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Deciphering the Puzzle of Hypobaric Hypoxia: Proteomics, Prophylaxis and Modelling Approach

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ABSTRACT

Hypoxia, particularly hypobaric hypoxia, is a multifaceted entity which includes certain molecular, patho-physiological and biochemical aspects. Any single aspect in itself cannot help us elucidate hypobaric hypoxia in its entirety. We observed three crucial lacunae within the existing literature. These include a lack of high-throughput investigations into redox PTMs, particularly NO-based PTMs; a prophylactic supplement with proven efficacy and safety which doesn't involve medical supervision and is not contraindicated in hepatic, renal and cardiac insufficiencies; and a clinically validated rodent model of HAPE without any genetic/pharmacological manipulations. In the present study, we present an antagonistic interplay between nitrosylation and carbonylation which shows an additional NO-based network that is active in acclimatised individuals. Then we present a micronised aqueous suspension of silymarin which is efficacious at low doses in providing antioxidant, anti-inflammatory and hypoxia-adaptive vascular responses in addition to being a free radical quencher itself. Silymarin has an excellent safety and efficacy profile in humans. Finally, we create a SD rat model of HAPE which was used to reverse-translate a previously known HAPE marker in humans (SULT1A1) and elucidate the synergistic occurrence of HAPE and inflammation cascades. This is the first radiologically validated rodent HAPE model. In conclusion, we were able to elucidate the molecular, biochemical and patho-physiological aspects of hypobaric hypoxia which were left out by previous studies.

Keywords: Hypoxia; High-altitude; Proteomics; Redox PTMs; HAPE model; Silymarin; Prophylaxis

1. INTRODUCTION

Hypobaric hypoxia, the most widespread form of environmental hypoxia encountered on this planet has fascinated the human psyche since the ancient times. Ancient folklore refers to the mountains as God's abode, a magical place with many gifts and pitfalls and a place to seek solitude and immortality. Indeed, with the advent of modern science and a deeper understanding of the effects of hypobaric hypoxia on human physiology, we now know that high altitude training is a gift to improve sports performance and tolerance to oxygen-deficient conditions in humans. High altitude is equally unforgiving in that it causes high altitude illnesses like acute mountain sickness (AMS) and high altitude pulmonary edema (HAPE) in those who ascend without rest. We have also found multiple herbal concoctions at high altitude that not only improve general health but also help prevent high altitude illnesses.

Our lab has been at the forefront of understanding how the proteome responds to hypobaric hypoxia at altitude. The proteome simply means collection of all the proteins that are present in a given cell/tissue/organ/organism at a particular time. As per the definition provided by Wilkins, proteome is defined as "the entire complement of proteins expressed by a genome, cell, tissue or organism." The study of the proteome is called proteomics. Proteomics has three cornerstones for the effective understanding of data and its conversion into knowledge. First is fractionation and separation of a sample into constituent proteins/peptides; second is its analysis using mass spectrometry (MS) (other older methods also may be used) and third is use of bioinformatics based network analysis tools to find the protein networks being indicated by the proteins identified and quantified during MS analysis. As is well known, the proteome has much greater complexity as compared to the genome of an organism. Also, the proteomic response is a real time response to prevalent stimuli while most of the genomic response is in the order of years and generations.

Since hypobaric hypoxia occurs with greater intensity and over longer durations as we ascend higher, the study of the proteome especially during acute and sub-chronic exposures, makes sense. In this regard, based on previous studies (of which a sizable chunk was contributed by our lab), we identified crucial missing links and then investigated these aspects for a better understanding of hypobaric hypoxia's effect on the proteome. During previous proteomics based studies[1-7], we observed that post-translational modifications (PTMs) brought about by hypoxia induced redox stress were not actively investigated for any sort of association. This is despite the fact that protein functions, signalling cascades are profoundly affected by redox PTMs.

