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Aging and hypobaric hypoxia induced skeletal muscle protein loss: A comparative experimental study using rat model

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Skeletal muscle protein concentrations are regulated by protein synthesis and protein degradation mechanisms. In the present study, we compared two muscle loss phenomenon i.e., hypobaric hypoxia and sarcopenia. Male SD rats were distributed into three groups: control, aged and hypobaric hypoxia (HH) exposed rats. Results showed elevated levels of oxidative stress marker, ROS and GSSG: GSH in both stresses which corroborated with decreased in thiol content. Ergo, protein and lipid oxidation also increased in aged rats as contrast to HH exposed and control rats. Moreover, protein degradative machinery and apoptosis related markers, 20S proteasome, caspase-3 and caspase-9 was also upregulated in the aged and HH-exposed rats. Interestingly, the activity of two important proteins i.e., p-Akt and heat shock protein-60 (HSP60) were found different in these two stresses. p-Akt and HSP60 protein were elevated in HH-exposed rats whilst decreased activity was noted in case of aged rats. These results suggest that age is natural phenomenon that resulted into muscle protein loss, whereas HH-exposure has entirely different degradation mechanism. In the event of HH-exposure, cell has mechanisms to cope up with the unfavorable stress condition but degradation mechanisms are highly activated which led to muscle protein loss.

Keywords: p-Akt, Heat shock protein, High altitude, Oxidative stress, Protein degradation/oxidation, Sarcopenia

Oxidative stress is an essential phenomenon for governing life process and if it crosses the threshold level of cells, it might lead to many chronic diseases[1-4]. Oxidative stress defines as an imbalance between oxidants and antioxidants in favour of the oxidants, leading to a disruption of redox signaling that ultimately lead to molecular damage[5].

Human aging process, from maturity to senescence, is a natural process leading to decline in muscular function and performance[6]. On the other hand, hypobaric hypoxia induced muscle protein loss is an environmental stress which under chronic exposure results in muscle protein loss and decreased human physical performance[7,8]. With advancing age, antioxidant defence system is compromised which lead to excess ROS accumulation and oxidative damage[9,10]. Further, excessive ROS contribute to sarcopenia via enhancing protein carbonylation, increasing proteolysis and decreasing muscle protein synthesis that ultimately results to muscle protein loss[11,12]. Sarcopenia is an age induced muscle mass loss associated with adverse outcomes such as physical disability, poor quality of life, morbidity, and death and it is highly prevalent in older populations[13-15]. On the other hand, approximately 81.6 million people reside at high altitude region permanently at altitudes above 2,500 metres (8200 ft)[16]. Due to military and strategic reasons, time to time, the armed forces are deployed at high altitude. High altitude associated hypobaric hypoxia also leads to elicit excess ROS generation, decreased antioxidant system, oxidative protein damage and loss in skeletal muscle[17-19].

Aging is a natural phenomenon which affects aged person; while high altitude associated hypobaric hypoxia is an environmental stress conditions which affects a healthy adult who is deployed at high altitude. While, high altitude stress affects not only older population but also healthy adult individuals. Interestingly, both the stresses end up with muscle protein loss and compromised physical activity. In the present study, we investigated the skeletal muscle loss phenomenon under two different conditions, hypobaric hypoxia and aging. The findings may provide better understanding of the mechanism responsible for age associated and hypobaric hypoxia induced muscle loss.

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Materials and Methods

Sprague-Dawley male rats, bred in the animal facility of Defence Institute of Physiology and Allied Sciences (DIPAS), Delhi, were maintained under controlled environment (25±1°C temperature, 55±10% humidity, and 12-h light-dark cycle on a bedding of rice husk in polypropylene cages) in the Institute's animal house. Animals consumed standard rodent pellet as feed and water *ad libitum*. Fifteen male Sprague Dawley rats were taken for the experimental purpose which was divided into three groups of five rats in each group. Gr. I, Untreated, unexposed and adult rats served as control (250±10 g); Gr. II, Aged rats (550±10 g; 18 months old); and Gr. III, hypobaric hypoxia (HH) exposure for 7 days (before exposure: 250±10 g; after HH exposure 220±10 g). The study was approved by the Institutional Ethical Committee on Animal Experimentations (IAEC), and the experiments were executed in accordance with the regulations specified by the IAEC and National Guidelines on the Care and Use of Laboratory Animals, India.

Hypobaric hypoxia exposure

Simulated high altitude exposure was given in an animal decompression chamber that maintained pressure of 282 torr which was equal to an altitude of 7620 m, representing an environmental partial pressure equivalent to 8% oxygen under normoxic conditions, coupled to mercury barometer, at 25°C for hypoxic group (Decibel Instruments, India). The airflow in the chamber was 2 L/min with relative humidity maintained at 45-55%. The animal decompression chamber/ hypoxia chamber was opened daily to assess the body weight of animals, refilling food and water dispensers. The retention period did not exceed 15-20 min[20-22]. Beside this, within the same laboratory, control group rats were sustained in the normoxic condition. Earlier studies from our lab have established that HH exposure at 7620 m for 7 days leads to skeletal muscle loss[23]. On completion of HH exposure, animals were anaesthetized with sodium pentobarbital (50 mg/kg, *i.p.*), rats were sacrificed and immediately whole hindlimb muscle was excised for biochemical, histopathological and protein expression studies. Muscle samples were snap frozen in liquid nitrogen and all samples were stored at −80°C. After completion of hypobaric hypoxia exposure, the rats were sacrificed and whole muscle from hind limb were isolated and weighed using a digital platform balance. Body weight was expressed in grams. The muscle to body index was also quantified which were expressed as ratio of wet muscle weight and body weight.

