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Role of Calpeptin in Amelioration of Hypobaric Hypoxia Induced Skeletal Muscle Damage in Rats: A Pilot Study

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ABSTRACT

Hypobaric hypoxia, a condition of low oxygen availability at high altitude, has a great impact on health via producing oxidative stress which could lead to protein modifications and skeletal muscle damage. Enhanced calpain activity has a major role in hypobaric hypoxia induced protein modifications and protein degradation in the skeletal muscle. The pilot study was designed to investigate the role of a calpain inhibitor, calpeptin in amelioration of hypoxia induced skeletal muscle damage. Male Sprague Dawley rats were exposed to hypobaric hypoxia (HH) with and without calpeptin (50 µg/kg, ip) treatment. HH exposure was given for 6 h at 25,000 ft. Following exposure, the animals were sacrificed and hind limb skeletal muscle was excised for analysis. Calpeptin administration inhibited protein oxidation (decrease protein carbonyl and AOPP content). Decrease in oxidized tryptophan and bityrosine content was also observed in calpeptin pre-treated group in comparison to HH exposed group. Beside this, HH induced reactive oxygen species (ROS) was also ameliorated via calpeptin treatment. Conclusively, calpeptin administration better maintained the oxidative homeostasis in skeletal muscle under hypobaric hypoxia and significantly protected against the protein damage and degradation. However, the exact mechanism is still unknown. Therefore, further research needs to be done to unravel the underlying mechanism.

Keywords: Hypobaric hypoxia; Protein modification; Calpain; Muscle loss

1. INTRODUCTION

Calpain (EC 3.4.22.17) is well known calcium regulated cysteine proteases[1]. Skeletal muscle expressed three members of this family, specially the ubiquitous m- and µ-calpains and the muscle-specific p94 calpain (also consider as calpains-1, -2, and -3)[2]. Calpains are calcium dependent proteases and once the intracellular calcium concentration increases inactive calpains which are usually located in cytosolic compartment, translocate to the cell membrane and converts into the active form.

A wide plethora of roles have been proposed for these proteases, extending from cellular survival and apoptosis[2]. Calpain have imperative role in numerous physiological operations such as cell proliferation, differentiation, apoptosis, membrane attachments, migration, cytoskeleton, aging and signal transduction pathways[4-5]. In contrary, enhanced calpain activity contributes in different pathologies too. Increased mRNA levels of calpain were observed in AH-130 hepatoma, skeletal muscle of animals[6]. Few other studies also exhorted the role of over-expression of muscle calpain of tumor-bearing rats which were treated with sorafenib[7]. Beside this cancer suffered patients showed both type of response either increased or unchanged calpain level[7,8]. Recently, upregulation of calpain activity have been observed in hypobaric hypoxia induced skeletal muscle atrophy[8,9].

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Calpeptin is a drug which specifically inhibits calpains[10] and reported to be beneficial for several cancers[11-15], inhibits pulmonary fibrosis[16], thromobosis[17] and cardiac hypertrophy[18]. Even then, till date no study explains the impact of calpeptin on hypobaric hypoxia induced skeletal muscle loss. Furthermore previous studies reported its role in control mechanical ventilation (CMV) induced muscle atrophy[19].

Since calpain activation has been reported as one of the proteases involved in hypobaric hypoxia induced muscle protein loss, the present study evaluates the role of calpeptin in amelioration of skeletal muscle damage.

2. MATERIALS AND METHODS

2.1 Experimental Design

2.1.1 Animals

Male Sprague-Dawley rats, 150± 20 g were maintained in the Institute's animal house facility under controlled conditions (25±1 °C, humidity 55±10 % with 12-h dark- light cycle). Animals had water ad libitum and access to standard rodent pellet feed. The approval of the Institutional Animal Ethical Committee (IAEC) was obtained for performing the study, and the experiments were performed in accordance with the regulatory guidelines of IAEC and conformed to National Guidelines on the Care and Use of Laboratory Animals, India.

2.2 Hypobaric Hypoxia Exposure

Animal decompression chamber (Decibel Instruments,

India) was used to provide simulated high altitude exposure to animals. The pressure was maintained at 282 torr (that is equivalent to 7620 m altitude and 8 % oxygen) at 25 °C for hypoxic group and humidity controlled at 50±5 per cent.