Secondly, we observed a great emphasis on drug

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repurposing during prophylaxis against hypobaric hypoxia. A great many drugs were present, including but not limited to, nifedipine, salmeterol, budesonide, tadalafil, acetazolamide and etc. on the market as prophylactics. However, almost all of these drugs were contra-indicated in people with cardiovascular, renal and hepatic insufficiencies. They were also contra-indicated in pregnant and breastfeeding females[8-10]. Some of these medicines (e.g. salmeterol) are also advised to be used with caution in individuals who have had recurrent HAPE[11]. Apart from this, all of these drugs require medical supervision for use. The dosage must be strictly adhered to and overdosing is an issue. Thus, the requirement for a general use health supplement that is not contraindicated in conditions like renal and hepatic insufficiencies, doesn't require medical supervision for dosage and can be taken during pregnancy and breastfeeding is the need of the hour. Also, this said prophylactic must be easy to manufacture and cost-effective.

Finally, we observed that although there was a fair understanding of the symptoms and their management in cases of high altitude illnesses, particularly HAPE, the underlying molecular reasons were yet to be fully elucidated. This lack of understanding of HAPE was due to the fact that it affected human lungs, which are not easily accessible and there existed no clinically validated animal model of HAPE. Hence, we strived to investigate solutions to these shortcomings in hypoxia research and eliminate them.

2. NITROSYLATION AND CARBONYLATION ARE INVOLVED IN A TUG-OF-WAR WITHIN A CELL: IMPLICATIONS FOR CELL FATE DURING HYPOXIA

Nitric oxide (NO) is central to the vasculo-endothelial response to hypoxia. It was initially identified as the endothelium derived relaxing factor[12,13]. Human studies focusing on high altitude native populations have found higher NO concentration as a major distinguishable characteristic during successful hypoxia adaptive response[14,15]. Previous studies show that lower exhaled NO is characteristic of maladaptive high altitude exposure[16-18] and HAPE susceptibility[19-21]. A previous study by Singh[22], *et al* from our lab showed that increased NO bioavailability during hypoxia helps activate antioxidant genes and control oxidative stress. NO is also crucial in being the sole proprietor of nitrosylation, a type of redox PTM where a S-nitrosyl group attaches to amino acid residue, particularly cysteine (Cys) and induces altered protein function. Also, hypoxia exposure induces excess oxidant/free radical generation in the organism. Its well known that CO moiety is added to proteins upon exposure to oxygen radicals and this leads to proteins being rendered inactive. As hypobaric hypoxia induces both NO production and free radical generation, it is imperative that the effects of nitrosylation and carbonylation be studied and their association elucidated.

Based on this, we exposed H9c2 cells to hypoxia (simulated; 0.5% O_2). In addition, to understand if there was any effect of nitrosylation on carbonylation and vice-versa, we also used L-NAME (2mM), an inhibitor of NO production. We observed that L-NAME individually and hypoxia in addition to L-NAME were the most successful in inhibiting both NO levels and nitrosylation (Fig. 1). This was then observed to cause increased carbonylation as compared to control group[23]. To summarize, we observed that blocking NO production led to decreased nitrosylation of proteins, increased ROS production and carbonylation of proteins, suppression of HIF1a (transcriptional master regulator of hypoxia adaptive response); Nrf2 (key transcriptional switch for antioxidant response) and GPx3 (central antioxidant) expression as well as thioredoxin reductase 2 activity and increased cell death during hypoxia exposure. Thus, we observed that nitrosylation and carbonylation are antagonistic even though both are triggered by hypoxia and reduced nitrosylation usually leads to poorer outcomes during hypoxia exposure at the cellular level.

3. MICRONISED SILYMARIN HELPS SD RATS SURVIVE EXTREME HYPOBARIC HYPOXIA BY AUGMENTING ANTIOXIDANT AND ADAPTIVE VASCULAR RESPONSES

In the next phase of our study, we wished to propose relevant prophylaxis options that were cost-effective, extremely safe to use without medical supervision, did not require complex formulations and were not contraindicated in cases of cardiac, renal and hepatic insufficiencies.

