IJPSR (2015), Vol. 6, Issue 12 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 26 June, 2015; received in revised form, 24 July, 2015; accepted, 30 September, 2015; published 01 December, 2015

SEA BUCKTHORN (*H. RHAMNOIDES* L.) MEDIATED ACUTE HYPOXIC TOLERANCE IN THE SKELETAL MUSCLE OF RATS BY DIFFERENTIAL ACTIVATION OF ENERGY METABOLISM AND ENHANCED ANTIOXIDANTS

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Keywords:

Antioxidants; energy metabolism; HIF-1α; hypobaric hypoxia; oxidative stress; Sea buckthorn, skeletal muscle

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ABSTRACT: Exposure to hypobaric hypoxia differentially affects physical performance and survival amongst individuals. The present study was designed to investigate the role of metabolic enzymes, antioxidants and bioenergetics molecular markers in the differential hypoxic tolerance of animals exposed to acute hypobaric hypoxia and the effect of herbal supplementation in augmentation of hypoxia tolerance. Adult rats were categorized as susceptible (<10 min), normal (10-25 min) and tolerant (>25min) on the basis of time taken for onset of gasping when exposed to a simulated altitude of 9754 m (~205 mm Hg). Animals susceptible to hypoxic stress showed significantly higher levels of reactive oxygen species and malondialdehyde, concomitant with lower endogenous antioxidants viz. superoxide dismutase (SOD) and catalase (CAT) levels. These groups of animals also showed increased lactate dehydrogenase activity. Conversely, tolerant animals displayed enhanced antioxidants (SOD, CAT and GSH), citrate synthase (CS) and glucose-6-phosphate dehydrogenase (G6PD) activities. Hypobaric hypoxia up-regulated the expression of key signaling proteins involved in energy metabolism viz. hypoxia inducible factor-1α (HIF-1α), AMP-activated protein kinase-α **(**AMPKα) and glucose transporter4 (GLUT4) in the tolerant group. Supplementation of Sea buckthorn (SBT) leaf aqueous lyophilized extract, distinctly improved the hypoxic gasping time in animals. This may be due to the SBT mediated increase in the CS, G6PD activity and AMPKα, GLUT4 expression in the treated group compared to hypoxia group. In conclusion, better bioenergetics and antioxidants status might be responsible for tolerance behaviour in rats under hypoxia. Further, SBT supplementation imparts tolerance to hypoxia susceptible animals by facilitating intracellular energy content and augmenting antioxidants under acute hypoxia.

INTRODUCTION: High altitude (HA)-induced hypobaric hypoxia has severely debilitating effects on cellular metabolism and performance of lowlanders at the HA. The decrease in cellular oxygen availability in hypoxic stress leads to many physiological responses in cells as oxygen plays a dominant role in the metabolism and viability of cells [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.6(12).5259-72

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.6(12).5259-72

Hypoxia induces the transcription of number of genes, among them those for the glycolytic enzymes plays a critical role in adaptation process. In-vitro studies have suggested that the transcription factor such as hypoxia-inducible factor 1-alpha (HIF1-α), is up-regulated in hypoxia, increasing glycolysis and thereby attenuating oxygen utilization and ATP synthesis [2, 3]. Another study suggests that the optimal use of oxygen in hypoxia is mediated by inducing metabolic pathways that favour the utilization of carbohydrates instead of lipids as substrate by up-regulating glycolytic and oxidative pathways for sustained supply of energy metabolites [4]. Skeletal muscle, like all oxidative tissues of the body, is critically dependent on a supply of oxygen to maintain energetic and redox homeostasis. ATP

can be synthesised in the skeletal muscle in an oxygen-dependent manner in the mitochondria via oxidative phosphorylation. The substrate for generating ATP could be glycolytically derived pyruvate, fatty acids, amino acids and ketone bodies. ATP synthesis in oxygen-independent manner take place in the cytosol, via glycolysis with the resulting pyruvate converted to lactate [3]. The up-regulation of aerobic metabolic pathway is required to sustain physical activity as cells need more energy to replenish their ATP during stress conditions [5]. The metabolic key regulatory enzymes such as hexokinase (HK), lactate dehydrogenase (LDH), citrate synthase (CS) and glucose-6-phosphate dehydrogenase (G-6-PD) play a critical role in metabolic function under pathophysiological stress conditions [6].

Oxidative stress, a known consequence of hypoxia is a critical factor in the adaptation to the stressors at HA. HA-induced hypoxia reduces the availability of oxygen to cells; this in turn results in an increase in superoxide production from all superoxide producing sites within the electron transport chain. Free radicals generated during hypobaric hypoxia causes oxidation of nucleic acids and proteins, it also damage biomembranes, reflected by increased lipid peroxidation, thereby compromising cell integrity and function [7]. Some studies demonstrated that endogenous antioxidants viz. superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase decrease in rats after hypobaric hypoxic exposure at 5500 m [8]. Another study reported a 3-fold increase in basal levels of lipofuscin, a marker of oxidative stress, in the vastus lateralis muscles of climbers after HA expedition at 5000 m [9].

However, not all the individuals respond similarly under such stressful conditions, the ability of an organism to respond and cope with the stressful conditions depends upon their genetic make-up. HA-natives (HAN) such as Tibetans and Andeans differ from their lowland counterparts in their ability to adjust to the hypobaric hypoxia. Studies comparing HAN and sea level population have shown that oxidative damage at HA is generally less in highlanders even though most of the antioxidants are reduced [10], which emphasize the importance of decreased reactive oxygen species (ROS) production in preventing oxidative damage. In our earlier studies, we demonstrated the significance of enhanced antioxidants and hypoxia responsive proteins in the better hypoxic tolerance within the rat myocardium [11, 12].

The management of unusual environmental stress can also be achieved by a food supplements, dietary elements, herbs and minerals for increasing physical and mental performance. Supplementation with various herbal extract has been shown to possess adaptogenic activity during exposure to stressful environment [13]. The medical benefits of Sea buckthorn (SBT) (*Hippophae rhamnoides* L., Elaegnaceae), a thorny nitrogen-fixing deciduous shrub, have been reported against mucosal injuries, skin disorders, cardiovascular diseases and flu that might be due to its immunomodulatory, adaptogenic, reparative and antioxidative activities [14]. Leaf extract of SBT, has potent anti-stress activity and has shown to protect against hypoxia-induced pulmonary vascular leakage and ROS-mediated damage [6, 15, 16].

The present study investigated the differential performance of rats under acute hypobaric hypoxia by evaluating key metabolic enzymes and antioxidants status in the gastrocnemius muscle and, further evaluated the effect of SBT supplementation in augmentation of hypoxic tolerance. Effect of key bioenergetics marker viz. glucose transporter4 (GLUT4), AMP-activated protein kinase-α (AMPKα) and HIF-1α on differential hypoxic performance has also been evaluated. Identification of regulatory biochemical and molecular markers can provide a better idea about HA-induced adaptation in the gastrocnemius muscle. Further, the possible associated mechanism of action of SBT-induced hypoxic tolerance was explored.

MATERIALS AND METHODS:

Animals:

The animal experiments were performed in accordance with the regulations specified by the Institute's Animal Ethical Committee (IAEC) and conform to the national guidelines on the care and use of laboratory animals, India. Male adult Sprague-Dawley rats (180 ± 20 g) were used in this study. The animals were maintained under

controlled environment at institute's animal house at 25 ± 1 ^{0}C, 12-h light-dark cycle with access to food and water *ad libitum*.