Twenty animals were taken and divided into following groups (n=5) each:

- Group I: Control group maintained in normoxic condition
- Group II: Calpeptin alone (50 µg/kg body weight; *i.p.*)
- Group III: Hypobaric Hypoxia exposure
- Group IV: Hypobaric Hypoxia exposure + pre-treatment of calpeptin (50 µg/kg body weight; *i.p.*)

Hypobaric hypoxia exposure was given at 25,000 ft for 6 h. Calpeptin was prepared in 0.1 per cent dimethyl sulfoxide (DMSO) in saline and Calpeptin (50 µg/kg body weight) was administered intraperitoneally 30 min prior to HH exposure. The dose of calpeptin was selected based on earlier references indicating its protective effects in various diseases[20-21]. Once exposure was completed, rats were sacrificed using sodium pentobarbital (50 mg/kg, *i.p.*) and hind limb skeletal muscles were excised and used for different biochemical and histopathological analysis, thereafter the tissues were stored immediately at −80 °C for further use.

3. BIOCHEMICAL ASSAYS AND HISTOPATHOLOGICAL ANALYSIS

Skeletal muscle tissue was homogenised in 0.154 M KCl-EDTA buffer for biochemical estimations and 4 per cent formalin was used for fixation of muscle for histopathological analysis.

4. OXIDATIVE STRESS RELATED MARKERS REACTIVE OXYGEN SPECIES

Free radical generation was determined by fluorometeric method using 2,7-dichlorofluoroscein diacetate DCFH-DA[22]. DCFH-DA, a nonfluorescent lipophilic dye, passively diffuses via cellular membranes which were cleaved by intracellular esterase and formed 2,7-dichlorofluorescein (DCF). Further, DCF reacts with reactive oxygen species (ROS) and produce 'fluorescein' that is highly fluorescent which was readable at wave length 485 nm (Ex) and at 530 nm (Em).

4.1 Thiol Content

Primary defense system is operated by thiol content and its oxidation is considered as oxidative stress in the body. 5, 5′-dithiobis (2-nitrobenzoate) (DTNB) is used to estimate total SH-groups (T-SH), non protein SH-groups (Npr-SH) and protein bound SH-groups (Pr-SH)[23]. For quantitation of T-SH, homogenate aliquot (0.125 ml) was added with incubation medium (0.375 ml) (100 mM KCl, pH 8.0, 40 mM Tris, 2 mM EDTA), followed by DTNB (25 µl) was added, incubated at 37℃ for 30 min and centrifuged at 2500 rpm for 10 min. Np-SH was quantified after addition of homogenate (0.5 ml) to H2O (1.75 ml) and trichloroacetic acid (50 %) (0.25 ml). After centrifugation, DTNB (25 µl) and 0.4 M Tris (1 ml and pH 8.9) were added to supernatant (0.5 ml) and incubated for 5 min. Yellow colour formazan was formed which was estimated at 412 nm. GSH was used as a standard.

5. OXIDATIVE PROTEIN DAMAGE MARKERS PROTEIN CARBONYL CONTENT

Protein carbonyl reacts with 2, 4 dinitrophenylhydrazine and form Schiff base to produce the corresponding hydrazone, and analysed spectrophotometrically[24]. Muscle tissue was homogenised in ice cold phosphate buffer (pH 7.2) and then centrifuged at 10,000 Xg for 15 min. The supernatant (200 µL) was added with 600 µL 10 mM 2, 4-dinitrophenylhydrazine (DNPH) and incubated for 1 h at room temperature. Further, protein precipitation was done with equal volume of 20 per cent TCA and was washed with 3times with ethanol/ethyl acetate (1:1 v/v). Finally, the precipitate was dissolved in 400 µL of 6 M guanidine hydrochloride (pH 2.3), and centrifuge to remove insoluble debris. Absorbance at 360 nm was measured for DNPH derivatives.

5. ADVANCE OXIDATION PROTEIN PRODUCT

One of the relevant markers for oxidant induced protein damage is advance oxidised protein product. Spectrophotometeric method of Witko-Sarsat[25], *et al.* was used for advance oxidation protein product (AOPP) estimation and chloramine T was used as a standard. The muscle was homogenised in 0.154 M KCl-EDTA and was diluted 1:5 with phosphate-buffered saline (PBS), pH 7.4. 200 µL of diluted samples were added with 10 µl of 1.16 M potassium iodide and 20 µL of acetic acid and absorbance was recorded at 340 nm immediately.