Since such a profile is usually found in herbal formulations, we began to search for a suitable herbal prophylactic. The milk thistle is a medium sized plant known to be abundant in a wide geographical area. It has been characterised since the times of Ptolemy as a general health supplement which was later proven to be effective against *Amanita* mushroom poisoning. In the present era, its extract (present in leaves, buds, flowers and seeds) is a widely sold herbal hepato-protectant and general health supplement named silymarin. Silymarin is proven to be safe for use in humans, has negligible side-effects and a practically unattainable LD_{50}. Its is a polyflavonolignan mixture comprising multiple constituents with polyphenolic and flavonoid rings suggesting excellent radical quenching capabilities. It is not contra-indicated in cases of renal and cardiac insufficiencies instead it's use is promoted in such cases. However, silymarin has its fair share of issues. Its polyphenolic ring structure makes its bioavailability low which is improved using complex formulations[24-27] that add both dosage intensity and cost to an otherwise cost-effective remedy. This drawback has impeded its use in general life as life becomes more and more hectic.

To improve upon this, we micronised silymarin using sonication and filtration in an aqueous solution (PBS). This led to a smaller median particle size as well as greater stability of the aqueous suspension as compared to the non-sonicated silymarin extract in aqueous suspension. The use of micronised silymarin also led to greater bioavailability as compared to the non-sonicated silymarin suspension. Further, we observed that sonicated silymarin suspension upon dosing for 5 days at 50 mg/kg bodyweight in SD rats who were subsequently exposed to hypobaric hypoxia (simulated; 7620 m for 6 h) resulted in reduced ROS and its byproducts like carbonyl content; increased antioxidant (glutathione, catalase, SOD etc.) expression and activity (Fig. 2); upregulated HIF1a, VEGF (improves vascularisation) and EPO (improves hematocrit)