Oxidative stress markers

Reactive oxygen species (*ROS*)

Free radical generation was determined by fluorescence method using 2,7-dichlorofluoroscein diacetate DCFH-DA[24]. DCFH-DA, a non fluorescent lipophilic dye, passively diffuses through cellular membranes where it is cleaved into 2,7-dichloro-fluorescein (DCF) by the action of intracellular esterases. The DCF then reacts with ROS to produce 'fluorescein' that is highly fluorescent and has a maximum absorption at 485 nm and maximum emission at 530 nm. The fluorescence emission is directly proportional to the free radical content in a sample. In detail, 150 μL of muscle homogenate was incubated with 10 μL DCFH-DA (100 μM) for 30 min in dark. After incubation fluorescence was read immediately with excitation at 485 nm and emission at 535 nm using a fluorimeter (Perkin Elmer, UK). Readings were acquired in arbitrary fluorometric units and results were reported as fold change in free radical generation.

Reduced glutathione and oxidized glutathione

For muscle reduced glutathione (GSH) and oxidized glutathione (GSSG) estimation, 0.25 g of tissue sample was homogenized on ice with 3.75 mL of phosphate-EDTA buffer and 1.0 mL of 25% HPO_3 which was used as a protein precipitation. The total homogenate was centrifuged at 100,000×g for 30 min at 4°C. For GSH estimation, 0.5 mL supernatant was added with 4.5 mL phosphate buffer (pH 8.0). The final assay mixture contained 100 mL supernatant, 1.8 mL phosphate-EDTA buffer and 100 mL O-phthaldehyde (1000 mg/mL in absolute methanol, freshly prepared). The fluorescence was read at 420 nm with an excitation wavelength of 350 nm via spectrofluorometer (Agilent Cary Eclipse Fluorescence Spectrophotometer, USA). For GSSG estimation, 0.5 mL supernatant was added with 200 mL of 0.04 mol/L Nethylmaleimide solution, and then incubated for 30 min at 25°C. Followed by, 4.3 mL of 0.1 mol/L NaOH was added and fluorescence was recorded at 420 nm with an excitation wavelength of 350 nm via spectrofluorometer (Agilent Cary Eclipse Fluorescence Spectrophotometer, USA)[25].

Antioxidant activity in terms of thiol content

Thiol Content

Thiol content considered as primary defense system in the body and its oxidation could be

correlated with the production of oxidative stress in the body. The concentrations of total thiol groups (T-SH), protein bound thiol groups (Pr-SH) and non protein thiol groups (Npr-SH) were determined using 5,5′-dithiobis (2-nitrobenzoate) (DTNB)[26]. For determination of T-SH, a 0.125 mL aliquot of homogenate was added to 0.375 mL of standard incubation medium (40 m M Tris, 2 mM EDTA, 100 mM KCl, pH 8.0). After addition of 25 µL of DTNB (10 mM in methanol) and 2.5 mL methanol, the mixture was incubated for 30 min and centrifuged at 2500 rpm for 10 min.

The concentration of Np-SH was determined after addition of 0.5 mL homogenate to 1.75 mL H_2O and 0.25 mL trichloroacetic acid (50%). Following centrifugation, 25 µL DTNB and 1.0 mL 0.4 M Tris (pH 8.9) were added to 0.5 mL supernatant and incubated for 5 min. The resultant 2-nitro-5-mercaptobenzoic acid was measured at 412 nm. GSH served as a standard. The concentration of Pr-SH was calculated by subtracting values for Npr-SH from that of T-SH.

Protein Oxidation and Modification marker

Protein carbonylation

Oxidative modifications of amino acid residues comprise derivatization of amino acid residues like proline, arginine, and lysine to reactive carbonyl derivatives. In brief, 2,4-dinitrophenylhydrazine reacts with protein carbonyl and form a Schiff base to produce the corresponding hydrazone that was analyzed spectrophotometrically[27]. Muscle tissue was homogenized in ice-cold 50 mM phosphate-EDTA buffer (pH 7.2) containing proteinase inhibitor cocktail. The homogenate was centrifuged at 10,000 ×g for 15 min, followed by absorbance was measured at 260 and 280 nm to check the presence of nucleic acids. In a reaction mixture, 200 µL sample, 600 µL 10 mM 2,4-dinitrophenyl hydrazine (DNPH) was added. The mixture was incubated for 1.0 h at 25°C. Then, 20% trichloroacetic acid (TCA) was added for protein precipitation and washing was done with ethanol/ethyl acetate (1:1 v/v) for three times. Finally, the precipitate was dissolved in 400 µL of 6M guanidine hydrochloride (pH 2.3), and the insoluble debris were removed by centrifugation. The absorbance of the DNPH derivatives was recorded at 360 nm. The quantitation of carbonyl groups was done using an absorbance coefficient 22 nM cm^{-1} and expressed as nmol carbonyl mg of $protein^{-1}$.