6. PATHWAYS OF PROTEIN DEGRADATION CALPAIN ASSAY

Calpains were estimated using *N*-succinyl-Leu-Tyr-7-amido-4-methylcoumarin (SLY-AMC), taken as substrate[26]. Buffer solution containing 25 mM HEPES (pH 7.5), 0.1 per cent CHAPS, 10 per cent sucrose, 10 mM DTT, 0.1 mg/ml ovalbumin and SLY-AMC, was incubated with muscle homogenate at 37 °C for 60 min. Fluorescence of free AMC released was observed in fluorimeter at excitation 380 nm and emission 460 nm.

7. DEGRADATION OF PROTEIN TRYPTOPHAN AND TYROSINE RESIDUES

Flourometeric method was used to estimate tryptophan content and tyrosine content respectively[27-28]. Muscle tissue was homogenised in ice-cold phosphate buffer (0.1 M, pH 7.4) and then centrifuged at 960 g for 10 min at 4 °C. SDS was added to sample aliquots. The tryptophan content present in solubilised proteins was quantified fluorimetrically at 280 nm (excitation) and 345 nm (emission) wavelengths and tyrosine content in solubilised proteins was quantified fluorimetrically at 277 nm (excitation) and 320 nm (emission) wavelengths, respectively.

8. CASPASE-9 SUBSTRATE CLEAVAGE ASSAY

Caspase-9, cysteine proteases engage in activating the apoptotic cell death machinery. Colorimetric substrate II, Ac-Leu-Glu-His-Asp-pNA, Ac-DEVD-pNA (Calbiochem) was used to estimate caspase-9 activity[29]. Muscle tissue

was homogenised in lysis buffer (10 mM Tris–HCl (pH 7.4), 150 mM NaCl and 1 % Triton X-100). The reaction mixture consisted of 100 mM HEPES–KOH (pH 7.3), 10 per cent sucrose, 0.1 per cent CHAPS, 10 mM dithiothreitol (DTT), 2 per cent dimethyl sulfoxide (DMSO), 0.2 mM substrate (Ac-LEHD-pNA) and 200 µg of protein from the extracts and incubated for 1 h at 37 °C. The amount of free pNa cleaved by caspases and released from the substrate was measured at the wave length of 405 nm. The results were expressed as nmol p-NA^{-1} min^{-1} mg $protein^{-1}$.

9. STATISTICAL ANALYSIS

The data were reported as mean±SEM. One-way analysis of variance (ANOVA) with post hoc Bonferroni analysis was used to determine statistical significance among groups. GraphPad Prism ver 7.00 software (GraphPad, CA, USA) was used for conducting the statistical analysis. The p value of ≤0.05, with a 95 per cent confidence interval was considered significant.

10. RESULTS

10.1 Reactive Oxygen Species (ROS) Produced Acute Hypobaric Hypoxia Induced Oxidative Stress

Generation of free radicals could be considered as one of the known causes of oxidative stress in cell. Figure 1 shows muscle ROS level in response to HH exposure, calpeptin alone and HH along with calpeptin. Hypobaric hypoxia exposure caused significant elevation in ROS as compared to control rats ($p < 0.05$) and this elevated ROS levels were curtailed by pretreatment with calpeptin. While, calpeptin alone treated rats showed no-significant change in ROS level as compared to control rats.

10.2 Acute Hypobaric Hypoxia Alters Intracellular Thiol Content

Cysteine and methionine are the amino acids that contain thiol group (-SH group) and these thiol groups have ability to undergo oxidation. Total thiol and protein thiol content decreased significantly in hypobaric hypoxia exposed animals ($p < 0.05$) which were recovered in calpeptin pre-treated rats. While no significant changes were observed in non-protein thiol content in calpeptin alone groups as shown in Fig. 2.

