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Endurance exercise causes adverse changes in some hematological and physio-biochemical indices in ponies under high altitude stress condition

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ABSTRACT

The ponies have immense relevance for logistic support for civil population and troops in hilly and high altitude areas. There is no information on specific biomarkers of endurance performance under high altitude stress condition, which could be supportive in the identification of elite ponies for deployment at high altitude. Therefore, the present study was conducted to evaluate the physiological responses, hematological, biochemical, metabolic, and antioxidant biomarker during endurance exercise in ponies at high altitude. For this study, total 5 mares were put on endurance exercise at 4–6 m/sec speed for 30 min on 30 m track situated at 3,500 m altitude for 28 days period. The result showed a significant change in physiological responses, and some hematological, biochemical, metabolic and antioxidant parameters viz. glutathione peroxidase, creatinine kinase-MB, lactic acid, total protein, glucose, hexokinase, cortisol, and interleukin-6 level at different phase of endurance exercise. In conclusion, this study showed the alteration in physiological responses and some hematological and physio-biochemical metabolic parameters during the endurance exercise. Hence, these parameters could be considered as biomarkers for evaluation of endurance performance in ponies at high altitude before putting them under load carrying deployment.

Key words: Biomarkers, Endurance exercise, High altitude, Ponies

The ponies and mules are extensively used for logistic support by civil population and military formations in hilly and high altitude areas due to prevalent rugged terrains and remote locations (Venkatesan *et al.* 2011). These animals need to undergo the acclimatization procedure before deployment under hypobaric-hypoxia and cold stress condition of high altitude. Several animals are culled if found sick, poor load carrying capacity and sudden death during acclimatization period, which incurs heavy economic loss to the establishment. Zanskar Pony is a native breed reared under tropical region (Talluri *et al.* 2016). Hence, there is need to identify biomarkers which can be helpful in identification of high-load carrying and fertile ponies to meet immediate logistics requirement.

A large number of physiological factors control metabolic pathways involved when animals are under work. Numerous studies indicated the modulation of certain blood physio-biochemical's, metabolic enzymes, inflammatory cytokines, and antioxidants levels in response to exercise performance of animals and human beings (Suzuki *et al.* 2002). Physical exercise increases the body's oxygen consumption and increases both cellular and tissue respiration, which causes an overproduction of free radicals (Avellini *et al.* 1999). When free radical generation exceeds the cell's antioxidant capacity, cellular and tissue damage develops due to oxidative stress (Halliwell *et al.* 2007). Hence, exercise-induced oxidative stress favours accelerated muscle fatigue and muscle fibre damage, leading to exercise intolerance and poor performance in animal (Sen *et al.* 2000). Physical work capacity at high altitude in the horse depends mainly on the rate of aerobic metabolism and the capacity of the anaerobic processes to supply energy for continued muscle contraction under hypoxic condition. Several factors including type, intensity and duration of exercise have been shown to influence these changes. Cardiovascular and respiratory performances are another indicatives of endurance of animals during exercise. Although numerous studies have been conducted in humans and treadmill based exercise protocol in horses, however not much study has been conducted in ponies at the high altitude about biochemical performance.

Treadmill based exercise usually involving a single bout of intensive exercise which precisely controls the exercise parameters, however, there are physiological and locomotion differences, and affect some measurements of performance when comparing exercise on the track with

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that on a treadmill (Jones *et al.* 2006) and until the equid would no longer maintain its position on the treadmill without humane support (Wickler and Anderson 2000). Therefore, variation in these parameters needs to be evaluated to assess the exercise performance of equid at high altitude on actual deployment. Hence, it is hypothesized how endurance exercise is altered at altitude and how conditioning at high altitude stress condition may improve endurance performance.