Protein and lipid hydroperoxide (*PrOOH and LOOH*)

Initial fractionation was performed for the simultaneous quantification of the main lipid and protein peroxidation product. Lipid hyderoperoxides (LOOH) and protein hyderoperoxides (PrOOH) was quantified on the basis of spectrophotometric analysis[28]. LOOH and PrOOH measurement was performed on the basis of reaction ie Fe^{+3} (resulting from the react ion of LOOH and PrOOH with Fe^{+2}) with xylenol orange (XO) and the spectrophotometric quantification of resultant XO–Fe complex. Concentrations were expressed as nmoles.

Inflammation

Pro- and anti-inflammatory cytokines

Pro- and anti-inflammatory cytokines were quantified in muscle homogenate using commercially available ELISA kit. IFN-γ and IL-10 was done by respective ELISA kits (Diaclone, France) as per manufacturer's instructions. The ratio of pro- and anti-inflammatory cytokine was presented in the study as TH1:TH2.

Heat shock protein-60 (HSP-60)

HSP60 were quantified in muscle by commercially available rat ELISA (Cusabio ELISA Kit, China) according to the manufacturer's instructions. The intensity of the colour reaction was read spectrophotometrically and the concentration was expressed in ng/mg protein.

Protein degradation machinery

20S Proteasome activity

The ubiquitin proteasome pathway was analyzed using chymotrypsin-like enzyme activity of 20S proteasome[29]. The muscle extract consist 60 µg protein was incubated at 37°C for 30 min with 50 µL of Tris-HCl buffer (pH 8.0) (100 mM Tris-HCl, 1.0 mM DTT, 5 mM $MgCl_2$, 1.0 mM Suc-LLVY-AMC, 2 mg mL^{-1} ovalbumin, and 0.07% SDS). After incubation, reaction termination was done using 25 µL of 10% SDS and then dilution was done by 2 mL of 0.1 M Tris-HCl (pH 9.0). Fluorescence of the liberated AMC was recorded in fluorimeter at excitation 380 nm, emission 460 nm.

Apoptosis

Caspase-3 and Caspase-9 substrate cleavage assay

An enzyme, caspase-3 is activated during apoptosis process. The activity of caspase-3 in skeletal muscle was determined by colorimetric substrate, Ac-Asp-Glu-Val-Asp-p-nitroaniline, Ac-DEVD-pNA (Calbiochem)[30]. Muscle tissue was homogenized in ice-cold lysis

buffer (50 mM HEPES, pH 7.4, 0.1% CHAPS, 5 mM DTT, 2 mM EDTA, 2 mM EGTA, 0.1% Triton X-100, 1.0 mM PMSF and protease inhibitors). Homogenate was centrifuged at 14,000×g for 15 min. For caspase-3 estimation, supernatant containing 100 mg protein was incubated with assay buffer (50 mM HEPES, pH 7.4, 100 mMNaCl, 0.1% CHAPS, 10 mM DTT, 2 mM EDTA, 2 mM EGTA, 0.1% Triton X-100) at 25°C with the substrate Ac-DEVD-pNA. For caspase-9 activity, colorimetric substrate II, Ac-Leu-Glu-His-Asp-pNA, Ac-DEVD-pNA (calbiochem) was used as substrate rather than Ac-Asp-Glu-Val-Asp-p-nitroaniline, Ac-DEVD-pNA (calbiochem). The other procedure was same as done for caspase-3 activity. Cleavage of the p-nitroaniline (pNA) dye from the peptide substrate was recorded by measuring pNA at 405 nm and results were expressed as nmol p-NA/minute/mg protein.

Immunoblotting

Cytoplasmic extract preparation

Muscle tissue was homogenized in an ice-cold buffer (0.5 M sucrose, 10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 10% glycerol, 1.0 mM EDTA, 1.0 mM DTT, 1 mM PMSF fortified with protease inhibitors). 0.6% Nonidet P-40 (NP-40) was added to homogenate and incubated for 15 min on ice. Followed by, centrifugation was done for 20 min at 5,000×g at 4°C. The supernatant collected was cytoplasmic fraction and total protein concentration was estimated.

Western Blotting

Protein (50 μg) was separated using 10% SDS-PAGE and transferred onto a nitrocellulose membrane (Millipore, Billerica, USA). The membranes were blocked with 3% bovine serum albumin (BSA) in PBS buffer consist 0.1% Tween-20, washed and probed with respective rabbit monoclonal antibodies. Primary antibodies p-Akt was purchased from Santa Cruz Biotech. Then, the membrane was incubated with anti-rabbit-IgG HRP conjugate (Sigma). The membrane was washed, incubated with chemiluminescent substrate (Sigma) and the bands were developed and captured by gel documentation system (UVP Bioimaging software, Upland CA, USA). Quantification was completed using densitometry using ImageJ software.