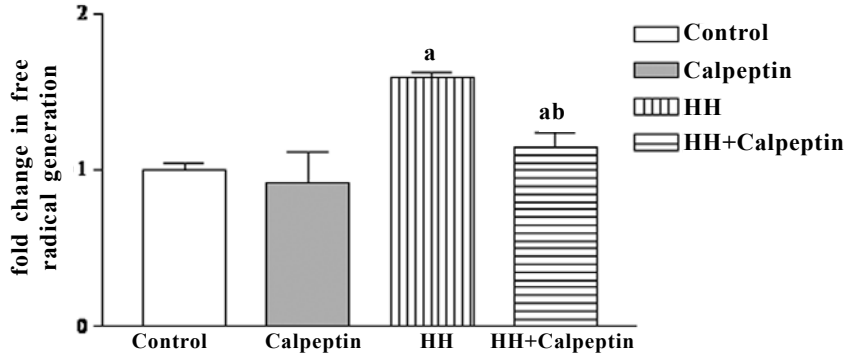

Figure 1. HH-induced oxidative damage via ROS generation and effect of calpeptin treatment alone or in combination with HH in rat muscle. Data represents the mean±SE; N=5.

[a]Statistically significant as compared with control ($p < 0.05$)

[b]Statistically significant as compared with HH ($p < 0.05$)

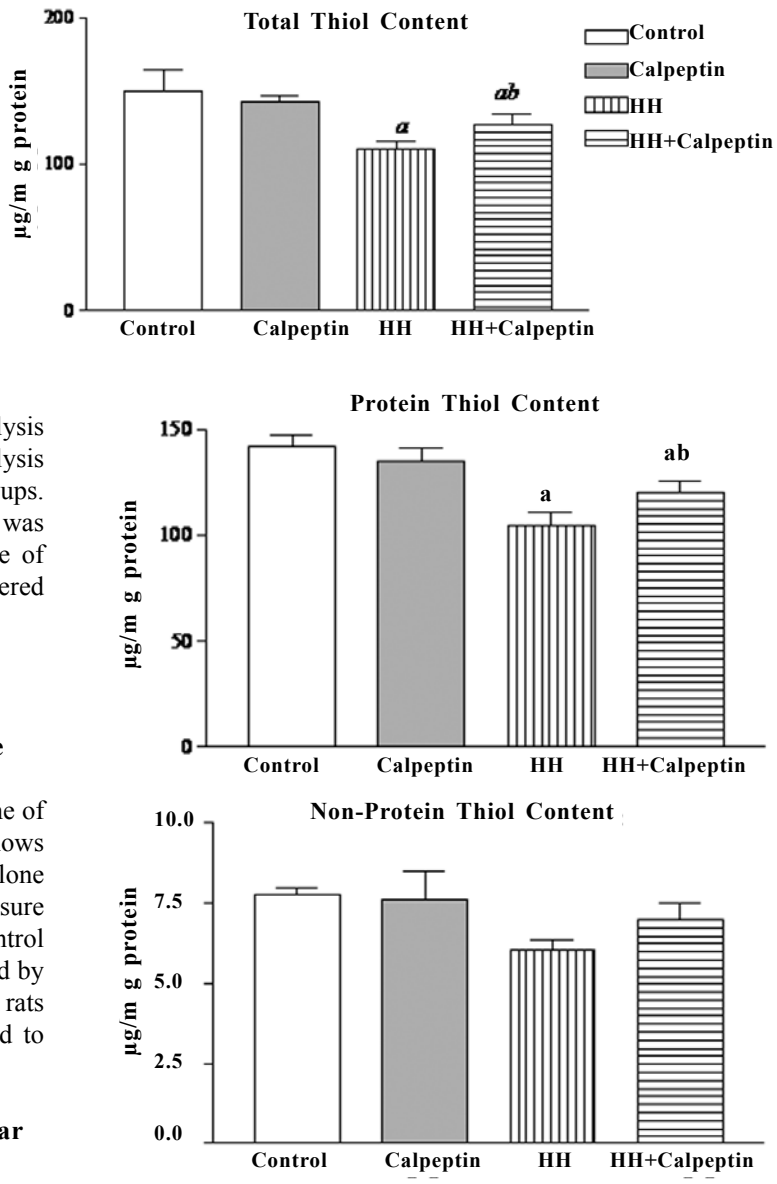

Figure 2. HH-induced oxidative damage via ROS generation and effect of calpeptin treatment alone or in combination with HH in rat muscle. Data represents the mean±SE; N=5.