Maintenance of breeding stock and working ponies and mules are very difficult at high altitude due to the harsh climate, unavailability of green fodders during long winter months, and poor reproductive health. At present, there are no studies which demonstrated the physiological or performance parameters or biomarkers to elucidate the physiological basis of endurance exercise which could be useful for selection of high performing ponies for high altitude region. Although there is a general consensus that high altitude affects the performance of ponies, however there is no study based evidence which relates to an acute change in hematological, physio-biochemical, and antioxidant parameters of ponies during endurance exercise under high altitude stress condition. This is a first study to identify biomarkers of endurance at high altitude, which would be helpful in selection of high performing ponies for deployment and future breeding.

MATERIALS AND METHODS

Experimental animals: Five adult female Zanskar ponies (*Equus Ferus caballus*) of 6 - 8 years old (Mean body weight 326.4 kg) were put before exercise training for six days to standardize endurance exercise. Zanskar ponies is a native breed of Zanskar valley located at high altitude terrain of Ladakh. These ponies are the hardiest to the hypobaric-hypoxia and cold environment conditions, and important pack animal for native population and military formations at high altitude.

Experimental design of endurance exercise: This experiment was conducted for the duration of 28 day during the winter season at a mean ambient temperature of – 6°C on the flat track of training facility of DIHAR located at 11,500 feet altitude. Diets were fed to all the ponies as per the standard ration scale of institute formulated by DIHAR and Army's RVC core, whereas lukewarm water was given *ad libitum*. Endurance exercise speed was at 6 m/sec for 30 min per day on 30 m long flat track, and total distance covered was 10800 m/day. A one day rest was given after every 6th day of continuous endurance exercise.

Observations and parameters recorded: The physiologic responses viz. heart rate, pulse rate, respiration, and rectal temperature were recorded before and immediate after the endurance exercise. Blood samples (10 ml) were collected at 45–60 min before and immediate after the endurance exercise on 1st, 14th, and 28th day for analysis of hematological parameters. However, serum isolated on 1st and 28th day was used for analysis of blood biochemical, inflammatory cytokines, and antioxidants parameters. Since, there is no apparent biochemical changes reported in native ponies on endurance exercise, therefore 14th day was not analyzed for different biochemical parameters.

All the blood samples were collected from the jugular vein in vacutainer tubes (REF 367812, BD Franklin Lakes, NJ, USA) and one aliquot used for serum separation at 37°C temperature. After that pooled serum was centrifuged at 5,000 rpm for 5 min to remove residual blood clot, supernatant was collected, and stored in sterile vials at -80°C.

Recording of physiological responses and analysis of hematological parameters: Evaluation of physiological responses is recommended along with biochemical analysis to investigate the level of stress on the animal. Therefore, heart rate, pulse, respiration, and rectal temperature were recorded 45-60 min before and immediate after the endurance exercise. All the parameters were recorded in tranquil position of animals to avoid any additional stress to the animals.

The complete blood cell counts were determined in one aliquot by using the PE-6800 VET fully automated hematology analyzer within 30–45 min of blood sampling.

Determination of physio-biochemical and metabolic biomarkers: Commercial kits used for analysis of serum alkaline phosphatase (ALP) (Cat. No. 105-000816-00), alanine aminotransferase (ALT) (Cat. No. 105-000814–00), aspartate aminotransferase (AST) (Cat. No. 105-000815-00), lactate dehydrogenase (LDH) (Cat. No. 105-000818-00), albumin (Cat. No. 105-000822-00), urea (UR) (Cat. No. 105-000824–00), uric acid (UA) (Cat. No. 105-000848-00), creatinine (Cat. No. 105-000852-00), and total protein (Cat. No. 105-000823-00) were procured from M/S Mindray Pvt. Ltd, Shenzhen, China; creatinine phospho kinase (CK-MB) (Cat. No. CK1296) from M/S Randox Pvt. Ltd, United Kingdom using fully automated BS-120 clinical biochemistry analyzer as per the manufacturer's protocol given in product brochure. Serum triiodothyronine (T_3) (Cat. No. CEA453Ge) and cortisol (Cat. No. CEA462Ge) hormones were estimated by using commercial ELISA kits procured from M/S USCN Life Science Inc, Wuhan, China as per the manufacturer's protocol. Hexokinase (Cat. No. MAK091) and phosphofructokinase (Cat. No. MAK093) estimated by using colorimetric assay kit procured from Sigma – Aldrich, St. Louis, MO, USA. Lactic acid assays were performed as per Pryce (1969).