Statistical analysis

All the experiments were performed on a minimum of three different occasions, and data are presented as Box-and-whisker plots. One-way analysis of variance with post hoc Bonferroni analysis was used to determine statistical significance between groups and the analysis was conducted on the means and SEMs, not on the median values. All analysis was performed using GraphPad Prism ver. 7 software (GraphPad, CA, USA). Statistical significance levels were set to **P* <0.05; ***P* <0.01; ****P* <0.001.

Results

Comparative effect of hypobaric hypoxia and aging on Body weight and Muscle Body Index

The body weights of the aged rats were increased while body weight of HH exposed rats decreased as compared to control adult rats (Fig. 1A). Whereas muscle to body index (ratio of muscle weight to body weight) decreased significantly as compared to control adult rats. The muscle/body weight ratio was 30.23% lower than in aged rats (*P* <0.001) and 16% lower in HH exposed rats (*P* <0.01) (Fig. 1B).

Effect of oxidative damage on hypobaric hypoxia and aging induced muscle loss

Oxidative stress is the phenomenon caused due to increase in reactive oxygen species (ROS) and

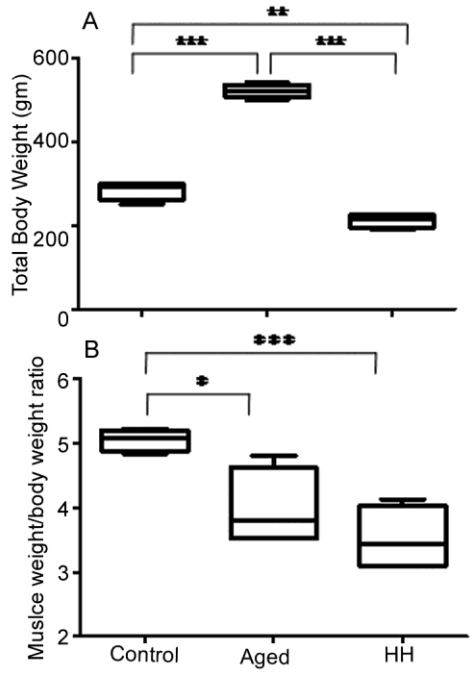

Fig. 1 — Effect of aging and hypobaric hypoxia exposure on skeletal muscle. (A) Body weights of control, aged and HH exposed rats (in gm); and (B) Ratio between muscle weight (mg) and body weight (g). [Data represents by Box-and-whisker plots (05 rats per group) and were statistically analyzed by one-way ANOVA with Bonferroni's post hoc test and presented as means±SEMs. Statistical significance levels were set to: **P* <0.05; ***P* <0.01; ****P* <0.001]

decrease in antioxidant activity. High altitude associates a number of environmental challenges in which the prime one is hypobaric hypoxia exposure which leads to oxidative stress and damage whilst aging also involved in producing oxidative stress and damage.

Reactive oxygen species (ROS) and GSSG:GSH

Reactive oxygen species are free radicals which have important role in production of oxidative stress and damage to macromolecules. ROS was observed 1.51 fold and more in aged rats (P <0.001) and 1.26 fold HH exposed rats (P <0.01) as compared to control rats. Aged rats were monitored to generate more ROS as compared to HH-exposed rats (F=1.404) (P <0.01) (Fig. 2A).

Increase in ROS leads to oxidation of GSH into GSSG which accumulates in the cell. Hence, increased ratio of GSSG-to-GSH indicates oxidative stress. GSSG: GSH ratio was found 1.29 fold (F=3.414) (P<0.01) and 1.17 fold (P<0.05) increased in aged and HH-exposed rats as compared to control rats (Fig. 2B).

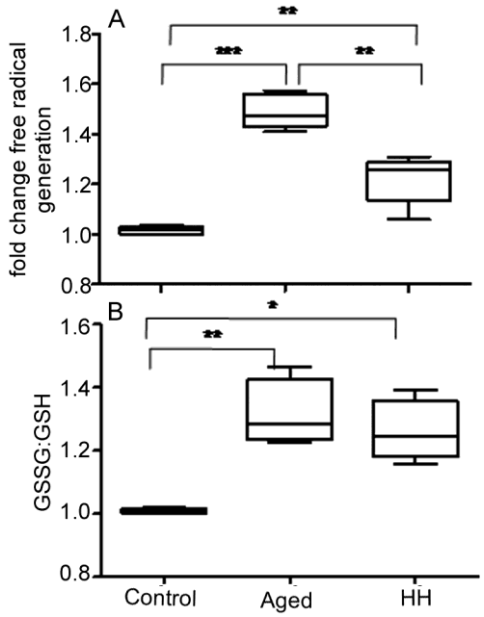

Fig. 2 — Oxidative stress in aged and HH exposed rats. (A) Reactive Oxygen Species generation measured using fluorescent dye DCFH-DA; and (B) GSSG:GSH. [Data represents by Box-and-whisker plots (05 rats per group) and were statistically analyzed by one-way ANOVA with Bonferroni's post hoc test and presented as means±SEMs. Statistical significance levels were set to: *P <0.05; **P <0.01; ***P <0.001]