Plant material and extract preparation:

SBT leaves were collected in the month of September, 2011 from hilly regions of North-West Himalayas (the region lies between latitude 32-36^o North and longitude 76-79^o), India. Plant material was characterized by Dr O. P. Chaurasia, an ethanobotanist at the Defence Institute of High Altitude Research (DIHAR), Leh, India. Fresh leaves of SBT were cleaned and washed thoroughly with nanopure water. Washed fresh leaves were dried under shade in a clean, dust free environment and crushed using laboratory blender. The aqueous extract was prepared by soaking powdered dry leaves in distilled water (1:5, w/v) at room temperature (25 ± 1 ^{0}C). After 24 h, the supernatant was decanted and the residue resoaked in fresh distilled water. The process was repeated 4-times for complete extraction. The supernatants were pooled, filtered through muslin cloth and centrifuged at 5000 g, 4 ^{0}C. The supernatants obtained after centrifugation were frozen at −20 ^{0}C and then lyophilized in Heto lyophilizer (HITOSICC, Heto-Holten A/S, Denmark). Lyophilized powder of the SBT leaf aqueous extract was stored at −20 ^{0}C in an airtight plastic container until further use (yield 17.3%, w/w). RP-HPLC fingerprinting of each batch of the extract was carried out and maintained throughout the experiment, to avoid batch-to-batch variation

Hypobaric hypoxia exposure and experimental design:

The present study was divided into two sets. For the first set of experiment, rats were divided into two groups viz. normoxic control (unexposed) and hypoxia exposed. The hypoxia exposed group rats were exposed once to simulated altitude of 9754 m (~ 32,000 ft) in an animal decompression hypobaric chamber (Decibel Instruments, Delhi) and their hyperventilation time noted [11]. The hypoxia exposed rats were further divided into 3 sub-groups (n=6 each) based on hyperventilation time: normal (10-25 min), tolerant (>25 min) and susceptible (<10 min) as described earlier [11, 12]. Normoxic control group rats (n=6) were maintained in the normoxic condition with the same laboratory conditions. In second set of experiment, rats were again divided into normoxic (unexposed) group and hypoxia exposed group. Normoxic group further subdivided into normoxic control (n=6) and SBT treated normoxic groups (n=6). The hypoxic exposed group also subdivided into two groups (n=6 each) comprises hypoxia and SBT treated hypoxia groups (rats were first exposed to decompression hypobaric chamber, noted their hyperventilation time then orally administrated with SBT leaf aqueous lyophilized extract (100 mg/kg body weight) for 7 consecutive days and again exposed to decompression hypobaric chamber and noted their hyperventilation time).

Animals were sacrificed using sodium pentobarbital and hind limb gastrocnemius muscles tissue was collected. A tissue homogenate (20%, w/v) was prepared in homogenizing buffer containing 0.15 M KCL and 5 mM EDTA using Polytron tissue homogenizer (PT 3100, Switzerland). Further, samples were sonicated (10 bursts of 5 second) using Ultra-Sonicator (VC-505, Sonics Vibra Cell, USA). The supernatant was collected for estimation of enzyme activities following centrifugation at 9000 x g in a refrigerated centrifuge for 20 min.

Assays of energy metabolising enzyme activities:

Hexokinase (HK) activity:

The HK (E.C.2.7.1.1) activity was measured in muscle tissue homogenates by the coupled enzyme assay system as described by Supowit and Harris [17] using glucose as substrate. The sample was mixed with assay buffer consist of 50 mM triethanolamine with 10 mM $MgCl_2$ (pH 7.4), 1M glucose, 50 mM NADP and 140 U/mL of glucose 6-phosphate dehydrogenase. The reaction was initiated by the addition of 200 mM ATP, and the rate of NADP reduction was recorded at 340 nm for 3 min using UV-Vis spectrophotometer (Smartspec 3000, BioRad, USA). Calculation of enzyme activity was based on the reduction of NADP (ε_{NADP} = 6220 M^{-1} cm^{-1}). The enzyme activity has been expressed as n mol/min/mg protein.

Lactate dehydrogenase (LDH) activity:

LDH (E.C.1.1.1.27), a marker of cell membrane permeability and anaerobic glycolysis, was estimated in muscle tissue homogenate as described

by Kornberg [18] using sodium pyruvate as a substrate. The tissue sample was mixed with LDH reaction solution containing 10 mM sodium pyruvate in 100 mM sodium phosphate buffer (pH 7.0). Reaction was initiated by the addition of 4.22 mM NADH and assayed at 340 nm for 3 min. The LDH activity was calculated based on the oxidation of NADH (ε_{NADH} = 6220 M^{-1} cm^{-1}) and expressed as n mol/min/mg protein.

Citrate synthase (CS) activity:

The CS (E.C.1.11.1.6) activity was measured using the method described by Shepherd and Garland [19]. The method was based on the chemical coupling of CoA-SH, released form acetyl-CoA, to Ellman's reagent 5, 5'-dithiobis [2-nitro-benzoic acid] (DTNB) during the enzymatic synthesis of citrate. The sample was added to 100 mM Tris-HCl buffer (pH 8.0) with 10 mM DTNB and 5 mM acetyl-CoA. Reaction was initiated by the addition of 50 mM oxaloacetate. The rate of change in extinction was measured at 412 nm. Calculation of enzyme activity was based on ε = 5.4 cm2/μ mole. CS activity was expressed as n mol/min/mg protein.

Glucose-6-phosphate dehydrogenase (G6PD) activity:

The G6PD (E.C.1.1.1.49) assay was performed by the method of Lohr and Waller [20] using glucose-6-phosphate as a substrate. The sample was mixed with assay buffer consist of 50 mM triethanolamine buffer with 5 mM EDTA (pH 7.5) and 30 mM NADP. Reaction was initiated by the addition of 42.6 mM glucose-6 phosphate. The G6PD-dependent reduction of NADP was measured as the increase in A340. The calculation of enzyme activity was based on reduction of NADP (ε_{NADH} =6220 M^{-1} cm^{-1}). The enzyme activity has been expressed as n mol/min/mg protein.

Assays of oxidative stress markers:

ROS measurement:

ROS levels were measured with a non-fluorescent lipophilic dye, dichlorofluorescein diacetate (DCFH-DA) as described earlier [21]. Briefly, 150 µl of muscle tissue homogenate was incubated with 10 µl of 100 µM DCFH-DA for 30 minutes in dark. Fluorescence was read using a fluorimeter (Perkin Elmer, UK) with excitation at 485 nm and emission at 535 nm. Readings were obtained in arbitrary flourometric units and results expressed as fold change in free radical generation over control.

Lipid peroxidation (LPO):

LPO was measured by direct estimation of MDA using the modified method of Dousset et al. [22]. Briefly, to 250 µl of muscle tissue supernatant, 750 µl of tri-chloroacetic acid (20%) and 750 µl of thiobarbituric acid (0.67% in 0.05 M NaOH) was added. The samples were incubated in a water bath at 85 ^{O}C for 45 min. The samples were then kept at room temperature followed by centrifugation at 1000 x g for 5 min. The absorbance of supernatant was measured at 531 nm. Results were expressed as n mol MDA formed/ mg protein.

Antioxidant assays:

Estimation of reduced glutathione (GSH) content:

GSH was measured in muscle tissue homogenates by the method of Beutler et al. [23]. Briefly, 0.1 mL of sample was added into 0.9 mL distilled water and 1.5 mL of precipitating reagent (3.34 g metaphosphoric acid, 0.4 g EDTA and 60.0 g NaCl). The mixture was then allowed to stand for 5 min at room temperature and centrifuged for 15 min at 3000 x g at 4 °C. Further 4.0 ml of 0.3 M phosphate solution and 0.5 mL 5-5′-dithiobis- (2-nitrobenzoic acid) (80 mg in 1% sodium citrate) were added to 1 mL of supernatant. The absorbance of developed yellow complex was read immediately at 412 nm. GSH concentrations were calculated using reduced glutathione as standard and expressed as n mol GSH/ mg protein.