Determination of anti-stress parameters and cytokines levels: The total antioxidant status in serum samples was measured by Ferric reducing antioxidant power (FRAP) assay as demonstrated by Benzie and Strain (1996) with slight modifications. Serum glutathione peroxidase (Cat. no. 353919) levels were measured through colorimetric method whereas reduced glutathione (Cat. No. ab65322) levels were measured through the fluorometric method as per the manufacturer protocol of Merck Millipore, Molsheim, France and M/S Abcam Pvt. Ltd, Cambridge, United Kingdom, respectively by the help of Molecular device (SpectraMax^R i3x). Serum cytokines markers such

as Interleukin-2 (IL-2) (Cat. No. 431007) and Interleukin-6 (IL-6) (Cat. No. 431307) were measured by ELISA kit procured from M/S BioLegend Inc., San Diego, US as per the manufacturer's instruction with the help of Molecular device (SpectraMax^R i3x).

Statistical analysis: All the data were analyzed using SPSS 22.0 statistical package programme (SPSS Inc, Chicago, Illinois USA) to compare differences between before and after the endurance exercise, a paired t-test was applied. All the values are expressed as means±SE. Statistical significance was considered to be significant at P<0.05.

RESULTS AND DISCUSSION

Physiological responses: The data were compared between before and after endurance exercise to evaluate the endurance performance. All the physiological responses were significantly (P<0.05) increased by 37–49% in heart rate, 42–49% in pulse, 36–51% in respiration rate, and 0.8–2.48% in rectal temperature during the endurance exercise (Table 1). However, physiological responses values were lower at 28th day as compared with 1st day value (Table 1).

Physiological responses to hypoxia occur at the systemic and cellular level. Hence, hypoxia directly affects the vascular tone of the pulmonary and systemic resistance vessels (Gore *et al.* 2007). Heart rate, pulse, and respiration rate vary with the change in the level of some humoral agents and local, spinal and bulbospinal chemo, baro and nociceptive reflexes. This could be reason of the significant increase in the heart rate, pulse rate, and the respiration rate in ponies. The systemic response is mediated by the chemoreceptor stimulation at the time of oxidative stress and activation of the central and peripheral nervous system causing changes in heart rate and respiration rate (Schuler *et al.* 2005). Respiratory rate is also related to lung capacity, with increased heart beat and pulse rate which may be attributed to increased cardiac output in this case also increase respiration rate. During endurance race, among the physiological parameters, rectal temperature plays an important role (Sinha *et al.* 2010). Since hypoxia and low humidity are major stimuli that induce acute production of heat in animals (Dobnikar *et al.* 2009). Therefore, this might be a reason for increases in rectal temperature in the present study.

Hematological parameters: The present study indicated interesting findings on hematological changes during the

Table 1. Change in physiological responses during different time intervals of endurance exercise in ponies at high altitude

Parameter	1st Day			28th Day		
	Before exercise	After exercise	% Change	Before exercise	After exercise	% Change
Heart rate (beats/min)	55.75±0.86	76.5*±0.72	37.21	48.20±0.80	72.00*±2.53	49.37
Pulse (pulse/min)	52.8±0.46	76.65*±1.00	42.78	48.60±0.98	72.60*±2.71	49.38
Respiration (cycle/min)	46±2.14	62.9*±2.49	36.73	41.60±0.98	63.20*±2.65	51.92
Rectal temp. (°F)	99.53±0.10	102*±0.18	2.48	100.20±0.22	101.04*±0.29	0.83

Values are presented as mean±SE; *indicates significant difference (P<0.05) within the same row on the particular day.