Protein and Lipid oxidation

Increased ROS further led to oxidation of lipids and protein. LOOH, considered as marker of lipid oxidation, was increased significantly in aged rats (P <0.01) and HH-exposed rats (P <0.05). No significant difference was observed between the aged and HH exposed rats (F=0.7012) (Fig. 3A). Protein hydroperoxide (PrOOH) and protein carbonyl content are biomarkers of protein oxidation. The results indicated the significant increase of PrOOH and protein carbonyl content in aged rats and HH-induced rats in relation to control rats (F=2.439 and F=1.968, respectively). While, the comparison of aged and hypoxia rats showed that aged rats had more protein and lipid oxidation (Fig. 3 B and C).

Antioxidant response

A significant decrease in total thiol and protein thiol content was observed in aged and HH-exposed rats as compared to control rats (Fig. 4 A and B). Whilst, no significant change was noted in non-thiol content in all groups (Fig. 4C), along with this aged and HH-exposed rat also showed no significant change in thiol contents.

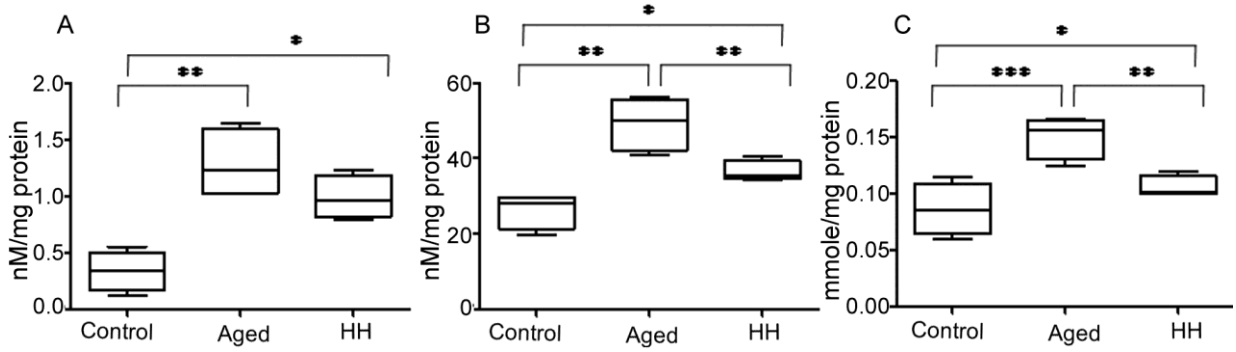

Fig. 3 — Aged and HH exposed rats showed decrease thiol content with comparison to control rats. (A) Total thiol content; (B) Protein thiol content; and (C) Non-protein thiol content. [Data represents by Box-and-whisker plots (05 rats per group) and were statistically analyzed by one-way ANOVA with Bonferroni's post hoc test and presented as means±SEMs. Statistical significance levels were set to: *P <0.05; **P <0.01; ***P <0.001]

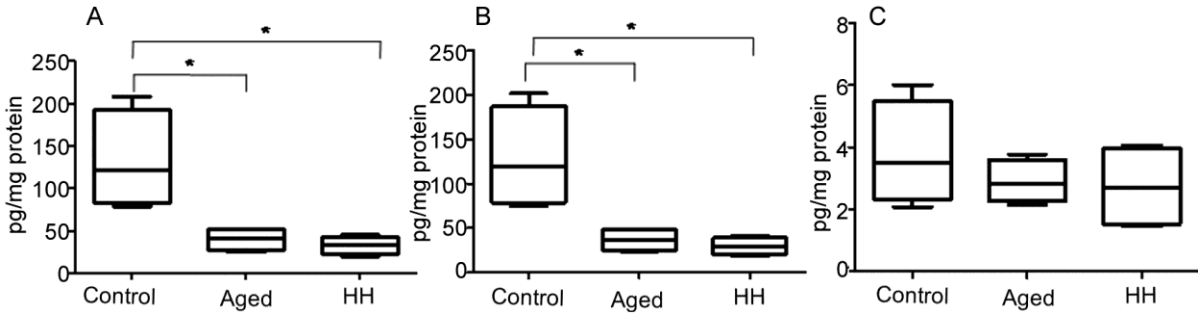

Fig. 4 — Aged and HH exposed rats showed increase lipid and protein oxidation with comparison to control rats. (A) LOOH represents lipid and other hydrophobic peroxides; (B) PrOOH representing protein hyderoperoxides; and (C) Protein carbonyl content. [Data represents by Box-and-whisker plots (05 rats per group) and were statistically analyzed by one-way ANOVA with Bonferroni's post hoc test and presented as means±SEMs. Statistical significance levels were set to: **P* <0.05; ***P* <0.01; ****P* <0.001]