Assay of superoxide dismutase (SOD) activity:

SOD (E.C.1.15.1.1) activity was estimated in muscle tissues by the method of Marklund and Marklund [24]. The total SOD assay volume (3.0 mL) consisted of 1.5 mL of 50 mM Tris-cacodylate buffer pH 8.2, 0.3 mL of nitro blue tetrazolium salt (NBT) (1mM in water), 0.3 mL of Triton-X-100 (0.01%), 0.8 mL of water, 0.1 mL of sample and 0.01 mL of pyrogallol (60 mM in water, substrate). Enzyme kinetic activity was recorded at 540 nm for 3 min and change in OD per minute. Δ OD was used to calculate % auto-oxidation inhibition to derive SOD units (U). One U of SOD was defined as 50% inhibition of the auto-oxidation caused by a

certain value of enzyme. The results of SOD activity have been expressed as unit/ mg protein.

Assay of catalase (CAT) activity:

The CAT (E.C.1.11.1.6) activity was measured in muscle homogenate by the method of Aebi [25]. The reaction mixture consisted of 2.9 mL buffer substrate (containing 0.1% H_2O_2 in 50 mM sodium potassium phosphate buffer, pH 7.0) and 0.1mL sample in the final 3.0 mL assay volume. Change in absorbance was recorded for 150 s (15 second interval) at 240 nm. Calculation of enzyme activity is based on εH2O2 = 43.6 M–1 cm–1. The results of CAT activity expressed as unit/ mg protein.

Total protein estimation:

Protein in the muscle tissue homogenates was determined by the method of Lowry et al. [26]. Bovine serum albumin (BSA) was used as a standard.

Immunoblotting for protein expression:

For nuclear and cytosolic fractionation, frozen muscle tissue was homogenized in ice-cold buffer containing 0.5 M sucrose, 10 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol, 1mM EDTA, 1mM PMSF and protease inhibitor cocktail with pH 7.6. Homogenates were kept on ice for 15 min and 0.6% Nonidet P-40 was added, and then centrifuged for 10 min at 2000 g at 4 ^{0}C. The supernatant with cytosolic fraction was collected, stored, and the pellet was dissolved in cold buffer B (20mM HEPES, 1.5mM $MgCl_2$, 0.3mM NaCl, 0.2mM EDTA, 20% glycerol, 0.5mM DTT, 0.5mM PMSF and cocktail of protease inhibitors) for the nuclear fraction. It was incubated for 30 min on ice followed by centrifugation at 20,000 x g at 4 ^{0}C for 15 min. The supernatant containing the nuclear fraction was aliquoted and stored at -80 ^{0}C for further analysis. Total protein concentrations were determined using the method of Lowry et al. [26]. Protein expressions of GLUT-4, AMPKα were quantified in cytosolic extract, whereas HIF-1α was analysed in the nuclear extract by Western blotting. Primary antibodies against GLUT-4 (#G4173), AMPK-α (#A3730) and HIF-1α (#H6536) were obtained from Sigma-Aldrich, St. Louis, MO. The protein (50 µg) was separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (Whatman GmbH, Germany). The membranes were blocked with 5% bovine serum albumin in phosphate buffer saline (PBS) containing 0.1% Tween-20 (Sigma, USA), washed and probed with respective rabbit polyclonal antibodies. The membranes were then incubated with anti-rabbit-IgG horseradish peroxidise (HRP) conjugated (Sigma-Aldrich, #A0545). The membrane was washed and incubated with chemiluminescent substrate (Sigma-Aldrich, #CPS-1-60) and the bands were developed using X-ray films (Kodak, Rochester, NY). The band densities were quantified using a scanning densitometric analysis and Fiji (Image J) software program (NIH, Bethesda, MD).

Statistical analysis:

Values are presented as mean ± SEM. One way analysis of variance (ANOVA) followed by post-hoc Bonferroni analysis was used to determine the statistical significance between groups (SPSS 17 software). A *p*-value < 0.05 was considered statistically significant. Densitometric data for Western immunoblots were expressed as a percentage of the control mean density after normalization to loading controls.

RESULTS:

Effect of acute hypobaric hypoxia on enzyme activities associated with energy metabolism:

Fig.1 depicts the *per se* effect of acute hypobaric hypoxia on differential enzyme activities of key regulatory energy metabolism in the skeletal muscle of rats. The result of present study showed significant ($p < 0.05$) higher HK activity in normal (43%) and susceptible (26%) hypoxia groups; however, non-significant change was recorded in tolerant group compared to the normoxic group in the skeletal muscle tissue (**Fig. 1A**). Similarly, the LDH exhibited significant ($p < 0.05$) increased activity in normal (50%) and susceptible (56%) groups, however its activity remained non-significantly changed in tolerant group compared to the normoxic control group (**Fig. 1B**).

The activity of CS significantly ($p < 0.05$) increased in tolerant (32%) group and significantly ($p < 0.05$) decreased in normal (23%) and susceptible groups (33%), as compared to the normoxic control group (**Fig. 1C**). G6PD exhibited significant ($p < 0.05$) increase activity in the

skeletal muscle of tolerant group (73%); however, it registered a significant ($p < 0.05$) decreased activity in susceptible group (23%) compared to the normoxic control animals (**Fig. 1D**).