Table 2. Change in hematological parameters during different time intervals of endurance exercise in ponies at high altitude

Parameter	1st Day		14th Day		28th Day	
	Before exercise	After exercise	Before exercise	After exercise	Before exercise	After exercise
WBC (×10^3/µl)	6.40±0.55	8.02*±0.42	6.66±0.43	7.04*±0.41	6.84±0.17	7.54*±0.30
LYM (%)	55.74±2.76	52.88±2.69	39.60±2.35	38.38±0.89	40.94±1.71	40.90±1.81
MID (%)	12.52±0.68	11.44±0.39	11.66±1.34	11.00±0.62	9.90±1.23	10.34±0.99
GRAN (%)	49.62±1.70	56.12*±1.48	51.60±1.77	55.73*±0.45	48.36±2.78	50.88*±2.22
LYM (×10^3/µl)	3.28±0.19	3.80±0.33	2.36±0.33	2.80±0.22	2.18±0.15	2.32±0.17
MID (×10^3/µl)	0.66±0.07	0.70±0.00	0.72±0.13	0.78±0.04	0.56±0.09	0.54±0.11
GRAN (×10^3/µl)	3.70±0.21	4.14±0.25	3.58±0.30	3.46±0.21	2.30±0.18	2.42±0.12
PLT (×10^3/µl)	235.60±2.54	288.40*±2.94	274.40±13.94	351.0*±9.09	266.80±19.12	368.40*±27.11
MPV (fl)	8.74±0.23	7.96±0.24	9.52±0.24	8.68±0.32	8.44±0.54	7.90±0.43
RBC (×10^6/µl)	8.80±0.25	9.66*±0.10	8.33±0.22	9.27*±0.20	8.59±0.08	9.37*±0.15
Hb (g/d)	11.62±0.14	13.08*±0.15	11.14±0.25	12.98*±0.20	10.80±0.17	12.32*±0.20
HCT (%)	38.68±0.88	43.06*±0.73	34.83±1.36	37.88*±1.02	30.78±2.64	33.27*±2.62
MCV (fl)	47.40±0.88	47.48±0.76	58.14±0.87	58.74±0.73	57.46±0.96	57.06±0.99
MCH (pg)	17.72±0.31	16.72±0.31	17.06±0.17	16.36±0.45	16.16±1.72	15.94±1.33
MCHC (g/dl)	36.44±0.48	35.10±0.25	35.46±0.68	34.44±0.43	37.34±1.22	36.89±1.12

*Values are presented as mean±SE; *indicates significant difference (p<0.05) within the same row on the particular day; WBC, white blood cells; LYM, lymphocyte; MID, mid-range absolute count; GRAN, granulocytes; PLT, platelet; MPV, mean platelet value; RBC, red blood cells; Hb, haemoglobin; HCT, hematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular hematocrit; MCHC, mean corpuscular hematocrit concentration.

exercise, which indicated significant (P<0.05) increase in WBC, GRAN, RBC, Hb, HCT, and PLT level after the exercise at 1st, 14th and 28th day as compared to before endurance exercise, whereas, other parameters did not show any significant changes (Table 2). However, decreasing trend was observed in extent of increasing WBC, GRAN, RBC, Hb level with the exercise period (Table 2). An elevated level of RBC, Hb, HCT, WBC, GRAN, and PLT are the indicative of stress or exercise mediated inflammation, which occurs during exercise and load carrying. However, minimal variations in WBC indices are indicative of the good performance of horses (Adamu *et al.* 2012). Exercises have variable effects on the hemogram depending on work intensity, fitness, training levels, environmental conditions and breed of horses (Satue *et al.* 2012). The increase in the value of erythrocyte indices in horses is caused, most of all, by a release of erythrocytes from the spleen (Satue *et al.* 2012). Increased number of platelets is essential for homeostasis (Yilmaz *et al.* 2004, Satue *et al.* 2012). Platelet count can be increased by exercise and this might also be associated with the fresh release of platelets from the spleen, bone marrow and another reservoir (Yilmaz *et al.* 2004). Hence, platelets measurement can reflect changes in platelet stimulation and hence might be an important marker of endurance performance at high altitude.