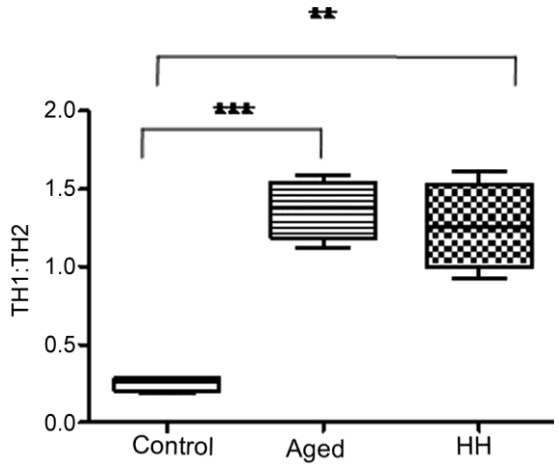

Fig. 5 — Aged and HH exposed rats showed increase TH1/TH2 ratio with comparison to control rats. [Data represents by Box-and-whisker plots (05 rats per group) and were statistically analyzed by one-way ANOVA with Bonferroni's post hoc test and presented as means±SEMs. Statistical significance levels were set to: **P* <0.05; ***P* <0.01; ****P* <0.001]

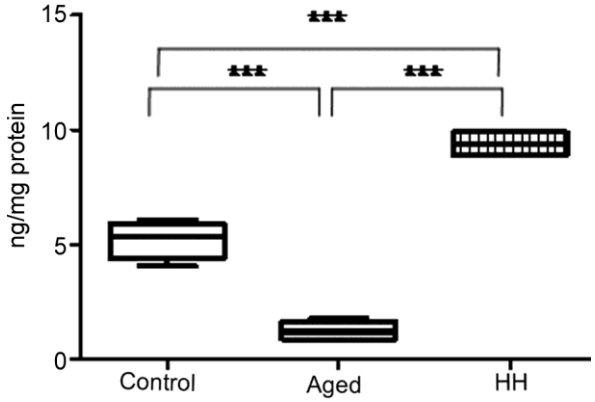

Fig. 6 — Aged showed decrease HSP60 while HH exposed rats showed increase HSP60 expression with comparison to control rats. [Data represents by Box-and-whisker plots (05 rats per group) and were statistically analyzed by one-way ANOVA with Bonferroni's post hoc test and presented as means±SEMs. Statistical significance levels were set to: **P* <0.05; ***P* <0.01; ****P* <0.001]

Role of Inflammation on hypobaric hypoxia and aging

TH1:TH2 (*IFN-γ:IL-10; ratio of pro-inflammatory and anti-inflammatory cytokine*)

TH1:TH2 ratio is basically ratio of pro- and anti-inflammatory cytokine which was increased in aged and HH-exposed rats in relation to control rats (F=40.10) (Fig. 5). TH1:TH2 ratio was noted ~12 fold increase in aged rats (*P* <0.001) and ~11 fold increase (*P* <0.01) in HH exposed rats with regard to control rats.

Role of HSP60 on hypobaric hypoxia and aging

HSP60 is hypoxia responsive as well as age specific protein which basically involved in rectifying misfolded proteins. An interesting finding was obtained in the present study as HSP60 decreased significantly (F=33.46; *P* <0.001) in aged rats while it was increased significantly (*P* <0.001) in HH-exposed rats (Fig. 6).

Protein Synthesis Marker, p-Akt during hypobaric hypoxia and aging

p-Akt is a key signaling protein of protein synthesis pathway and also involved in general tissue growth. p-Akt was observed ~8 fold increase in HH-exposed rats (*P* <0.01) while ~6 fold decrease in age rats (*P* <0.001) as compared to control rats (Fig. 7).

Effect on protein degradation and apoptotic machinery during hypobaric hypoxia and aging

Protein degradation

The catalytical core of 26S proteasome is 20S proteasome which is involved with the degradation of accumulated misfolded proteins. The activity was assayed by fluorogenic substrate, Suc-LLVY-AMC. A significant increase in chymotrypsin-like protease activity of 20S proteasome was observed in aged (*P* <0.01) and HH-exposed rats (*P* <0.001), while

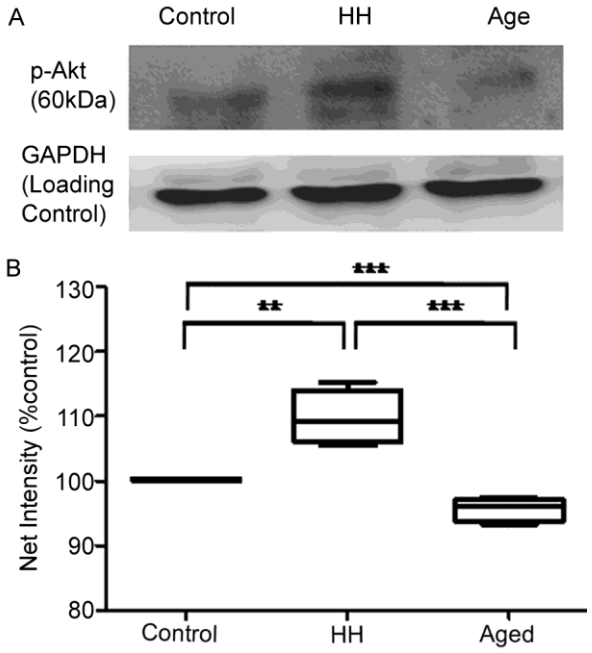

Fig. 7 — Aged rats showed decrease p-Akt protein expression while HH exposed rats showed increase p-Akt expression with comparison to control rats. (A) Representative western blots for expression of p-Akt in cytosolic fraction; and (B) Densitometric analysis using ImageJ software [Data represents by Box-and-whisker plots (05 rats per group) and were statistically analyzed by one-way ANOVA with Bonferroni's post hoc test and presented as means±SEMs. Statistical significance levels were set to: **P* <0.05; ***P* <0.01; ****P* <0.001]