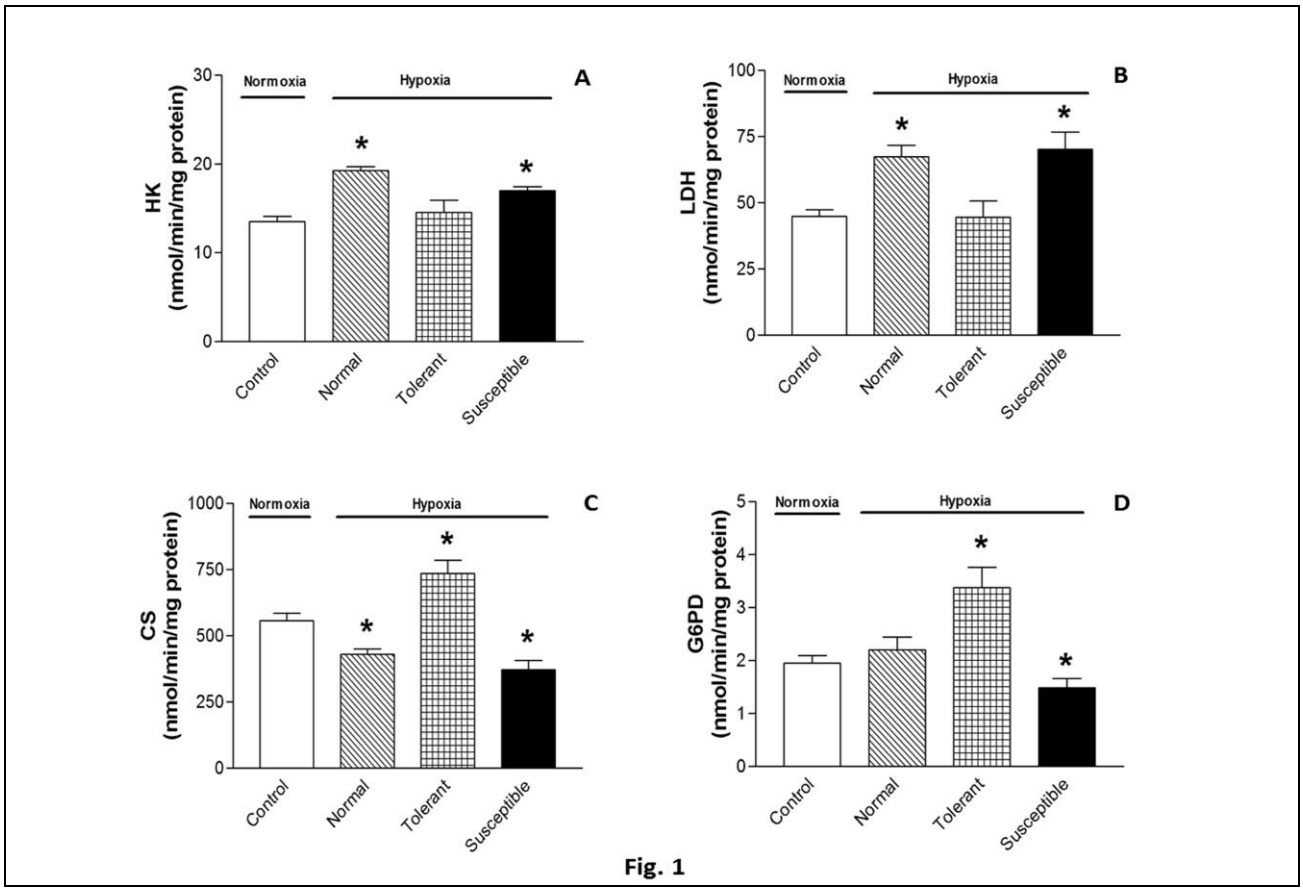

FIG.1: EFFECT OF ACUTE HYPOBARIC HYPOXIA ON KEY REGULATORY ENZYMES ASSOCIATED WITH ENERGY METABOLISM IN SKELETAL MUSCLE OF RATS. (A) HEXOKINASE (HK), (B) LACTATE DEHYDROGENASE (LDH), (C) CITRATE SYNTHASE (CS), (D) GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) ACTIVITIES IN SKELETAL MUSCLE OF RATS.

Values are mean ± SEM, n = 6 animals per group. ∗ $p < 0.05$ compared to the normoxic control group.

Effect of acute hypobaric hypoxia on oxidative stress and endogenous antioxidant levels:

Exposure to acute hypobaric hypoxia resulted in a significantly ($p < 0.05$) enhanced ROS level (3.5-fold) in susceptible group compared to the normxic control group (**Fig. 2A**). The MDA level in the skeletal muscles of susceptible group was significantly ($p < 0.05$) increased compared to the normoxic control group, (**Fig. 2B**). In normal and tolerant group of animals, the change in these oxidative stress parameters was non-significant. GSH level showed a significant ($p < 0.05$) elevation in tolerant animals vs. the normoxic control animals (**Fig. 2C**).

Evaluating the antioxidant enzyme activity, we found that while SOD activity did not increase significantly in the susceptible group, a significant ($p < 0.05$) increase in SOD activity was observed in the normal and tolerant groups as compared to the normoxic control group (**Fig. 2D**). The CAT activity was also found to be significantly ($p < 0.05$) higher in tolerant as compared to the normoxic control group (**Fig. 2E).**

Effect of acute hypobaric hypoxia on protein expression of GLUT4, AMPKα and HIF-1α:

Western immunoblot analysis of acute hypobaric hypoxia exposed skeletal muscle revealed differential protein expression of GLUT 4, AMPKα and HIF-1α. The Western immunoblot results showed significant ($p < 0.05$) up-regulated expression of GLUT4 in tolerant and normal group animals, while non-significant change observed in susceptible and animals. Protein expression of AMPKα in tolerant animals was significantly ($p <$

0.05) up-regulated, however, insignificant changed found the susceptible and normal group animals (**Fig. 3A, 3B** and **4A, 4B**). Expression of HIF-1α was measured in the nuclear extracts of skeletal muscle tissue homogenates by immunblotting. A significant ($p < 0.05$) upregulation was seen in the protein expression of HIF-1α in the tolerant and normal groups. However the expression of HIF-1α in the susceptible animals remained largely unchanged (**Fig. 3C** and **4C**).

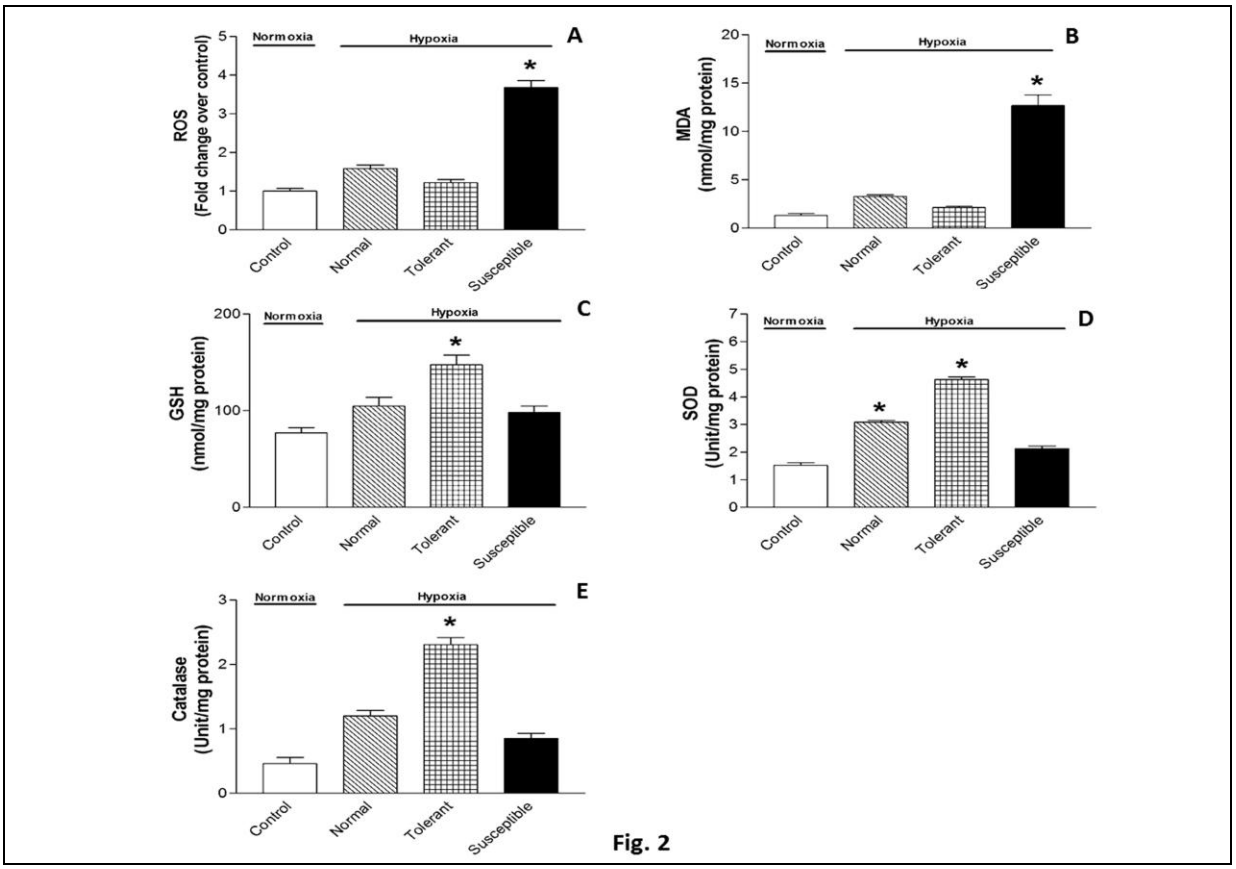

FIG.2: EFFECT OF ACUTE HYPOBARIC HYPOXIA ON OXIDATIVE STRESS MARKERS IN SKELETAL MUSCLE OF RATS. (A) ROS LEVEL, (B) LIPID PEROXIDATION INDICATED BY MALONDIALDEHYDE (MDA) LEVELS, (C) REDUCED GLUTATHIONE CONTENT (GSH), (D) SUPEROXIDE DISMUTASE (SOD) AND (E) CATALASE ACTIVITIES IN SKELETAL MUSCLE OF RATS.