However, the increase in hematocrit could also be attributable to changes in plasma volume, in relation to thermoregulatory processes, mainly sweating and evaporation from the respiratory mucosa and to fluid shift derived from physical activity (Munoz *et al.* 2008, Adamu *et al.* 2012). However, numerous studies have shown that horses subjected to high altitude possessed significantly higher RBCs, Hb and HCT values, compared to animals that lived at less altitude (Wickler *et al.* 2000, Satue *et al.* 2012). Long term hypoxic exposure or stress to altitude can lead to an increment of RBC, Hb, the density of capillary blood vessels, and myoglobin density in skeletal muscle, resulting in enhancement of oxygen delivery capacity at high altitude (Rodriguez *et al.* 2000).

Physio-biochemical and metabolic parameters: Physio-biochemical and metabolic biomarkers like ALT, AST, CK-MB, hexokinase (HK), lactic acid (LA), total protein (TP), urea, glucose (Glu), and cortisol were significantly (P<0.05) elevated immediately after the endurance exercise (Table 3). However, no significant (P<0.05) change in triiodothyronine (T_3), phosphofructokinase, lactate dehydrogenase, alkaline phosphatase, albumin, uric acid, and creatinine were found (Table 3). Some studies involving long distance exercise have shown increases in plasma protein and/or albumin, indicating a degree of dehydration (Kerr and Snow 1982). In this study, total protein increased significantly, while albumin values did not change. Several investigators have been assumed that any increase in total protein or decrease of plasma water is a disadvantage during strenuous exercise, and this assumption has been challenged (Kronfeld *et al.* 2001). In our study serum urea increased

Table 3. Change in biochemical parameters, metabolic enzyme and hormone activity during different time intervals of endurance exercise in ponies at high altitude

Parameter	1st Day		28th Day	
	Before exercise	After exercise	Before exercise	After exercise
ALP (U/l)	157.86± 9.77	176.14± 11.80	155.82± 7.67	169.04± 8.62
ALT (U/l)	11.20± 1.17	12.12*± 0.94	13.26± 0.61	18.48*± 1.33
AST (U/l)	253.82± 8.99	266.82*± 11.50	259.58± 5.13	288.12*± 10.84
LDH (U/l)	387.38± 16.39	513.10± 27.43	339.88± 18.57	397.92± 20.26
CK-MB (U/l)	255.80± 5.18	316.00*± 7.67	266.40± 16.15	368.80*± 15.70
Glucose (mg/dl)	89.16± 3.70	92.92± 3.98	90.88± 3.73	102.04*± 4.72
Hexokinase (mU/ml)	1.39± 0.18	1.76*± 0.09	0.92± 0.10	1.84*± 0.54
Phospho fructokinase (mU/ml)	1.80± 0.49	1.05± 0.05	1.41± 0.15	1.01± 0.08
Lactic acid (mmol/l)	0.77± 1.00	7.00*± 1.00	0.98± 0.3	10.0*± 3.0
Total protein (g/dl)	6.58± 0.09	6.62*± 0.30	6.28± 0.36	6.76*± 0.08
Albumin (g/dl)	4.10± 0.08	3.84± 0.02	3.92± 0.14	3.74± 0.09
Urea (mg/dl)	35.36± 1.02	36.08*± 0.61	34.14± 1.21	36.90± 1.05
Uric acid (µmol/l)	0.28± 0.02	0.42± 0.04	0.24± 0.04	0.32± 0.02
Cortisol (ng/ml)	6.00± 1.05	9.00*± 1.33	6.00± 1.33	10.00*± 1.46
T3 (pg/ml)	26.00± 1.16	28.00± 0.73	30.00± 0.64	32.00± 1.73

Values are presented as mean±SE; *indicates significant difference (P<0.05) within the same row on the particular day. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; LDH, lactate dehydrogenase; CK-MB, creatine kinase isotype; T_3, triiodothyronine.

significantly (P<0.05) and also observe sweating and dehydration during endurance race which may be due to electrolyte balance. Creatinine and urea concentrations elevation may also result from higher metabolic rate (Bayly 1987).