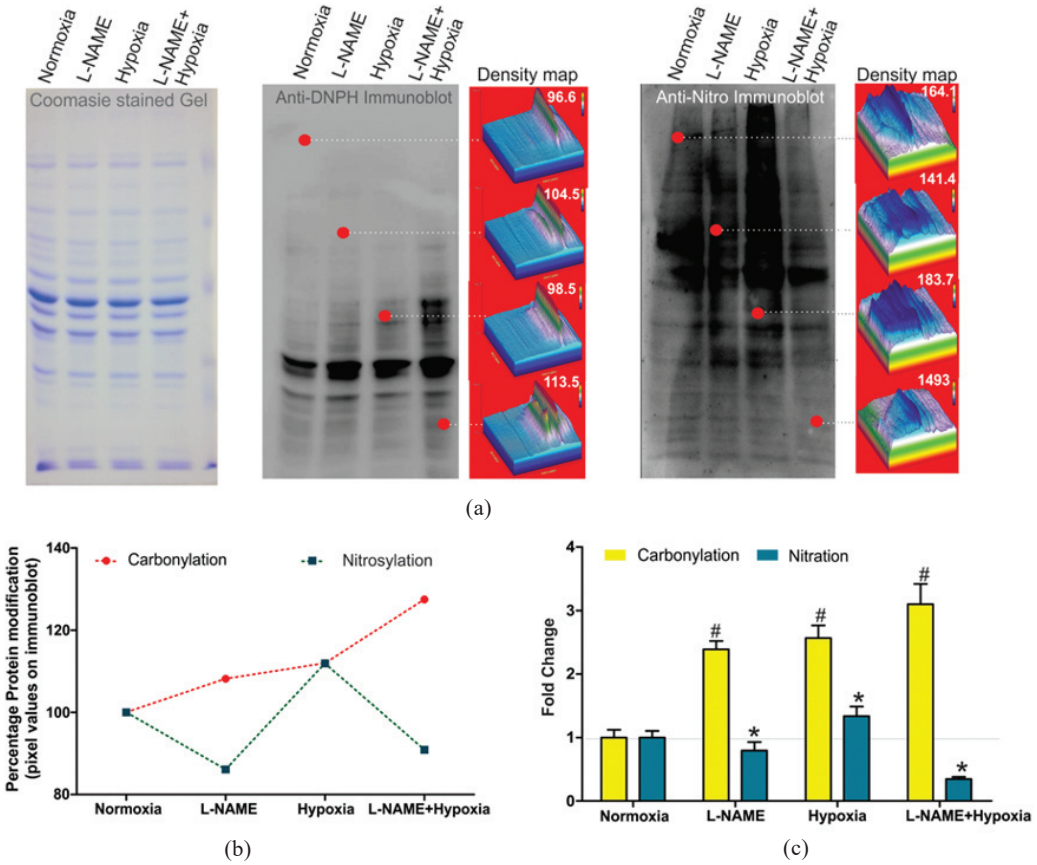

Figure 1. **Comparison of trend of carbonylation and nitrosylation: (a) Using a Coomassie stained gel as loading control, oxy-blots and nitroblots assessing carbonylated and nitrosylated proteins (3-D density maps) across Normoxia, Normoxia+L-NAME, Hypoxia and L-NAME+Hypoxia groups (Mean±SEM), (b) Relative trendlines for nitrosylation and carbonylation. Each data point per group represents percent nitrosylation/carbonylation (derived from Mean±SEM of 3-D density maps for both nitroblots and oxyblots), and (c) Bar-graphs comparing fold-change in protein carbonylation and nitration. ELISA was used to determine levels of nitration and carbonylation (Mean±SEM). * represents p-value<0.05. # represents p-value<0.01.**

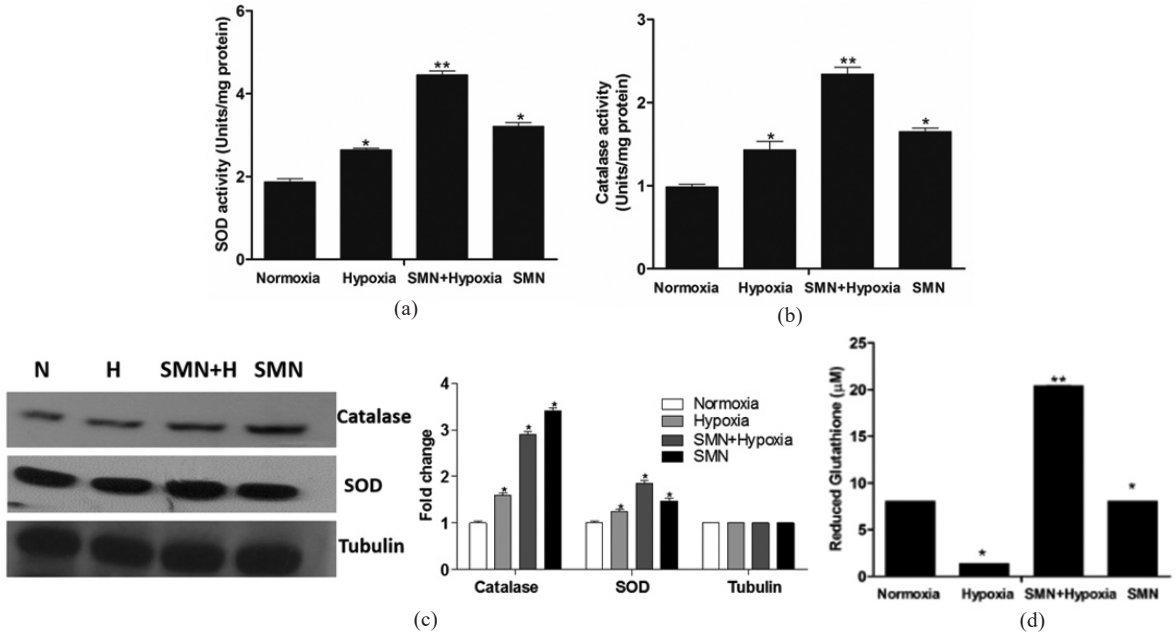

Figure 2. **Status of key antioxidants: (a) Superoxide dismutase (SOD), (b) catalase in Normoxia, Hypoxia, SMN+Hypoxia and SMN groups, (c) Representative immunoblots of Catalase and SOD in Normoxia, Hypoxia, SMN+Hypoxia and SMN along with bar graphs depicting their respective levels across experimental groups. Tubulin was used as loading control, and (d) Bar graph depicting reduced glutathione concentrations across experimental groups. Significance represented as *p-value<0.05; **p-value<0.01. Results represented as Mean±SEM.**

at both transcript and protein levels and also helped diffuse hypoxia induced inflammation via repression of cytokines like TGF-beta, IL-6 and TNF-alpha[28] (Fig. 3). Thus, we created a cost-effective micronised silymarin solution that is already proven to be safe-for-use in humans and doesn't require medical supervision for use effective at mitigating acute hypoxia induced damage.