[a]Statistically significant as compared with control ($p < 0.05$)

[b]Statistically significant as compared with HH ($p < 0.05$)

10.3 Disturbed Oxidative Homeostasis Induces Oxidative Protein Modifications

10.3.1 Advanced Oxidation Protein Products (AOPP)

Advanced oxidation protein products are relevant marker for oxidised protein. The results indicated a significant higher level of AOPP in HH-exposed animals with respect to control animals which were recovered in calpeptin pre-

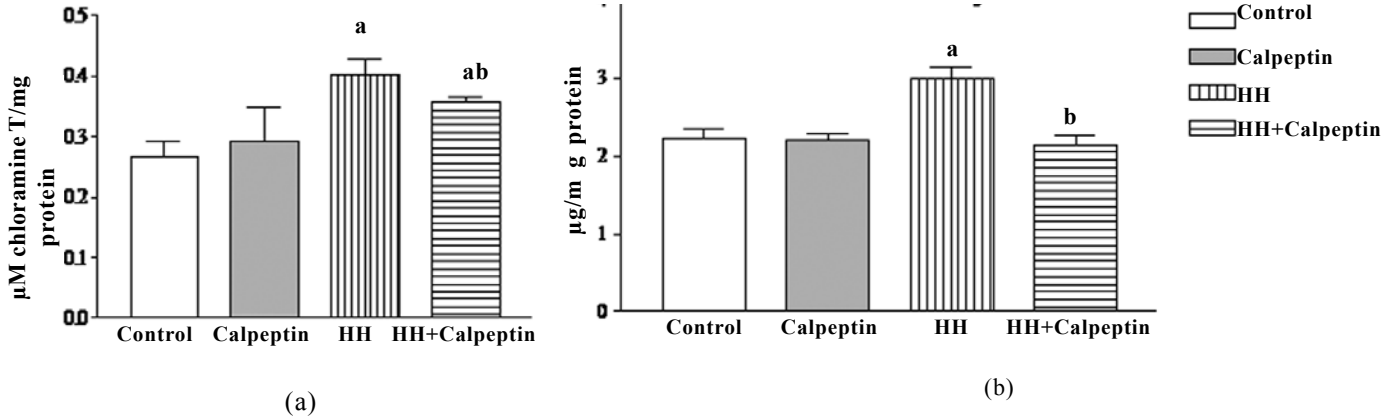

Figure 3. HH-induced protein oxidation and effect of calpeptin treatment alone or in combination with HH in rat muscle. (a) Advance Oxidized Protein Products (AOPP) (b) Protein Carbonyl Content. Data represents the mean±SE; N=5.

[a]Statistically significant as compared with control ($p < 0.05$)
[b]Statistically significant as compared with HH ($p < 0.05$)

treated animals ($p< 0.05$). While, AOPP non-significantly increased in calpeptin alone treated animals as compared to control animals as shown in Fig. 3(a).

10.3.2 Protein Carbonyl Content

Protein carbonyl content was also found to increase in HH-exposed animals with reference to control animals which were ameliorated in calpeptin pre-treated animals ($p<0.05$). However, no-significant changes were observed in calpeptin alone treated animals as shown in Fig. 3(b).

10.4 Protein Degradation

10.4.1 Oxidised Proteins Lead to Cellular Proteolysis and Protein Degradation

Higher degree of oxidised proteins was observed in hypobaric hypoxia exposed animals which further led to intracellular proteolysis. A significant increase of calpain activity was noted in hypobaric hypoxia exposed rats ($p< 0.05$). Calpeptin administration significantly curtailed the hypoxia induced increase in calpain activity and, calpeptin alone treated rats did not show any adverse effects as shown in Fig. 4.