Numerous studies have supported the hypothesis that increased availability of glucose to muscle increases capacity for prolonged exercise. We also observed an elevated level of glucose after the endurance exercise, which is due to increased glucose uptake as the liver increases both glycogenolysis and gluconeogenesis. This is essential to meet instant energy requirement, therefore, efficient glucose metabolism is an important indicator for better endurance (Coggan *et al.* 1991). Interestingly, the very high increase in lactic acid level in the present study may be

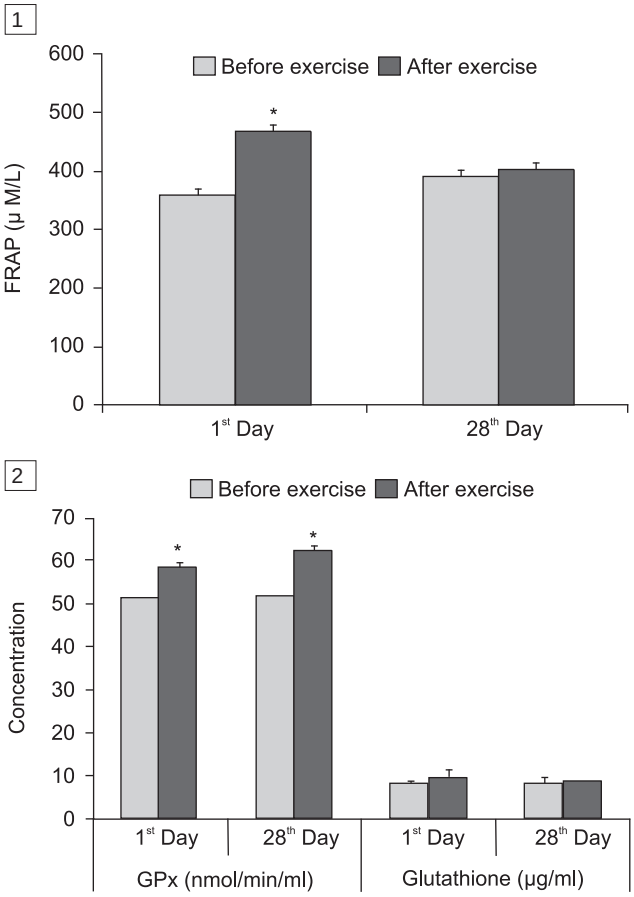
linked to poor oxygenation of muscles and reduction of muscle glycogen (Newsholm *et al.* 1992).

One of the studies had shown that increase in ALP, ALT as well as AST in the serum during the endurance race in horse (Larsson *et al*. 2013). In this study, there was significant (P<0.05) increase in the serum levels of AST and ALT, whereas ALP varied non-significantly. Creatine kinase as an index of "fitness" in horses is also being considered since studies have shown that changes in the CK levels, along with other serum enzymes – lactate dehydrogenase and aldolase reduced by repeated exposure to exercise (Lippi *et al.* 2008). In fact, elevated CK levels in the blood are said to be associated with muscle cell damage and disturbance following strenuous exercise (Baird *et al.* 2011). In our study also significantly increase CK-MB level after an endurance exercise because due to the high intensity of endurance exercise at high altitude stress condition resulting in muscle cell damage.

We found increasing pattern in serum hexokinase activity after the exercise, which may be due to increased in glucose metabolism and phosphorylation on high glucose flux after endurance exercise (Richter *et al.* 2001). In our study, cortisol level increased without any change in T_3 level after the exercise. Hormonal responses in hypoxic conditions may be more intensive and even unpredictable, comparing to normoxia, due to reduced maximal oxygen consumption and increased workload. Increased serum cortisol levels have been reported in studies at 3,500–5,200 m heights, while at higher altitude, serum cortisol levels had no difference with the sea levels (Benso *et al.* 2007). Moreover, exercise variables including type, intensity, volume, duration and rest periods and previous training status of subjects can influence on serum cortisol (Sanavi *et al.* 2013). Where there is an intensive exercise usually accompanied with reduced muscle glycogen, the body supplies energy requirements from triacylglycerol breakdown and free fatty acid production by increasing serum cortisol concentrations (Struder *et al.* 1996). Thus, the exact mechanism of the rise in serum cortisol levels after exercise at high altitude is not very well elucidated and is a good future prospect for further research.