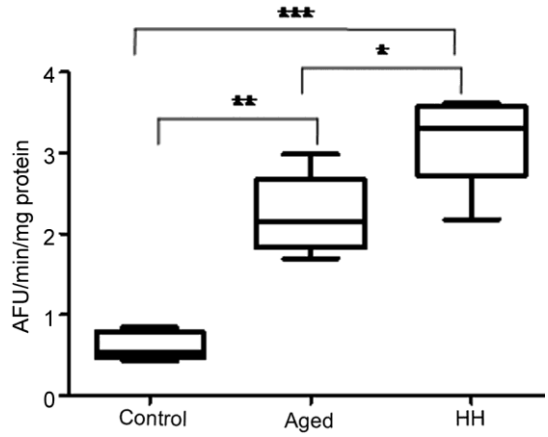

Fig. 8 — Aged and HH exposed rats showed increase 20S proteosome activity with comparison to control rats. [Data represents by Box-and-whisker plots (05 rats per group) and were statistically analyzed by one-way ANOVA with Bonferroni's post hoc test and presented as means±SEMs. Statistical significance levels were set to: **P* <0.05; ***P* <0.01; ****P* <0.001]

HH-exposed rats showed more 20S proteasome activity as compare to age rats (F=1.486) (*P* <0.05) (Fig. 8).

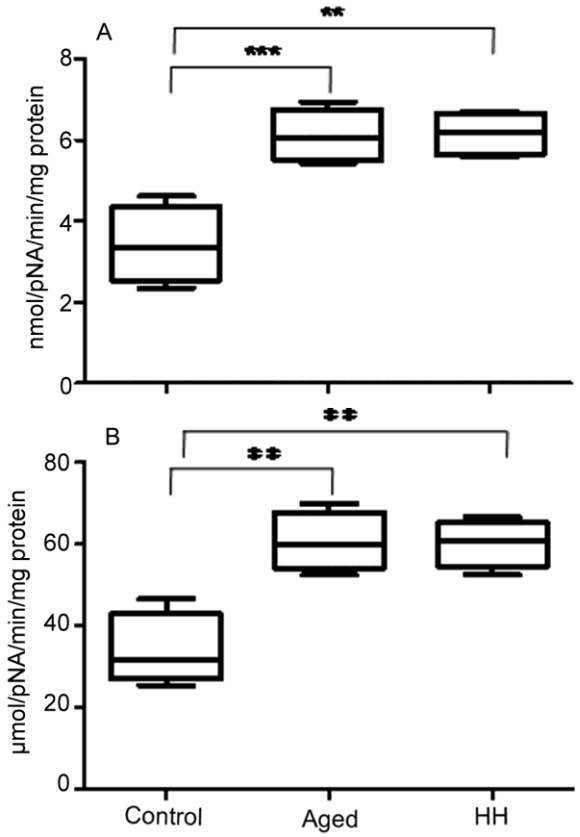

Fig. 9 — Aged and HH exposed rats showed increase cellular degradation and apoptotic machinery with comparison to control rats. (A) Caspase-3 activity; and (B) caspase-9 activity. [Data represents by Box-and-whisker plots (05 rats per group) and were statistically analyzed by one-way ANOVA with Bonferroni's post hoc test and presented as means±SEMs. Statistical significance levels were set to: **P* <0.05; ***P* <0.01; ****P* <0.001]

Caspase 3 and Caspase 9

The comparison among all the groups showed a significant increase in caspase-3 and caspase-9 activity in aged and HH-exposed rats with regard to control rats (F=26.29 and 14.29). While comparison between aged rats and HH-exposed rats showed no change in caspase 3 and caspase 9 (Fig. 9 A and B).

Discussion

The present study explored a comparative mechanism of hypobaric hypoxia and age associated muscle loss. The detailed mechanistic study provided the fact of skeletal muscle protein loss in both the stresses however the mechanism is slightly different.

Oxidative stress believed a common and prime culprit for both the conditions as it sequenced into adverse effects in skeletal muscle[31,32]. In the field of

free radical biology, ROS is considered as the primary factor which is toxic and mostly involved in disturb redox signaling that further leading to direct damage of macromolecules, proteins, lipid, DNA and RNA and the present study made an agreement with the fact[33,34]. High altitude associated hypobaric hypoxia leads to hypoxemia and oxidative stress due to overproduction of reactive oxygen species (ROS). Oxidative stress concluded in overproduction of ROS during aging which consequent into functional alterations, pathological conditions, and even death[35,36]. Ergo, ROS generation led to increased GSSG:GSH ratio in aged rats and HH-exposed rats. In spite of this, a decline in total thiol and protein thiol content contributed to the oxidative stress production in cells. The increase GSSG/GSH ratio as the indicator of oxidative stress due to consequent alternation of the redox state of glutathione[37,38].

