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Reverse translating SULT1A1, a potential biomarker in roentgenographically tested rat model of rapid HAPE induction

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ABSTRACT

Aims: HAPE remains the most common lethal high-altitude disease. Although its pathophysiology and other associated causal factors have been partially uncovered along with some potential biomarker proteins, it has not been completely elucidated. A major hindrance to improving the understanding of HAPE pathophysiology and associated molecular events has been the absence of a quick, reliable and definitive animal model of HAPE. This study is aimed at development of a rapid and reliable SD rat model of high altitude pulmonary edema (HAPE) that can be roentgenographically confirmed and be used to study protein markers of HAPE.

Main methods: In this study, we detail the process of rapidly inducing HAPE in male SD rats within 18 h of simulated high-altitude exposure without causing high rates of mortality. Thereafter, we confirmed HAPE using roentgenography. We assessed Sulfotransferase 1A1 (SULT1A1), IL-1 beta, TNF- alpha and IFN-gamma using ELISA. Finally, H&E staining of lung tissues was also performed.

Key findings: A roentgenographically confirmed HAPE model was demonstrated. SULT 1A1 levels are found to be highest in rats suffering HAPE, as previously confirmed in human patients. Inflammation was also assessed based on levels of inflammatory proteins like IL-1b, TNF-a, and IFN-g in addition to H&E staining of lung tissues. Inflammation and HAPE were observed to be synergistic events and not cause and effect of each other.

Significance: This rat model of HAPE will help researchers and clinicians in evaluating performance of therapies, potential biomarker and also further elucidate underlying molecular processes causing HAPE.

1. Introduction

High altitude environment causes systemic stress to all organisms primarily due to deficient ambient oxygen as the barometric pressure decreases with increasing altitude. Secondary factors like cold, harsh terrain and low humidity also contribute towards it. High altitude exploration or sojourns require physically demanding acclimatization to prevent chances of high-altitude illnesses. High altitude illnesses include Acute mountain sickness, High altitude cerebral edema and High altitude pulmonary edema [1]. High altitude pulmonary edema, abbreviated as HAPE, refers to the accumulation of extra vascular fluid in the lungs in an individual upon ascent to altitudes of 8000 ft or above [2]. HAPE incidence usually increases as one ascends higher and faster [3]. The accumulation of this exudate (originating from leaking capillaries) also causes inflammatory consequences as the exudate is rich in inflammatory proteins like interferon gamma, tumor necrosis factor and interleukins [4,5]. This aspect is debated in context of its faithful replication in animal models [5–8]. The cause of fluid accumulation is non-cardiogenic in nature. Rather, increased hypoxic pulmonary vasoconstriction causing increased pulmonary artery pressure and uneven distribution of this increased pressure across the capillary beds, termed stress failure of the alveolar capillaries, in the lungs are the causal factors [9]. Secondly, the reduced rates of fluid clearance from the lungs due to insufficient ENaC activity add to the edema and result in destruction of alveolar integrity [6,10,11].

Many drugs are available for prophylaxis or treatment of HAPE including nifedipine and acetazolamide. However, oxygen and descent are still essential for treating HAPE. Many previous studies have provided potential protein markers for diagnosis of HAPE [12,13]. These and other studies [5,9,10] have also elucidated, although only partially, the underlying pathophysiology of HAPE. For further improving the understanding of the underlying pathophysiology and more importantly the molecular events (particularly in lung tissue), an animal model of HAPE is essential. Previous attempts at establishing an animal model, more particularly a rat model of HAPE, have provided inconsistent results. Other animals used for creation of HAPE or closely related pulmonary hypertension model include pigs, mice, cows, primates, sheep and swine [14–17]. However, such large and relatively

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more evolved animals not only involve incurring public wrath but also lead to greater logistics, higher expenditure, difficult handling and longer life cycles leading to longer wait periods. Rat model provides the following advantages for studies in context of HAPE: economy, safe handling, quicker life cycles and mechanistic certainty. Most procedures cause exercise fatigue but over long durations like 48 h [9], partly nullifying the quicker turn over times associated with a rat model due its shorter life cycle. These procedures also lack in terms of reliable induction of HAPE as no clinically accepted techniques are used to assess HAPE in animals subjected to the particular protocol. Certain aspects of HAPE like hypoxic pulmonary vasoconstriction, pulmonary hypertension and lack of ENaC activity are highlighted and mechanisms or therapies related to these are discussed [18–21]. Again, logistical requirements (e.g. a treadmill within a hypoxia simulation chamber) for such long lasting experimental procedures are a definite burden on the experimenter. Procedures involving running may not be the best suited to HAPE induction because running is typically known in humans and rats to relieve hypertension and improve circulation [22,23]. This is obviously not feasible in a model of HAPE. Another major lacuna of previous procedures to induce HAPE has been reluctance in checking HAPE induction with the clinically accepted chest X-ray test and instead performing gravimetric analysis and estimating lung water content. Since it is reported that lung vital capacity and lung size are lower in those susceptible to HAPE [24,25], it seems counterproductive to estimate lung water content in rat exposed to hypobaric hypoxia against a normoxic control as the measures required for lung function are never evaluated in rats prior to grouping. Indirect measures should be avoided when a direct test is possible. In this study, male SD rats underwent forced swim till exhaustion in a circular tank. They were then dried with a towel and immediately placed in a hypoxia simulation chamber for 18 h at simulated altitude of 25,000 ft. The ascent rate was kept extremely quick. After simulated exposure to 25,000 ft, the rats were kept in normoxic conditions for 30 min. They were then subjected to chest X-rays to determine edema in their lungs. Mortality was very low. Upon confirmation of pulmonary edema after X-rays, the rats were sacrificed and their lungs and blood plasma harvested. We reverse translated a previously reported potential biomarker protein associated with HAPE as well as certain inflammatory markers and histologically assessed lung tissue.

