.00	4.46	1.91
Range		12.44	0.09	12.34	5.29
Minimum		23.75	0.05	43.21	9.04
Maximum		36.19	0.14	55.55	14.33
Percentiles	25%	28.02	0.07	45.13	10.32
	50%	30.43	0.08	46.10	11.05
	75%	33.19	0.10	47.59	12.20

Note: MDA – malondialdehyde, SOD – superoxide dismutase, GSH – glutathione, GPx – glutathione reductase, Std. Deviation – standard deviation, Std. Error of Mean – standard error of mean

Table 3 depicts reference intervals for MDA, SOD, GSH, and GPx in the combined group (n = 190) and in the individual game-specific populations. Reference intervals were calculated with a 90% confidence interval limit for all the sport disciplines. Reference intervals of the total athletic population/combine group were calculated using the non-parametric percentile method and individual sports-specific groups using the robust method (CLSI guideline, C28-A3). Reference intervals in the combined group for MDA, SOD, GSH and GPx were 24.55-35.58 μmoles/100 ml serum, 0.06-0.13 U/min/mg protein, 43.27-51.86 mg/100 ml serum, and 9.07-14.12 μmol/min/mg protein, respectively. Reed's method was applied for the outlier measurement and GSH only has three outlier measures.

The following figures [1(A), 1(C), 1(E), 1(G)] represent histograms for MDA, SOD, GSH and GPx, respectively. On the other hand, figures [1(B), 1(D), 1(F), 1(H)] show the Box-and-Whisker plots of MDA, SOD, GSH and GPx, respectively, for the median, lower quartile, upper quartile, lower extreme and upper extreme values. Histogram data show MDA (mean = = 30.29, Std. Dev. = 3.246); SOD (mean = 0.08, Std. Dev. = 0.016), GSH (mean = 46.43, Std. Dev. = 2.114), and GPx (mean = 11.36, Std. Dev. = 1.385).

Discussion

Functional and non-functional overreaching may result from physical training. Physical activity alters the antioxidant status in an athlete's body and MDA, SOD, GSH, GPx serve as biomarkers to monitor the athlete's antioxidant status and redox equilibrium [13,

Table 3. Reference interval for oxidative stress variables of young male athletes of football and hockey sports discipline

Variable wise reference interval			Combine (n = 190)	Football (n = 89)	Hockey (n = 101)
MDA (μmoles/100 ml serum)	Reference interval		24.55-35.58	23.82-35.87	23.75-37.50
	90% CI	lower limit	24.30-25.38	23.05-24.65	22.85-24.67
		upper limit	35.22-36.19	34.97-36.77	36.71-38.36
SOD (U/min/mg protein)	Reference interval		0.06-0.13	0.05-0.11	0.04-0.11
	90% CI	lower limit	0.05-0.06	0.04-0.05	0.04-0.05
		upper limit	0.10-0.14	0.10-0.11	0.11-0.12
GSH (mg/100 ml serum)	Reference interval		43.27-51.86	43.26-48.82	41.03-51.45
	90% CI	lower limit	43.21-43.50	42.78-43.70	39.98-42.17
		upper limit	49.30-55.55	48.29-49.24	50.36-52.47
GPx (μmol /min/mg protein)	Reference interval		9.07-14.12	8.64-14.17	8.24-13.91
	90% CI	lower limit	9.07-9.29	8.29-9.02	7.85-8.57
		upper limit	14.02-14.33	13.66-14.63	13.33-14.35

Note: MDA – malondialdehyde, SOD – superoxide dismutase, GSH – glutathione, GPx – glutathione reductase, 90% CI – 90 percentage confidence interval