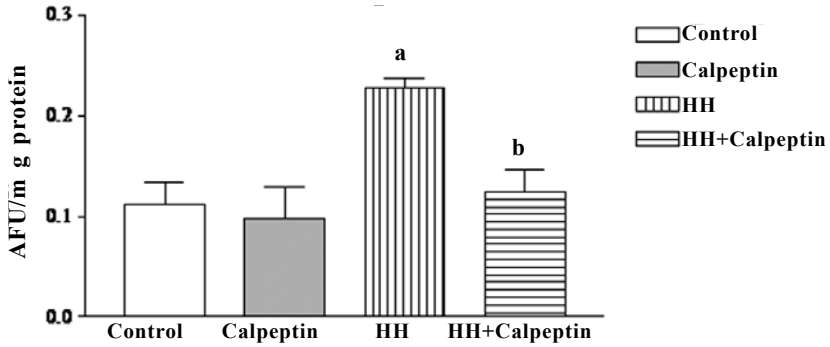

Figure 4. HH-induced increase in calpain activity and effect of calpeptin treatment alone or in combination with HH in rat muscle.

[a]Statistically significant as compared with control ($p < 0.05$)
[b]Statistically significant as compared with HH ($p < 0.05$)

Aromatic amino acids such as tryptophan and tyrosine in the polypeptide chain are highly prone to attack by free radical species like ROS, RNS etc. Higher protein oxidation observed in hypobaric hypoxia exposed rats resulted into degradation or release of oxidised amino acids like tryptophan and tyrosine.

In the present study, a marginal increase in released tryptophan and tyrosine residues were noted in HH-exposed animals and no significant change was observed in calpeptin pre-treated rats as shown in Figs. 5.

10.4.2 Oxidative Protein Damage and Degradation Enhances Apoptosis

A significant increment in caspase-9 activity was observed in HH exposed rats ($p<0.05$). Along with this a decrease was also noted in HH exposed and calpeptin treated rats. While calpeptin alone treated rats didn't show any changes in caspase-9 activity as shown in Fig. 6.

10.4.3 Creatinine Phosphokinase (CPK)

Creatinine phosphokinase (CPK) is a marker of muscle damage which was also estimated in skeletal muscle tissue. CPK activity was decreased significantly in hypobaric hypoxia exposed rats while no significant change was observed in calpeptin alone administered rats. Along with this, CPK activity was also restored in HH+calpeptin pretreated rats as compared to hypobaric hypoxia exposed rats as shown in Fig. 7.

11. DISCUSSION

Chronic high altitude exposure leads to loss of body weight and skeletal muscle atrophy even at moderate altitudes. Upregulation of calpain plays a major role for the enhanced protein degradation and hypobaric hypoxia induced skeletal muscle atrophy. The present study investigated the role of calpeptin in the prevention of skeletal muscle damage induced by hypobaric hypoxia.

Reactive oxygen species play a major role in hypobaric hypoxia induced oxidative stress via mitochondrial electron transport system[30].

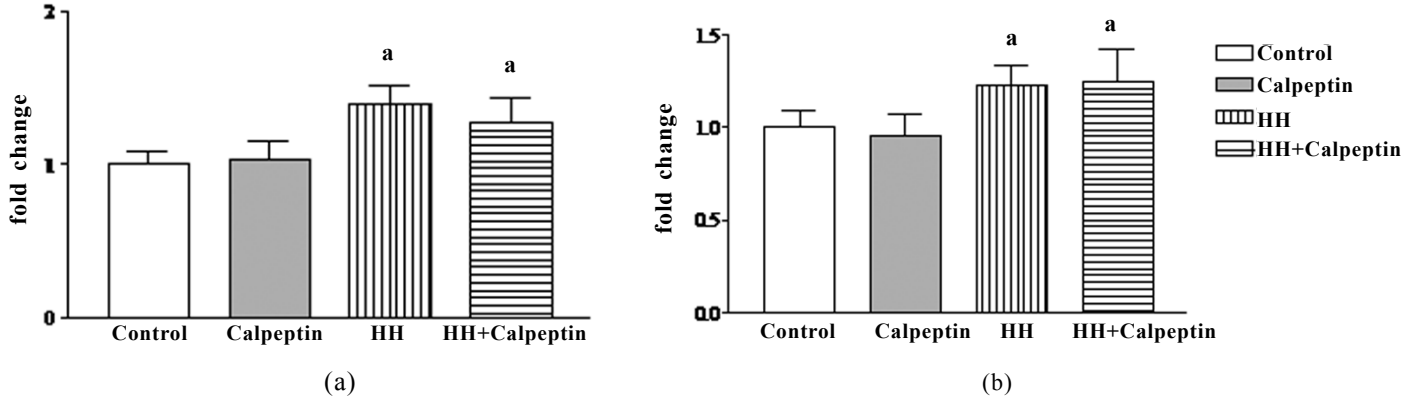

Figure 5. HH-induced increment in oxidized amino acids and effect of calpeptin treatment alone or in combination with HH in rat muscle. (a) release of tryptophan, (b) release of tyrosine products.