4. A CLINICALLY VALIDATED SD RAT MODEL OF HAPE

In the third phase of our study, we wanted to create a clinically validated HAPE model using SD rats. Our previous study had already revealed that the proteome response to hypoxia of SD rats and humans share a lot of homology[29]. Thus, we chose the SD rat as our model. In previous studies attempting to model HAPE using animals, long effort-intensive, equipment-intensive, pharmacological and indirect methods to create and confirm HAPE were used[30-35]. Most of these previous studies measured HAPE via ratio of wet:dry weight of lung and by estimating the fluid content of lungs. Also, the exercise protocols employed were of long durations. In a previous study, it has been shown that low intensity exercise actually relieves hypertension in rats when done over long temporal scales[36]. Since HAPE has the cardinal feature of pulmonary hypertension, the exercise protocol is inherently flawed when used for HAPE induction. None of these studies attempted establishing HAPE via chest x-rays in their models, which is the clinical gold standard. None of these previous studies definitively dealt with the question of whether HAPE induces inflammation or vice-versa.

In our study, we first exhausted the rat by forcing them to swim in a circular tank. At the point of exhaustion, the rats were dried off and placed in a hypobaric hypoxia simulation

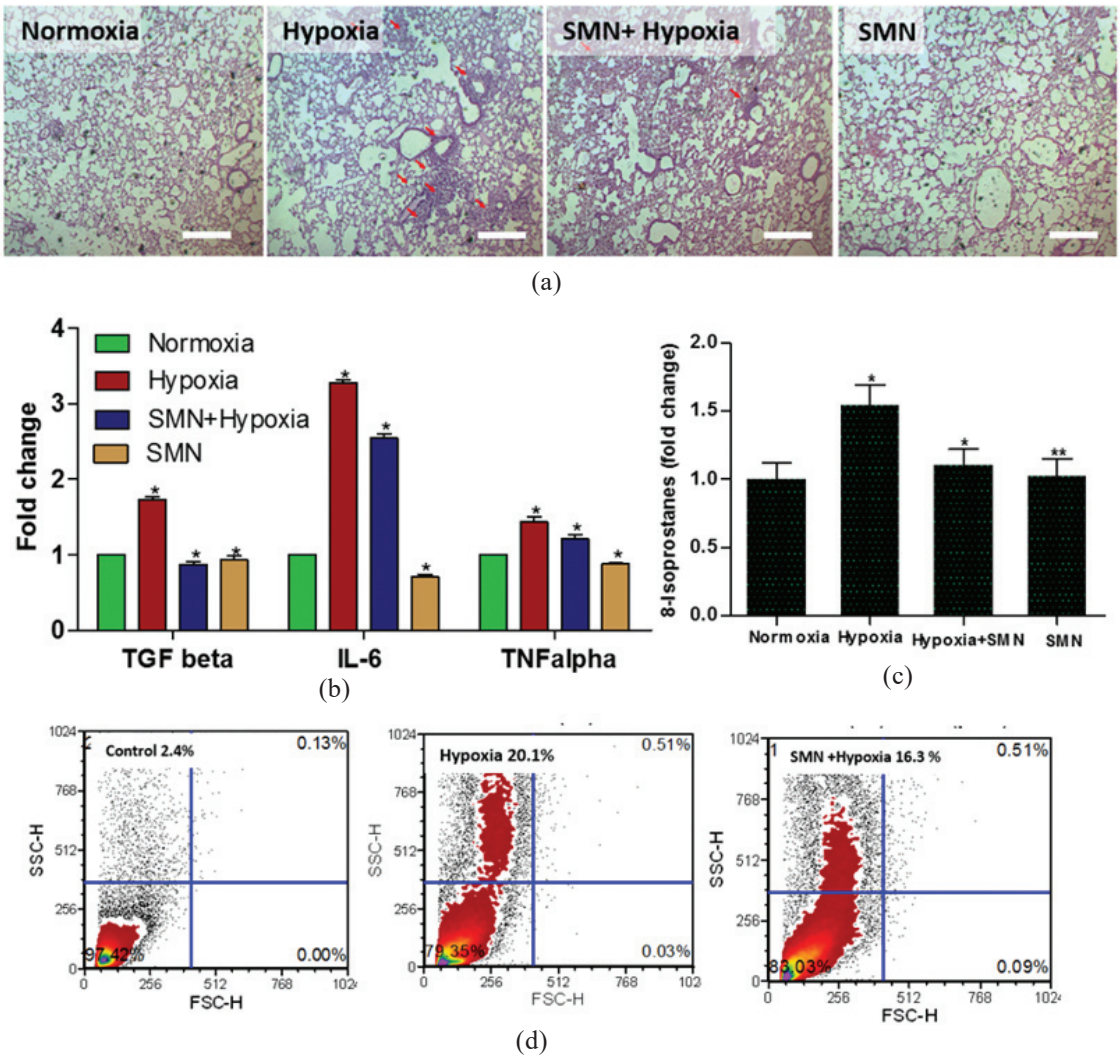

Figure 3. SMN prevented loss of liver function and ameliorated inflammatory response in hypoxia: (a) Representative lung tissue sections of Normoxia, Hypoxia, SMN+Hypoxia and SMN groups stained with hematoxylin and eosin to visualize qualitative inflammatory changes, (b) Fold change across experimental groups in plasma concentrations of cytokines TGF- beta, IL-6 and TNF-alpha represented as bar-graphs, (c) Bar-graph depicting fold change in 8-isoprostanes' concentration in plasma across experimental groups, and (d) FACS analysis of bronchoalveolar lavage fluid of Normoxia (Control); Hypoxia and SMN+Hypoxia represented as Forward scatter and side scatter plots.

chamber for 18 h at 7620 m. After 18 h, the rats were examined using a chest x-ray for signs of edema in the lungs. We observed all rats in our swimming+hypoxia group developed pulmonary edema. After confirmation of pulmonary edema, we harvested the lungs and plasma of the rats. Upon the analyses of lungs and plasma, we observed sulfotransferase 1A1 (SULT 1A1) levels to be the highest in HAPE-afflicted rats (Fig. 4). SULT 1A1 was previously reported by our lab to be a biomarker for HAPE in humans[4]. Thus, we successfully reverse translated our marker in this study. We also observed levels of pro-inflammatory cytokines (e.g. TNF-alpha) to increase sharply while dual-role cytokines (e.g. IFN-gamma) declined sharply in HAPE-afflicted rats (Figs. 4(b)-4(d)). In both cases, hypoxia did not elicit sharp changes in the levels of these cytokines. Thus, HAPE and inflammation can be termed as synergistic events rather than the cause and effect of each other. In addition, H&E staining of lung tissues revealed that exudate accumulation only occurred in HAPE afflicted lung tissues (Fig. 5). To summarize, our study led to a clinically relevant protocol for inducing HAPE using which we found that inflammation and HAPE are synergistic events[37].