Values are mean ± SEM, n = 6 animals per group. ∗ $p < 0.05$ compared to the normoxic control group.

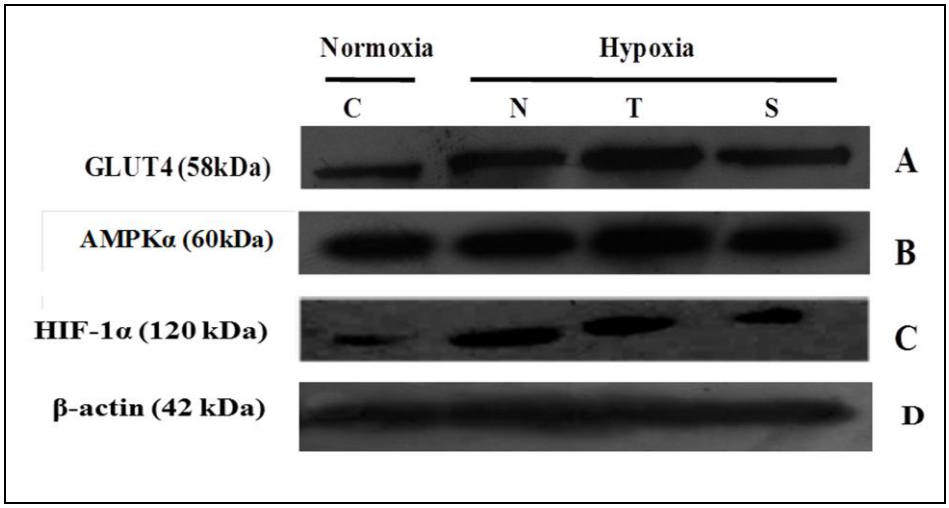

FIG.3: EFFECT OF ACUTE HYPOBARIC HYPOXIA ON DIFFERENTIAL PROTEIN EXPRESSION OF BIOENERGETICS MARKERS (A) GLUCOSE TRANSPORTER4 (GLUT4), (B) AMP-ACTIVATED PROTEIN KINASE α (AMPKα) AND (C) HYPOXIA INDUCIBLE FACTOR-1α (HIF-1α) IN SKELETAL MUSCLE OF RATS. (D) β-ACTIN TAKEN AS LOADING CONTROL.

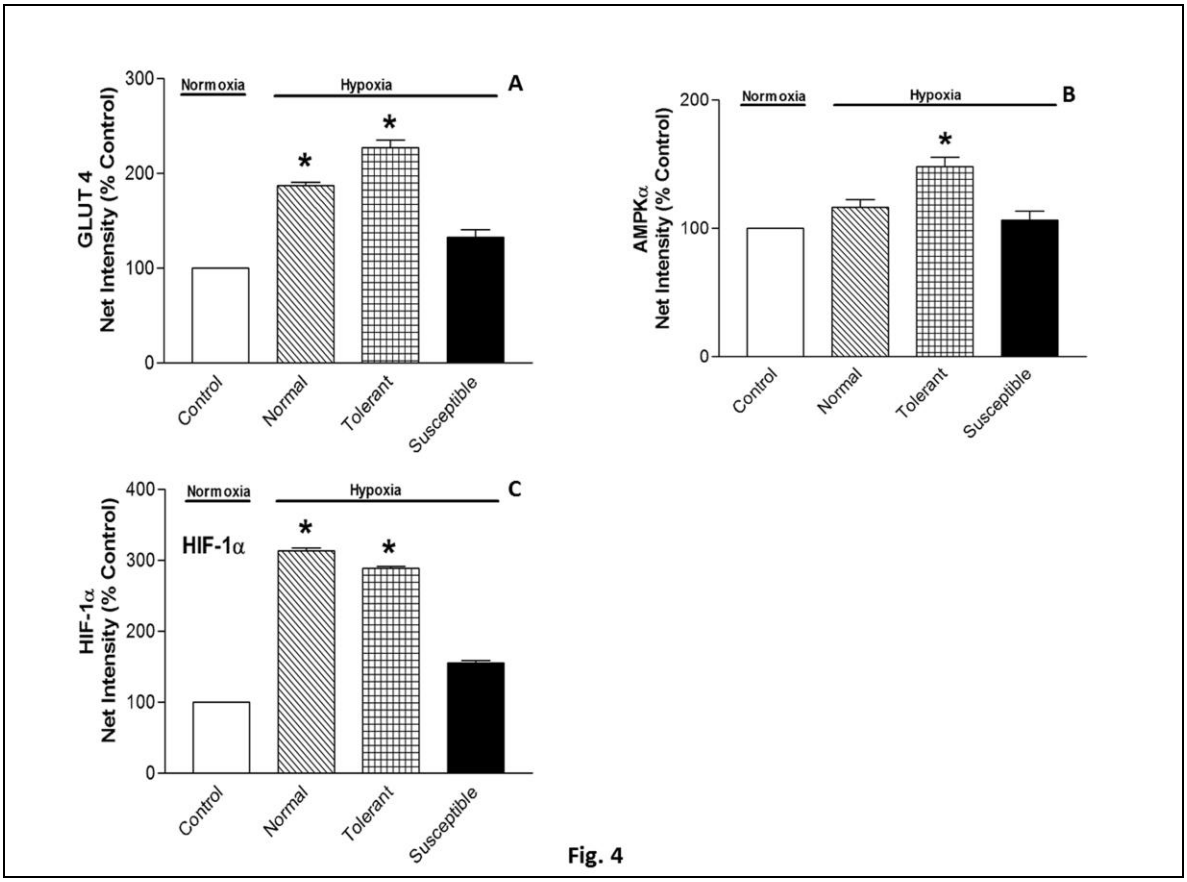

FIG.4: DENSITOMETRIC ANALYSIS OF WESTERN BLOTS USING FIJI (IMAGE J) SOFTWARE. DIFFERENTIAL PROTEIN EXPRESSION OF (A) GLUCOSE TRANSPORTER4 (GLUT4), (B) AMP-ACTIVATED PROTEIN KINASE α (AMPKα) AND (C) HYPOXIA INDUCIBLE FACTOR-1α (HIF-1α) IN RESPONSE TO ACUTE HYPOBARIC HYPOXIC STRESS IN NUCLEAR AND CYTOSOLIC EXTRACTS. CHANGE IN PROTEIN EXPRESSION EXPRESSED AS NET INTENSITY (% NORMOXIC CONTROL GROUP).

Values are mean ± SEM, n = 6 animals per group. ∗ $p < 0.05$ compared to the normoxic control group.

Effect of SBT administration on hyperventilation time of rats under acute hypobaric hypoxia:

Treatment with SBT leaf aqueous lyophilized extract showed increase in the hyperventilation time. The hyperventilation time of rats under acute hypobaric hypoxia (simulated altitude of ~ 32,000 ft.) was recorded 12 min ± 2 min prior SBT administration. However; after oral administration with SBT aqueous extract for constitutive 7 days treatment increased their hyperventilation time significantly ($p < 0.05$) to 32 min ± 2 min under acute hypobaric hypoxia.

Effect of SBT administration on enzyme activities associated with energy metabolism under acute hypobaric hypoxia:

SBT leaf aqueous lyophilized extract administration showed insignificant increase in the HK activity in SBT treated normoxic and SBT treated hypoxia groups, however a significant ($p < 0.05$) increased activity was recorded in hypoxia group compared to normoxic control group **(Fig. 5A).** Similarly, the LDH activity significantly ($p < 0.05$) increased in hypoxia group, however its activity remained unchanged in SBT treated normoxic and SBT treated hypoxia groups compared to normoxic control group **(Fig. 5B**). The activity of CS significantly ($p < 0.05$) increased in SBT treated hypoxia group whereas significantly ($p < 0.05$) decreased in hypoxia group and remained unchanged in SBT treated normoxic group compared to normoxic control group (**Fig. 5C**).