[a]Statistically significant as compared with control ($p<0.05$)

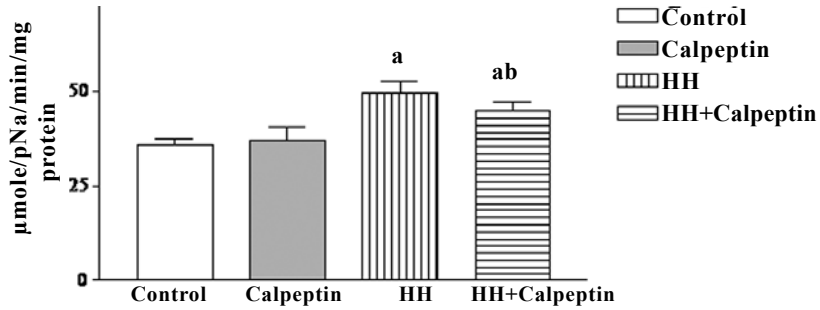

Figure 6. HH-induced increase in caspase-9 activity and effect of calpeptin treatment alone or in combination with HH in rat muscle.

[a]Statistically significant as compared with control ($p < 0.05$)
[b]Statistically significant as compared with HH ($p < 0.05$)

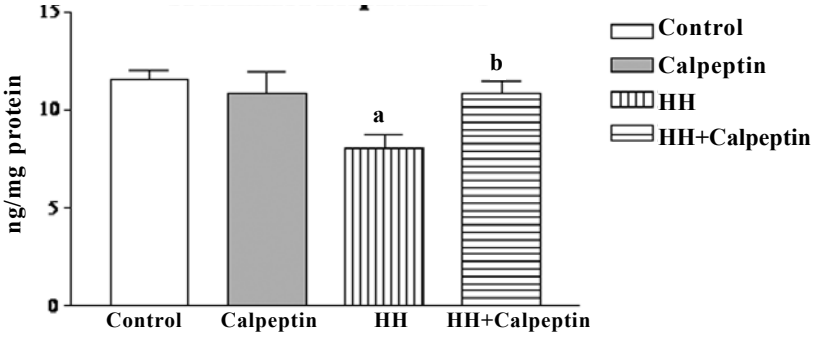

Figure 7. HH-induced decrease in CPK activity and effect of calpeptin treatment alone or in combination with HH in rat muscle.

[a]Statistically significant as compared with control ($p < 0.05$)
[b]Statistically significant as compared with HH ($p < 0.05$)

Other macromolecules such as protein, lipids and DNA are also reported to be damaged due to high altitude associated hypoxia[31]. The previous studies by us and others also reported that reactive oxygen species (ROS) was also increased in HH exposed rats and that was accompanied by decrease in thiol content[32-33]. The condition is considered as oxidative stress or disturbed oxidative homeostasis[34].

Disturbed oxidative homeostasis further results protein oxidation and our data depicted the same as protein carbonyl content and advance oxidised protein products (AOPP) increased in HH exposed animals. An increase in protein carbonyl residues and advanced oxidation protein products (AOPP) are considered as indicator of protein oxidative damage[35]. Further, oxidised protein structure leads to modification in amino acid residues viz. proline, arginine, and lysine and produce increase protein carbonyl content and advance oxygen protein products[36]. Oxidised amino acid leads to protein misfolding or unfolded protein response (UPR). Disturbed homeostasis and protein oxidative modification at high altitude disturb their physiological activity, making them highly sensitive to proteolysis[37]. In this respect, several lines of evidences suggest that calpain plays a substantial role in degrading major myofibrillar proteins. Calpain degrades titin and nebulin, major architectural / cytoskeletal proteins of the sarcomere which attaches to the Z-disk. The removal of oxidised proteins through enhanced calpain activation contributes to the alleviation of the misfolding load on the cell.