Helper T (TH) cells may be divided into TH1 and TH2 cells. TH1 and TH2 cells are responsible for production of pro-inflammatory and anti-inflammatory cytokines, respectively. In normal scenario, a balance used to maintain between TH1 and TH2. However, a disturbance of this ratio leads to generation of inflammation[39]. The present study demonstrated an increase in TH1/TH2 ratio in aged and HH exposed rats, depicted inflammation in both the stress which involved in oxidative stress generation and these results also presented a harmony with our previous reports[40].

All these processes trigger the disruption of the redox-regulated signaling mechanism and oxidation of macromolecules like protein and lipids. The present study also made an attribution to the same reality as increased protein carbonyl content, protein hypdroperoxide and lipid hydroperoxide was noted in aged and HH-exposed rats but the comparison depicted that aged rats were more susceptible for oxidized proteins and lipids.

Heat shock proteins (HSPs), induced during stressful stimuli, engaged with crucial cellular processes like protein synthesis, facilitate protein transport, facilitate protein folding, protect newly synthesized polypeptide chains against misfolding and protein denaturation and prevent apoptosis[41]. Regarding HSP60, an interesting finding was observed in the present study that HSP60 level was increased in HH-exposed rats while it was declined in the case of aged rats. These results are an agreement with the previous reports[42,42]. These results portrayed that increment in HSP60 could be observed as adaptive mechanism in the case of hypobaric hypoxia exposure; on the other hand decrement in HSP60 could be considered as a natural degenerative phenomenon of aging. In simple words, we could speculate that hypobaric hypoxia is the stress in which cell tried to cope up with the situation via HSP60 to maintain protein structure and folding. But aging is the stress condition in which HSP60 was declined which depicted that cell was not able to cope with the stress condition.

Beside HSP60, another interesting finding of this study was expression of phosphorylated-Akt (p-Akt) in hypoxia and aged rats. Akt, involved in cellular survival pathways via inhibiting apoptotic processes and enhancing protein synthesis pathways[44]. Therefore, it is considered as key signaling protein for skeletal muscle hypertrophy[45]. Our observations indicated that p-Akt expression declined in aged rats, which demonstrate a decrease in protein synthesis pathway. While, p-Akt expression was upregulated during hypobaric hypoxic exposure, indicating increase protein synthesis as compared to control rats; even then skeletal muscle protein loss occurred. The reason is protein degradation was 5.0-fold higher and protein synthesis was 1.5-fold higher during hypobaric hypoxic stress and this disturbed the protein turnover leading to excessive protein degradation and muscle protein loss[20]. This different expression detailed that HSP60 activity declined during aging that result into misfolding protein machinery and decline protein synthesis rate. Nevertheless, HH exposure activated several signaling mechanisms to cope up with the stress condition but protein degradation pathway was too high that concluded into muscle protein loss.

At this stage if misfolded protein is rectified by HSPs then cell could behave properly. But if does not happen so, the misfolded and unfolded proteins accumulate continuously and activate ubiquitin proteasome pathway (UPS) and protein degradation. In this regard, 20S proteasome was quantified which was upregulated more in aged rats as compared to HH exposed rats. The proteasome is a major intracellular proteolytic system involved in the removal of oxidized and ubiquitinated protein and the induction of certain stress response pathways[46].

Apoptosis related biomarkers, caspase-3 and caspase-9 was also increased significantly in both the stress conditions. Akt activated via phosphorylation

and converted into phosphorylated Akt (p-Akt) which targets a number of downstream substrates, including transcription factors, pro-apoptotic Bcl-2 family members, and caspases, promoting cell survival and blocking apoptosis[47]. Additionally, p-Akt also targeted caspase-9, downregulated its activity that result into decrease apoptosis[48]. Ultimately, all the factors are attributed to muscle protein loss in both the stress condition.

Conclusion

The present study provides a detailed representation on a natural aging phenomena and another environmental stressor hypobaric hypoxia, both of which are associated with skeletal muscle protein loss. Although both the conditions lead to loss of skeletal muscles, the mechanisms are slightly different. During hypobaric hypoxia exposure, the skeletal muscle tissue activates several adaptive signaling mechanisms such as increase in HSPs, protein folding, protein synthesis pathway for coping with the adverse stress. However due to severe and chronic hypoxia, there is increased accumulation of misfolded and oxidized proteins which leads to enhanced protein degradation and apoptosis. In contrast, aging induced sarcopenia is characterized by the slow and progressive loss of muscle mass in the absence of any underlying disease or condition. During aging there is a decline in the antioxidants, protein folding machinery and the protein synthesis pathway which is the major reason for skeletal muscle loss. Further detailed studies will be able to decipher molecular targets for designing drugs/interventions for amelioration of muscle loss.

Conflict of interest

Authors declare no conflict of interest.

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