G6PD exhibited significant ($p < 0.05$) increase activity in the SBT treated hypoxia group; however, it registered a significantly ($p < 0.05$) decreased activity in hypoxia group and remained unchanged in SBT treated normoxic group compared to the normoxic control animals (**Fig. 5D**).

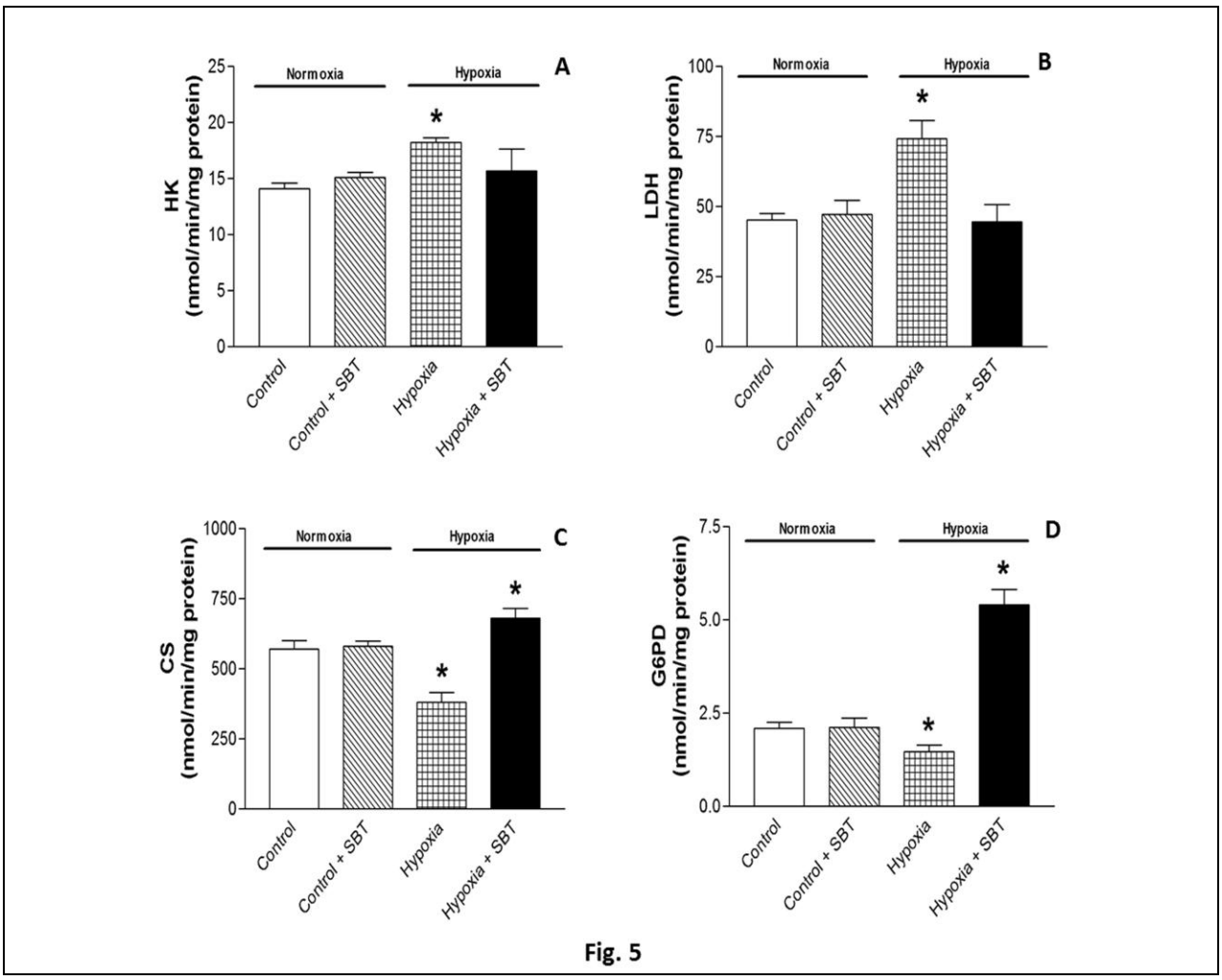

FIG.5: EFFECT OF SEA BUCKTHORN (SBT) LEAF AQUEOUS LYOPHILIZED EXTRACT ADMINISTRATION ON ENZYME ACTIVITIES ASSOCIATED WITH ENERGY METABOLISM UNDER ACUTE HYPOBARIC HYPOXIA IN SKELETAL MUSCLE OF RATS. (A) HEXOKINASE (HK), (B) LACTATE DEHYDROGENASE (LDH), (C) CITRATE SYNTHASE (CS), (D) GLUCOSE-6-PHOSPHATE DEHYDROGENASE (G6PD) ACTIVITIES IN SKELETAL MUSCLE OF RATS.

Values are mean ± SEM, n = 6 animals per group. ∗ $p < 0.05$ compared to the normoxic control group.

Effect of SBT administration on oxidative stress and endogenous antioxidant levels under acute hypobaric hypoxia:

ROS level was significantly ($p < 0.05$) increased in hypoxia group; however its generation was nil in other groups compared to the normoxic control group (**Fig. 6A**). The MDA level in the skeletal muscles of hypoxia group was significantly ($p < 0.05$) increased in compare to the normoxic control group. In SBT treated normoxic and SBT treated hypoxia animals; the change in these oxidative stress parameters was non-significant (**Fig. 6B**). GSH level showed a significant ($p < 0.05$) elevation in SBT treated hypoxia animals vs. the normoxic control animals (**Fig. 6C**). The SOD activity did not increase significantly in other groups except SBT treated hypoxia animals that showed a significant ($p < 0.05$) increase in SOD activity as compared to the normoxic control group (**Fig. 6D**). The enzyme activity profile of CAT was similar as SOD activity which showed significantly ($p < 0.05$) higher activity SBT treated hypoxia group and remained unchanged in other groups as compared to the normoxic control group (**Fig. 6E**).

Effect of SBT administration on protein expression of GLUT4 and AMPKα under acute hypobaric hypoxia:

The Western immunoblot results showed significant ($p < 0.05$) up-regulated expression of GLUT4 and AMPKα in SBT treated hypoxia group whereas, non-significant changes were observed in SBT treated normoxic and hypoxia groups as compared to the normoxic control group (**Fig. 7**).