Thus, the present study was planned to observe the ameliorative effect of calpeptin, a calpain inhibitor, on hypobaric hypoxia induced skeletal muscle damage. The basis of selection of calpeptin as preventive measure is because of its restricted activity against calpain. Calpeptin is well known inhibitor of calpain[38-39] and calpain activity could be one of the main protease responsible for HH induced muscle proteolysis[40]. Calpeptin is dipeptide aldehyde and membrane permeable thus, capable of penetrating in the cytosol of the cell. Calpeptin binds to the cysteine residue of the active site of the calpain[41].

Our data depicted that calpeptin not only inhibited calpain activity in HH exposed rats but also able to restore oxidative homeostasis to some extent. The exact mechanism for the same is still unexplored but it could be due to presence of free hydrogen atom in its chemical structure (Fig. 8.) which plays role in decreasing ROS and increasing thiol content. Further, oxidative protein damage induced by hypobaric hypoxia were also found to be decreased in calpeptin treated rats and suggested its importance in maintaining oxidative homeostasis.

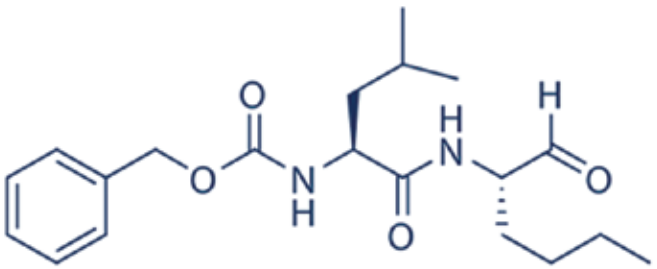

Figure 8. Chemical Structure of Calpeptin

In our study, the results demonstrate that calpeptin, as a calpain inhibitor might play a key role in ameliorating muscle protein degradation under hypobaric hypoxia exposure. Calpeptin inhibits calpain, one of the central proteases involved in not only the removal of misfolded proteins but also in the proteolytic modification of crucial proteins in the

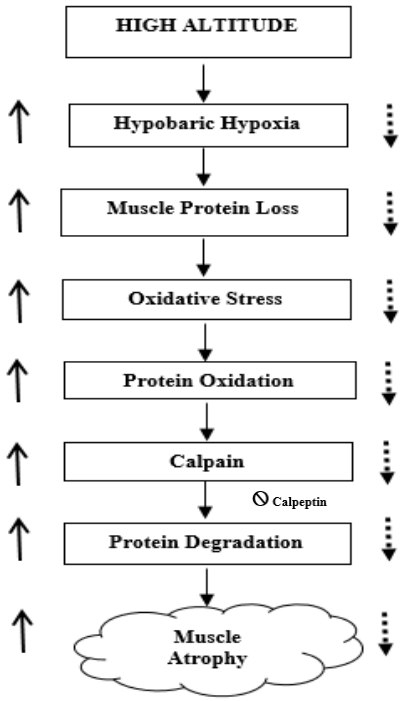

Figure 9. Diagrammatic representation of mechanism approach to elucidate the effect of hypobaric hypoxia (HH) (solid arrow) and preventive efficacy of Calpeptin treatment (dotted arrow).

cell. In the present study, calpeptin could ameliorate hypobaric hypoxia induced reactive oxygen species (ROS), which could suggest its role in defence mechanism against oxidative stress as shown in Fig. 9. Further, we also analysed the apoptotic activity via estimating caspase-9 activity. Caspases, the main enzymes involved in both the initiation (caspase-8, caspase-9, caspase-12) and execution of apoptosis (caspase-3, caspase-6, caspase-7). In skeletal muscle, caspase-3 was involved in protein degradation, especially of filamentous actin, and this process contributed to muscle weakness in response to endotoxin[42]. In this respect, we evaluated the activity of caspase-9 as it activates caspase-3 and apoptotic signaling gets initiated. Our findings suggest the significant decrease in caspase-9 activity in calpeptin administered group which has been exposed for 6 h of hypoxia as compared to other groups. In skeletal muscles, apoptosis is a highly regulated process with a subsequent activation of caspase-3 and caspase-9 under pathophysiological conditions, so, to counteract the effect of apoptosis detailed research is needed with this drug. This defence mechanism further suggested its role in decrease in protein modification. However, the exact mechanism is still unknown. Therefore, further research needs to be done to unravel the underlying mechanism in the future.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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