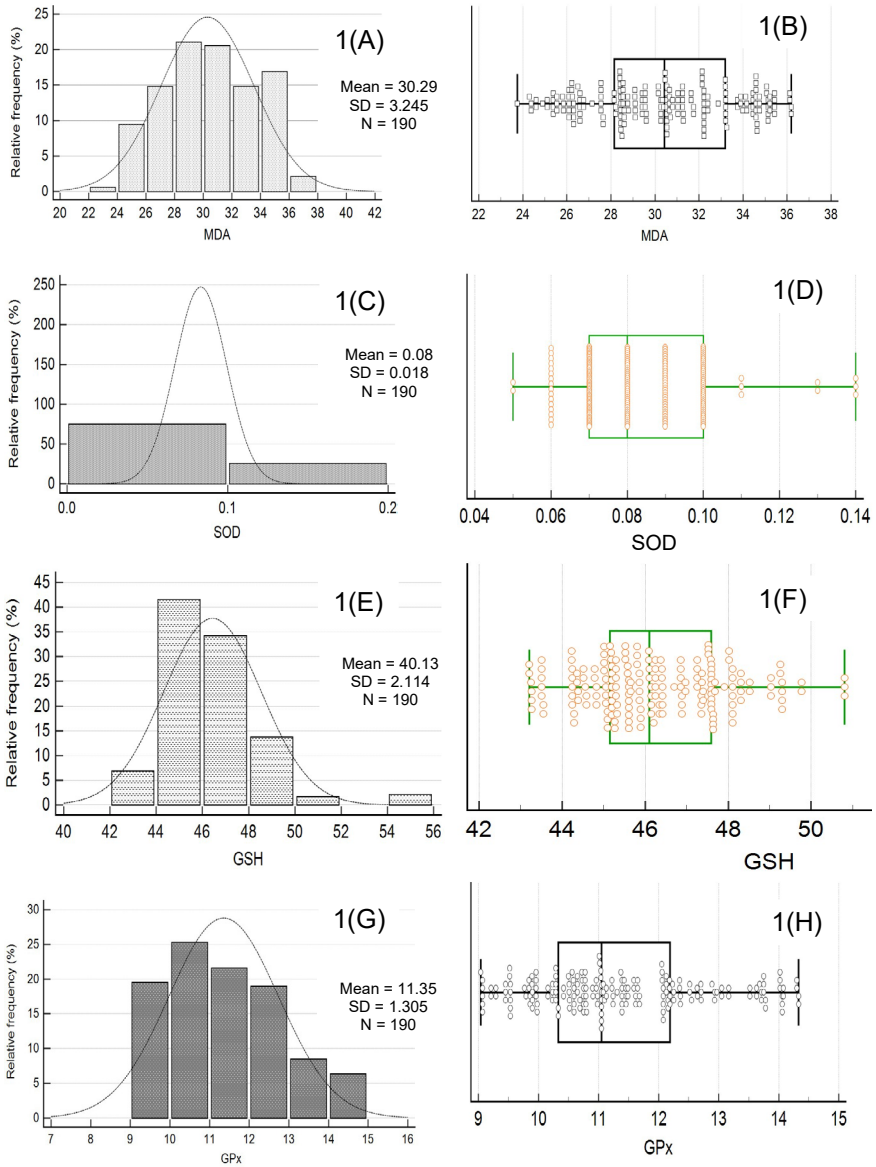

Figure 1. Representing histogram (A, C, E, G) and Box-and-Whisker plot (B, D, F, H) of MDA, SOD, GSH and GPx, respectively

17, 23]. Elevated SOD and GPx activities along with a reduced GSH level were assumed as the indication of oxidative stress specially when associated with a rise in the MDA level. An excessive training load and training intensity results in exercise-induced oxidative stress via ROS generation leading to redox equilibrium imbalance [13, 23]. Sometimes the altered antioxidant status during the oxidative stress condition of overtraining/overreaching was reported to be associated with limiting and/or hampering the physical performance [4, 9, 23]. Thus, to establish the reference intervals of antioxidant variables is crucial for the resting range of antioxidant status and to maintain the same for endurance team-game athletes.

In the present study, the 95% RI ranges for muscle damaged indices (i.e., MDA, SOD, GSH, and GPx) of all athletes (n = 190) were calculated following the guidelines of IFCC and CLSI (C28-A3) of the non--parametric percentile method (CLSI standard C28-A3) using reference limits at the 0.025 fractile (2.5^{th} percentile) for the lower reference limit and 0.975 fractile (97.5^{th} percentile) as the upper reference limit. Data were transformed to the Gaussian distribution using the Box–Cox transformation and then outliers were detected and removed using Dixon's method [22]. However, for individual groups (i.e., football, hockey) the interval was calculated according to the 'robust method', as

those groups had a smaller sample size (<120) [3, 8]. One sample Kolmogorov–Smirnov test clarifies the distribution of data set and depicts statistical significance values (2 tailed) for MDA, SOD, GSH, and GPx. Skewness and kurtosis were for MDA (0.009, –1.036), SOD (0.780, 1.315), GSH (1.725, 5.512), and GPx (0.455, –0.605), respectively, whereas the Kolmogorov–Smirnov test, histogram and the Box-and-Whisker plot show that MDA has a normal frequency distribution.