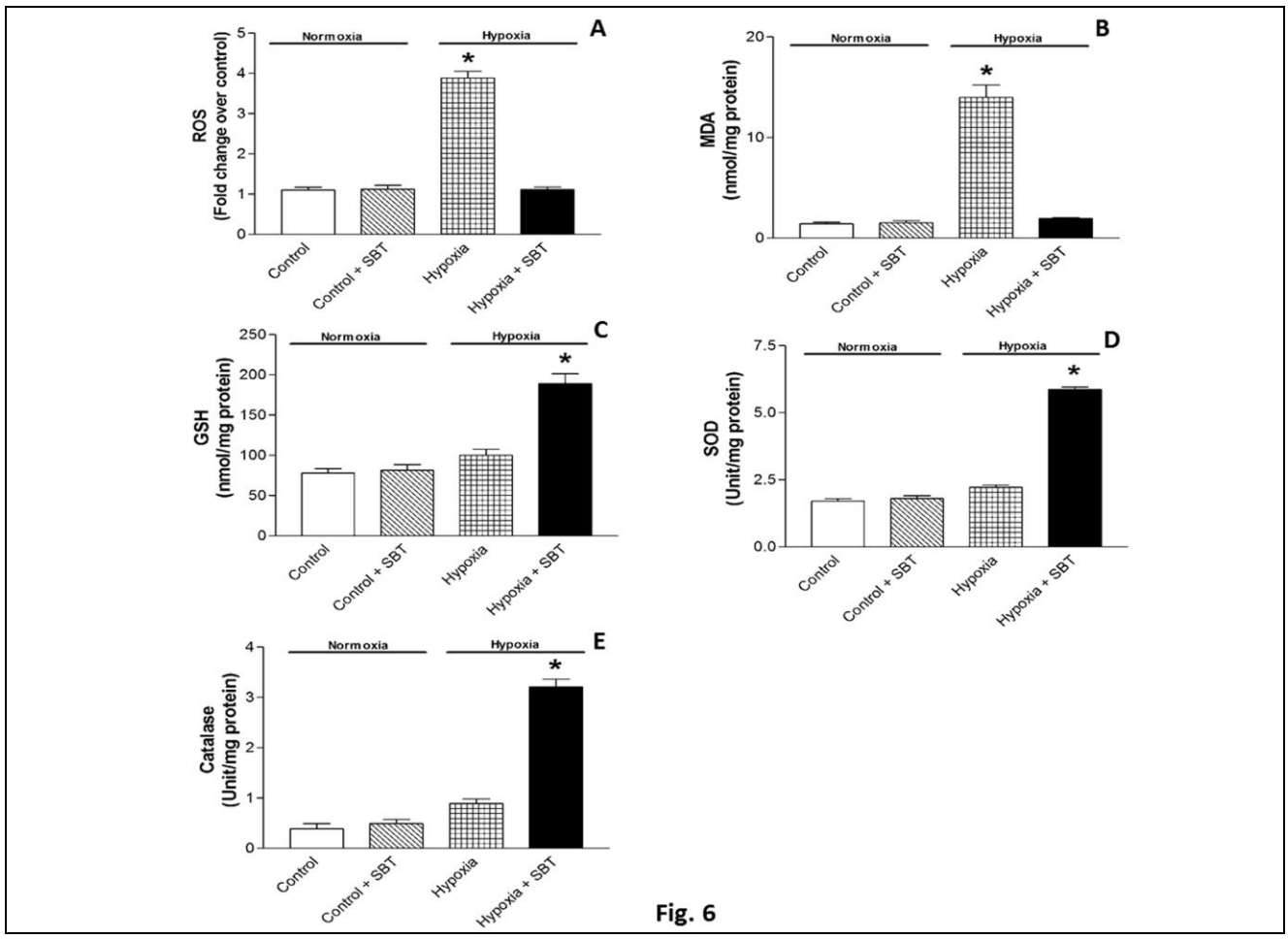

FIG.6: EFFECT OF SEA BUCKTHORN (SBT) LEAF AQUEOUS LYOPHILIZED EXTRACT ADMINISTRATION ON OXIDATIVE STRESS AND ENDOGENOUS ANTIOXIDANT LEVELS UNDER ACUTE HYPOBARIC HYPOXIA IN SKELETAL MUSCLE OF RATS. (A) ROS GENERATION, (B) LIPID PEROXIDATION INDICATED BY MALONDIALDEHYDE (MDA) LEVELS, (C) REDUCED GLUTATHIONE CONTENT (GSH), (D) SUPEROXIDE DISMUTASE (SOD) AND (E) CATALASE ACTIVITIES IN SKELETAL MUSCLE OF RATS. Values are mean ± SEM, n = 6 animals per group. ∗ $p < 0.05$ compared to the normoxic control group.

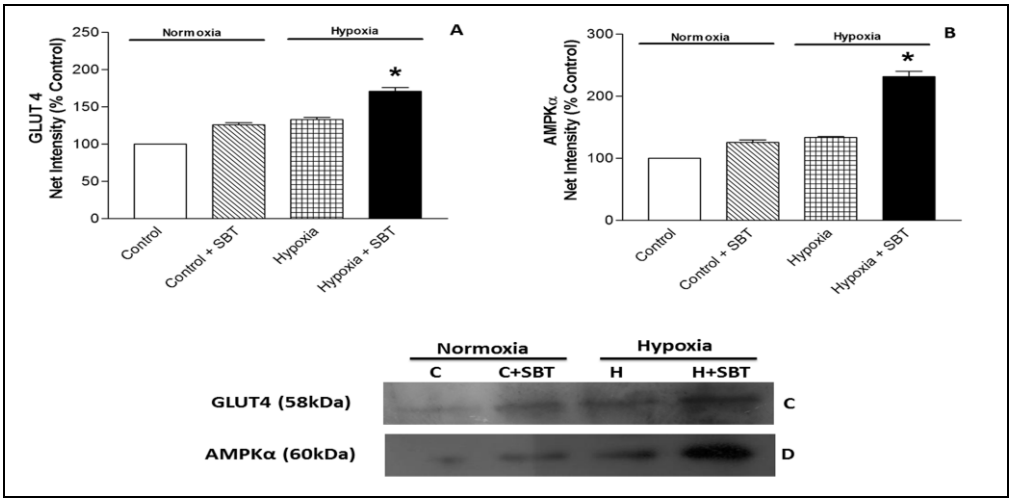

FIG.7: DENSITOMETRIC ANALYSIS OF WESTERN BLOTS USING FIJI (IMAGE J) SOFTWARE. EFFECTS OF SEA BUCKTHORN (SBT) LEAF AQUEOUS LYOPHILIZED EXTRACT ADMINISTRATION ON PROTEIN EXPRESSION OF BIOENERGETICS MARKERS UNDER ACUTE HYPOBARIC HYPOXIA IN SKELETAL MUSCLE OF EXPERIMENTAL RATS IN CYTOSOLIC EXTRACTS. (A) GLUCOSE TRANSPORTER4 (GLUT4) AND (B) AMP-ACTIVATED PROTEIN KINASE α (AMPKα). CHANGE IN EXPRESSION EXPRESSED AS NET INTENSITY (% NORMOXIC CONTROL GROUP).
Values are mean ± SEM, n = 6 animals per group. ∗ $p < 0.05$ compared to the normoxic control group. (C) And (D) depicts the representative Western blot images of GLUT4 and AMPKα.

DISCUSSION: The ability to respond with any kind of environmental stress differs from individual to individual, depending on the inherent threshold of stress with which they can deal. HA-induced hypobaric hypoxia stress causes a fall in the availability of oxygen to the cells leading to a shift in the energy metabolic pathway from aerobic to anaerobic mode. Study reported by Hoppeler and Vogt [4] have shown that the indigenous HA population have evolved mechanisms to withstand hypoxic conditions by using more closely relate oxygen supply and subsequent energy generation. It has been reported that food supplements, dietary elements, herbs and minerals increase the physical and metal performance of the body by supporting the adaptation process during stressful situations [6]. The plants cultivated in adverse climatic conditions, such as Sea buckthorn, have acquired biomolecules which help them to sustain life under harsh environments [27].

The present study investigated the effect of hypobaric hypoxia on the energy metabolizing enzymes (HK, LDH, CS and G6PD) in the skeletal muscle of the experimental rats. Glucose is of central metabolic importance in virtually all organisms, from microbes to humans. Glycolytic metabolism of glucose is a major pathway for the generation of energy (ATP), and glycolytic intermediates also serve as precursors for biosynthesis of other cellular constituents. The first step in metabolism of glucose is usually phosphorylation, catalyzed by HK. This study demonstrated that HK and LDH activity significantly increased in normal and susceptible groups and its activity remain unchanged in rest of all groups. Increased activity of LDH indicates anaerobic regulation of cellular metabolism in susceptible and normal hypoxic rats.