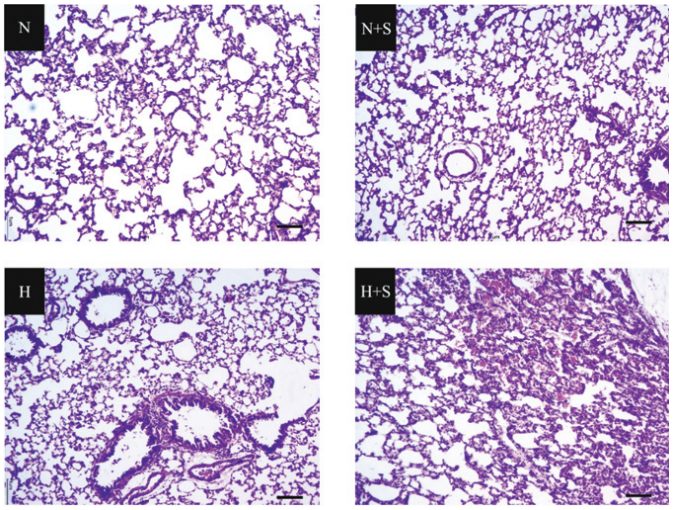

Figure 5. Representative H&E stained lung tissues showing extensive damage in HAPE model lung tissues. H group shows increased aggregation of inflammatory cells however H+S group, suffering from HAPE, shows greater inflammation along with accumulation of exudate in the lung tissues.

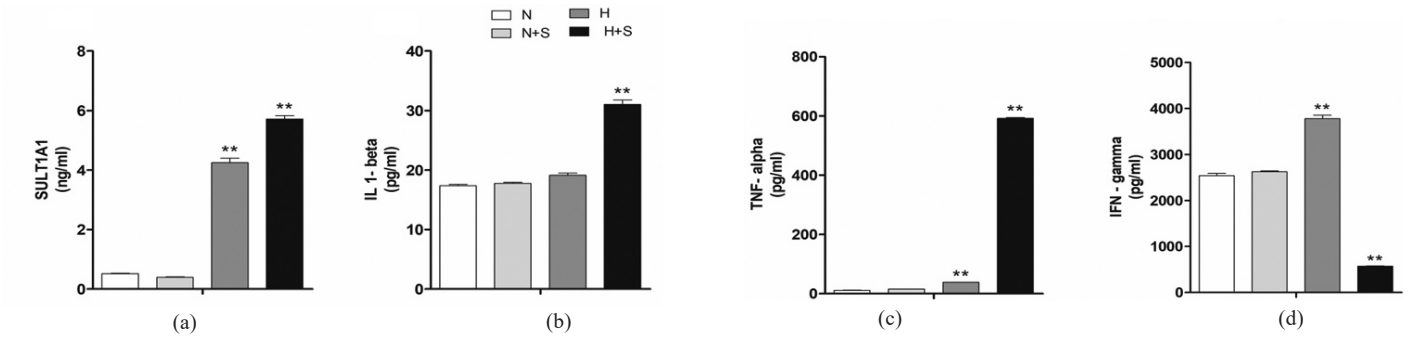

Figure 4. Reverse translation of protein markers and inflammatory proteins in HAPE model: (a) Sulfotransferase 1A1 (ng/ml), (b) IL 1-beta (pg/ml), (c) TNF-alpha (pg/ml), and (d) IFN-gamma (pg/ml) concentrations in plasma of SD rats. Results are expressed as Mean±SEM. ** represents p-value<0.0001.

5. CONCLUSION

Our studies revealed that hypoxia exposure causes a crosstalk between nitrosylation and carbonylation, adding the dimension of PTMs to standard protein response in hypoxia. In addition, we were able to provide a novel cost-effective, prescription-free, side-effect free prophylactic choice in silymarin and a clinically validated, reliable and non-pharmacologic HAPE model for detailed studies on HAPE.

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CONTRIBUTORS

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Dr Subhojit Paul received his PhD in Life Sciences He is a recipient of CSIR-SRF fellowship.
In this study, he has performed the experiments, compiled and analysed the data for studies on silymarin and creation of HAPE model.

Ms Shikha Jain is persuing her PhD from DIPAS, DRDO, Delhi. Shehas submitted her PhD thesis in Bharathiyar University.
In this study, She has performed part of the experiments and analysed data.

Dr Yasmin Ahmad is currently working as Scientist D in Peptide and Proteomics Division, DIPAS, Delhi.
In the present study she has guided the research related to hypobaric hypoxia and related maladies and effect of silymarin on hypoxic condition.

Dr Kalpana Bhargava is currently working as Scientist F at High Energy Material Research Lab (HEMRL), Pune. Prior to this she worked in DIPAS, DRDO, Delhi.
In the current study she has conceptualised the idea and guided the research work on hypobaric hypoxia related studies.