In the present study, the RI of resting serum MDA was 24.55-35.58 μmoles/100 ml serum with the mean of 30.29 ± 3.24 and the median around 30.43. The RI of resting serum SOD was 0.06-0.13 U/min/mg protein with the mean of 0.08 ± 0.016 IU/L and the median around 0.08. In turn, the RI of resting serum GSH was 43.27--51.86 mg/100 ml serum with the mean of 46.43 ± 2.11 and the median around 46.10. The RI of resting serum GPx was 9.07-14.12 μmol/min/mg protein with the mean of 11.35 ± 1.38 and the median around 11.05. Present studied RI data was in agreement with the report of Sarkar et al. [17], where control group data falls into the presently discussed reference ranges of antioxidant variables. Excess high intensity exercise or overtraining leads to a temporary hypoxic condition, which includes the overproduction of ROS, induces oxidative stress and challenges redox equilibrium, further disrupting cellular homeostasis and leading to increased lipid peroxidation [13, 23]. Excess ROS generation mainly occurs in the mitochondria and diffuses into the cytoplasm, where as a result a molecular cascade is initiated, which involves AMP-activated protein kinase (AMPK), proliferator activated receptor-gamma (PPARγ), PPARγ coactivator--1α (PGC-1α), etc. [25].

Several studies such as e.g. Miyazaki et al. [14], Azizbeigi et al. [1], and Metin et al. [13] reported that even a single bout of intense exercise can alter the antioxidant equilibrium and induce lipid peroxidation with an increased MDA level. According to Sarkar et al. [17], high-intensity training (HIIT for 8 weeks) commonly used during the preparatory phase might lead to a significant rise in oxidative stress indicated by increased levels of MDA, SOD, GSH, and GPx biomarkers along with muscle damage and inflammation. Additionally, Sousa et al. [21] by summarizing 38 studies reported that pro-oxidants, i.e., TBARS/MDA, PC, myeloperoxidase and H_2O_2, etc. tend to decrease significantly in association with an increase in SOD, GPx, catalase, TAC and GSH as a physical training-induced effect. On the other hand, Sarkar et al. [17] reported a significant positive correlation of maximal oxygen consumption ($\dot{V}O_{2max}$) and explosive strength with MDA and a significant negative correlation of $\dot{V}O_{2max}$, anaerobic power and explosive strength with the GSH level. All those previous studies could establish the importance of monitoring the antioxidant enzymatic profile of an athlete to know the status of training conditioning, fatigue limit, etc.

The presently studied summary data of MDA, SOD, GSH, and GPx indicates that endurance team-games such as football and hockey have any game specific references in terms of antioxidant variables, which might be due to the nature of energy requirement for the game and the high demand of recovery with a higher level of endurance capacity with a high burst of intense energy for short running sprints [17, 23, 25]. However, a single high-intensity exercise and/or even a long duration moderate-high intensity training of endurance team-games such as football and hockey were observed to induce oxidative stress via altering antioxidant biomarker enzymes and can create redox imbalance [1, 14, 17]. The present study can help to monitor the overreaching/overtraining condition of athletes by observing the resting data compared with well-defined reference intervals of antioxidant biomarkers (i.e., MDA, SOD, GSH, and GPx), which can finally lead to ROS induced damage to muscle and create exercise-induced fatigue and might limit sports performance.

This is one of the pioneer studies to access the RI of antioxidant biomarker variables in a sports/athletic population of the Indian subcontinent/origin. The study results will not only help to set guidelines to the athletes and coaches, but also maintain the balance between increasing training load and oxidative stress conditioning within the physiologic fatigue limit to reach the optimal performance. The major limitation of the present study was the moderate sample size (n = 190), as a bigger sample size will facilitate a more accurate standardization of the RI values in any given population. Another limitation of the study is the ethnicity of the study sample, i.e. the south-east Asian part. Total training volume in terms of % HR_{max} was not recorded and thus it is considered as a limitation of the study. The diet of the players during the study period was not thoroughly evaluated. So, the exact nutritional pattern and availability of daily ingested antioxidant levels could not be presented in the study and this is another major limitation of this investigation. All the players were considered homogeneous in nature in terms of their diet and socio-economic condition, since all of them were residing in the campus hostel and were having same diet throughout the day.

Conclusions

The present study concluded the RI range for antioxidant variables, i.e., MDA (24.55-35.58 μmoles/100 ml serum), SOD (0.06-0.13 U/min/mg protein), GSH (43.27-51.86 mg/100 ml serum) and GPx (9.07-14.12 μmol/min/mg protein) with precise limits. Both the games, football and hockey, did statistically differ in terms of resting antioxidant status and oxidative stress condition. An altered antioxidant enzymatic condition is an indirect prediction for training intensities and/or overtraining, which can be monitored with the help of reference values. Further, there is a close correlation of antioxidant variables with performance variables, i.e., endurance capacity, anaerobic power and explosive strength measure and thus the reference value will help to monitor the performance indirectly. Thus, the present study will help the athletes and coaches directly to avoid overreaching or overtraining and to maintain/maximize their performance within the physiologic fatigue limit. The study will also help to clarify the misinterpretation of antioxidant biomarker variables during any competitive training phase of endurance team-game. Lastly, the study will help to standardize and enrich the population data set of reference value, which can be used for future researches.

Conflict of Interest

The authors declare that there is no conflict of interest.

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