Although anaerobic glycolysis is an instant source of energy during hypoxic stress but this metabolic pathway does not provide enough energy to manage with hypoxic stress, hence both normal and susceptible hypoxic group showed minimal resistant to hypoxia. The increased LDH activity under acute hypobaric hypoxia in normal and susceptible groups correlates with the findings of Yoshino et al. [28] that showed markedly increased plasma LDH activity in rats when exposed to high altitude. The increased CS activity indicated better regulation of TCA cycle in the tolerant and SBT treated hypoxia rats that favour the complete oxidation of substrate for maximum energy output in mitochondria in terms of ATP. More production of ATP compensates the energy requirement needed by the cells to overcome hypoxic stress. The adaptive role of increased CS activity in diving muscle has been reported earlier [29] but for the first time we are reporting the role of increased CS activity in tolerance behavior of skeletal muscle under acute hypobaric hypoxia.

Increased activity of CS in SBT treated hypoxia group is correlate with the previous study done by Saggu et al 2007 which showed increase in CS activity in Seabuckthorn treated C-H-R exposed rat in compared to the unexposed control. On the basis of CS activity level, we conclude that tolerant and SBT treated hypoxia rats follow aerobic glucose metabolism for better adaptation process than normal and susceptible hypoxic rats. Our results are in agreement of the earlier reports indicating increased aerobic activity in skeletal muscles of high altitude Guinea pig and human HA natives versus lowlanders [30, 31]. In another study, Kanatous et al. [29] has reported aerobic energy metabolism in the muscle of Weddell seals under hypoxia condition imposed by deep sea diving.

Metabolism of glucose through the pentose phosphate pathway (PPP) generates NADPH and precursors, required for a variety of anabolic pathways. G6PD is the first enzyme in the PPP and the main intracellular source of NADPH, involved in diverse physiological processes. In the present study, enhanced activity of G6PD in the tolerant and SBT treated hypoxia groups indicates that increased production of NADPH helps to maintain redox state of cell; which further supports animal to cope up with oxidative stress. The result of G6PD correlate parallels with increased GSH contents in the tolerant and SBT treated hypoxia rats as increase NADPH levels helps to maintain intracellular GSH content to overcome hypoxic stress. These findings were consistent with some previous studies suggesting increased NADPH production in pulmonary artery and lungs exposed to hypoxia [32, 33]. The more oxidative damage in normal susceptible and hypoxia groups may be due

to decrease NADPH production and less endogenous GSH contents.

Hypoxia is known to mediate an elevation in free radical generation [34, 35]. The increase in anaerobic respiration also generates excess free radicals causing an imbalance between ROS and antioxidant defence mechanism. These harmful free radicals species are known to cause oxidative damage to various molecules in cells including membrane lipids, proteins and nucleic acid [7, 36]. The increased generation of ROS damage mitochondria and muscular structure [37], compromising the functional integrity of the cell and reducing its capacity to maintain cellular energy levels [38].

All these situations make cell susceptible to sustain in hypobaric hypoxia stress. Hence, an adaptive mechanism must be obtained by cell to overcome the hypobaric hypoxia stress. In the present study, the enhanced free radical generations in the susceptible and hypoxia groups, along with a lower antioxidant enzyme activity provide evidence of the interplay of energy metabolizing pathways and ROS production in skeletal muscle tissue. The result of present study is supported by our previous finding in which, we measured the ROS levels in the myocardial tissue and found that the animals susceptible to hypoxic stress showed a significantly greater production of such reactive species [11]. It follows that the damage mediated by these highly reactive molecules would be maximal in the susceptible and hypoxic animals. The elevated antioxidant lelvel in the tolerant and SBT treated hypoxia animals explains the reduced oxidative damage in these animals and is indicative of better adaptive mechanisms. Similar elevated antioxidant activity has also been seen in the sojourners as compared to natives who are exposed to high levels of free radicals throughout their lives and have developed adaptive mechanisms [39].

The increased antioxidants enzyme in the SBT treated hypoxia group is indicative of an acclimatization process in the adverse situation of oxidative stress. These finding are in agreement with the previous observation of increased antioxidant levels in SBT treated C-H-R exposed rats [6].

GLUT4 is a transmembrane protein that facilitates the intracellular glucose transport. GLUT4 expression in muscle tissue is inducible by insulin through exocytosis. Cellular stress such as hypoxia inhibits the ATP production and increases the intracellular AMP concentration that is sensed by AMPKα [40]. AMPKα is an intracellular heterotrimeric complex composed of a catalytic alpha subunit and regulatory beta and gamma subunits. AMP binds the regulatory gamma subunit to allosterically open the alpha subunit for phosphorylation and activation. In our study we observed an up-regulated expression of GLUT4 and AMPKα protein level in the tolerant and SBT treated hypoxia animals that may contribute to adaptive response in hypoxic stress via maintaining intracellular glucose homeostasis. Our results support previous finding by Cartee et al. [41] who found up-regulated expression of GLUT4 in rat hind limb muscle after hypoxia exposure. In another study, Klip et al. [42] explained adaptive response in condition of low oxygen availability that involves AMPK activation by the initial drop in ATP then recovers rapidly through compensatory increase in glucose uptake by GLUT4.

HIF-1α, a key regulator of cellular response to the fall in oxygen levels, serves to fulfill the energy requirement of the cell by up-regulating enzyme activities associated with glucose metabolism [43]. Hypoxia responsive nuclear factor, HIF-1α degraded under normoxic conditions; its stabilization under low oxygen results in the activation of a transcriptional program with effects on metabolism, redox homeostasis, vascular remodeling, inflammation, and other processes [44, 45]. Results of our study showed that tolerant animals have a greater stabilization of HIF-1α as compared to susceptible animals which could be attributed to tolerance behavior. Previous study has identified a key role played by hypoxia responsive proteins such as HIF-1α and its regulated proteins in enhancing hypoxic tolerance [46].

In our earlier study we have shown the tolerance behavior of animal impart by significantly higher HIF-1α level and better antioxidant defence system in cardiac tissue [11]. Collectively, the optimum maintenance of cellular redox state, enhanced

activity of aerobic energy metabolizing pathways, better antioxidant levels, adequate amount of intracellular glucose transportation and their metabolization in the tolerant and SBT treated hypoxia groups enable them to sustain in hypoxic stress.

CONCLUSION: The ability of an organism to respond and cope up with the stressful conditions differs from individual to individual and the survival capability depends on various physiological as well as genetic factors. The present study provides knowledge on biochemical and molecular marker responsible for differential hypoxic tolerance in animals exposed to acute hypobaric hypoxia. Supplementation with plant extract, which grow in adverse climatic conditions can help to sustain in hypoxic stress. In conclusion, our results demonstrated that aerobic glycolysis, activated PPP (reducing equivalent), better endogenous antioxidant defence machinery, up-regulated expression of molecular markers including those involved in glucose utilization (GLUT4, AMPKα) and optimizing metabolic process (HIF-1α) in the tolerant and SBT leaf aqueous lyophilized extract treated hypoxia groups imparts the tolerance behaviour under acute hypobaric hypoxia.

CONFLICT OF INTEREST: None

ACKNOWLEDGEMENTS: The study was funded by the Defence Research and Development Organization (DRDO), Ministry of Defence, Government of India. Authors are thankful to the Director, DIPAS for encouragement and support to the study.

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How to cite this article:
Keshri GK, Gupta A, Jain K, Suryakumar G, Sharma P, Gola S, Yadav A, Verma S and Ganju L: Sea Buckthorn (*H. Rhamnoides* L.) Mediated Acute Hypoxic Tolerance in the Skeletal Muscle of Rats by Differential Activation of Energy Metabolism and Enhanced Antioxidants. Int J Pharm Sci Res 2015; 6(12): 5259-72.doi: 10.13040/IJPSR.0975-8232.6(12).5259-72.