Variation in anti - stress parameters and cytokines levels: Anti-stress parameters like ferric reducing antioxidant power (FRAP), glutathione peroxidase (GPx), and cytokines interleukin-6 significantly (P<0.05) elevated immediately after endurance exercise (Figs 1–3).

These changes indicated that if these parameters are correlated with load carrying performance of ponies, these would be important biomarkers of endurance at high altitude. We observed the significant increase in IL-6 after the endurance exercise. Hagobian *et al.* (2006) also reported the increase in plasma IL-6 and CRP concentrations in response to exercise during acute high-altitude exposure. The hypoxia may initiate an acute phase response during exercise, which is characterized by alterations in immune and inflammatory responses including increases in plasma interleukin-6 (Lundby *et al.* 2004). Elevations in serum



Figs 1, 2. Variation in anti-stress level recorded in Ponies at different time intervals before and after exercise. Values are presented as mean±SE; *indicates significant difference (P<0.05).

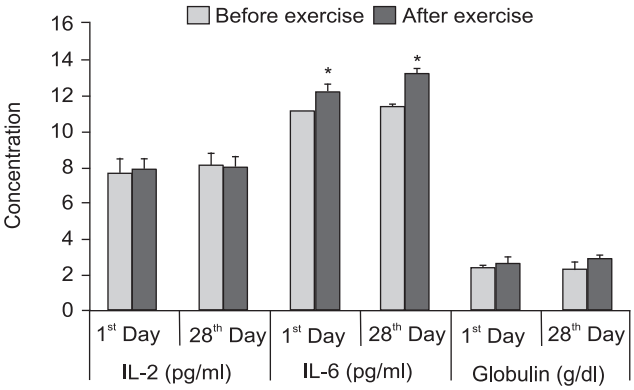


Fig. 3. Variation in cytokines level recorded in ponies at different time intervals before and after exercise. Values are presented as Mean±SE; *indicates significant difference (P<0.05).

IL-6 concentrations may contribute to altitude-associated illnesses (e.g., acute mountain sickness and high altitude pulmonary edema) and therefore may compromise physical work capacity (Smith *et al.* 2000). There is not much evidence on the serum interleukin-2 levels after exercise at high altitude. However, IL-2 levels in the serum have been found to decline in rats under hypoxic stress (Chunlan *et al.* 2014). In the present study, IL-2 levels in serum were

slightly elevated after endurance exercise on 28th day of running but difference was statistically significant. Therefore, cytokine would be good biomarker to assess endurance performance of ponies at high altitude.

It has been well demonstrated that endurance exercise training results in an increase in the antioxidant capacity (Powers *et al.* 2011). Ferric reducing ability of plasma (FRAP) and GPx increased significantly during, before and immediately after the endurance exercise. These findings were similar to other studies reported by Pialoux *et al.* (2006) and Krumrych (2010). Thus, the rise in FRAP values in these horses was probably caused by increased uric acid and GPx concentration, because it possesses free-radical-scavenging properties (Waring *et al.* 2003). It can be concluded that FRAP provides a better understanding of the stress reaction and metabolic processes in ponies. Our observation also correlates the FRAP concentration and uric acid concentration. Since, this study has brought important information on physio-biochemical and inflammatory cytokines relationship between physiological stress and endurance exercise in ponies, which need further examinations and correlation with load carrying capacity on larger population size.

In conclusion, this study provides important information to identify the hematological, physio-biochemical, and immune parameters that varies during endurance exercise in pony. Physiologic response and haemogram is a good indicator of endurance test. This study indicated up-regulation of antioxidants, metabolic enzymatic activity and IL-6 cytokines. Hence, these biomarker may be used to examine endurance performance in ponies before putting on load carrying work. However, considering variations in environmental factors, further mechanistic studies are essential for detailed investigation at molecular level for nutritional intervention to improve their performance.

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