2. Materials and methods

2.1. Experimental design

Male SD rats (age matched) weighing 220–250 g were used for the study. All rats were divided randomly into the following groups:

Normoxia (N): SD rats that were exposed to normoxic conditions only.
Normoxia + swimming (N + S): SD rats that underwent normoxia after exhaustive swimming.
Hypoxia (H): SD rats that underwent hypobaric hypoxia exposure.
Hypoxia + swimming (H + S): SD rats that underwent hypobaric hypoxia exposure after exhaustive swimming.

Hypoxia exposure lasted for different durations of 6 h, 12 h, 15 h and 18 h in both H and H + S groups. For all exposures in H and H + S groups, each group had 6 rats each initially while later in the 18 h group 10 rats were used in each group based on initial assessment of edema. N and N + S groups also had 10 rats in each group. The animals were housed in polypropylene cages with sterilized straw. They were provided food and water ad libitum. A light/dark cycle of 12 h each was provided. Temperature and humidity were 25 °C and 50% respectively.

2.2. Method of HAPE induction

The N + S and H + S groups were given 5 min of swim in a 3 ft diameter round tank filled with water at 20–25 °C for 3 consecutive days in the morning. On the fourth day, both groups were subjected to exhaustive swimming at the same time as on previous days. The point of retrieval for the rats was inability to keep snout above water for 10 s continuously. Upon retrieval, the rats were dried using towels. The H and H + S group underwent simulated hypoxia exposure corresponding to 25,000 ft (282 mmHg) for different time intervals (6 h, 12 h, 15 h and 18 h). The rate of ascent was kept extremely high at > 550 m/min. Airflow was kept at 2 l/min. Temperature and humidity throughout the hypoxia exposure were kept at 25–30 °C and 50–55%, respectively. A hypoxia simulation chamber was used for the same (7 Star Systems, New Delhi). The N + S group underwent normoxic exposure for the same time. Animals were sedated after the normoxic/hypoxic exposure using a heavily diluted solution of barbiturates and lignocaine injected intraperitoneally and a chest x-ray was performed on each animal. In the H and H + S groups, chest x-ray was performed after approximately 30 min of normoxic exposure. All groups had their lungs and blood plasma harvested after chest x-rays were performed. Animal procedures and experimental protocols were approved by Institutional Animal Ethics Committee and we followed the standards set forth in the Guide for the Care and Use of Laboratory Animals (National Academy of Science, Washington, D.C.) in addition to ARRIVE guidelines.

2.3. Roentgenographic assessment of HAPE in rats

Rats were placed in supine anteroposterior position for chest X-ray. The X-ray dose given to each rat was 2 rem (0.02 mSv) per X-ray (Siemens Klinoskop H/Heliophos D X-ray and Carestream CR Imager, India). All rats were sedated prior to being subjected to X-rays. A blinded observer specialized in roentgenography categorized the roentgenograms into two groups: lungs with edema and lungs without edema.

2.4. ELISA

Sulfotransferase 1A1 (SULT1A1; Cat no. SEB268Ra, Cloud Clone, Hubei, China), Tumor necrosis factor alpha (TNF-a; Cat no. CSB-E11987r, Cusabio, Houston, USA), Interleukin 1-beta (IL-1b; Cat no. SL0402Ra, Sunlong Biotech, Hangzhou, China) and Interferon-gamma (IFN-g; Cat no.ab113349, Abcam, USA) were estimated using ELISA kit as per the manufacturers' instructions. Briefly, plasma samples were added to individual wells and incubated for 2 h at room temperature. The wells' content was aspirated. Biotin-antibody was added to each well and incubated for 1 h. The wells were then washed thrice and HRP-avidin was added to each well. Incubation was done for 1 h. The wells were again washed thrice. TMB Substrate was added to each well in dark and incubated for 10–30 min. Finally, Stop solution was added to each well and OD_{450} measured immediately.

2.5. H&E staining of lung sections

The posterior lobes of the left lung were fixed in formalin solution for 24 h. The fixed lung tissue was then sectioned and processed for H&E staining. After the staining lung tissues were observed under a light microscope.

2.6. Statistical analysis

Data sets were analyzed using Graph pad prism (v 5.0). For statistical significance, 1-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison test was applied. Significance was set at $p < 0.05$. In all cases, three independent experiments were carried out. Results were presented as mean value ± standard error of mean (SEM).

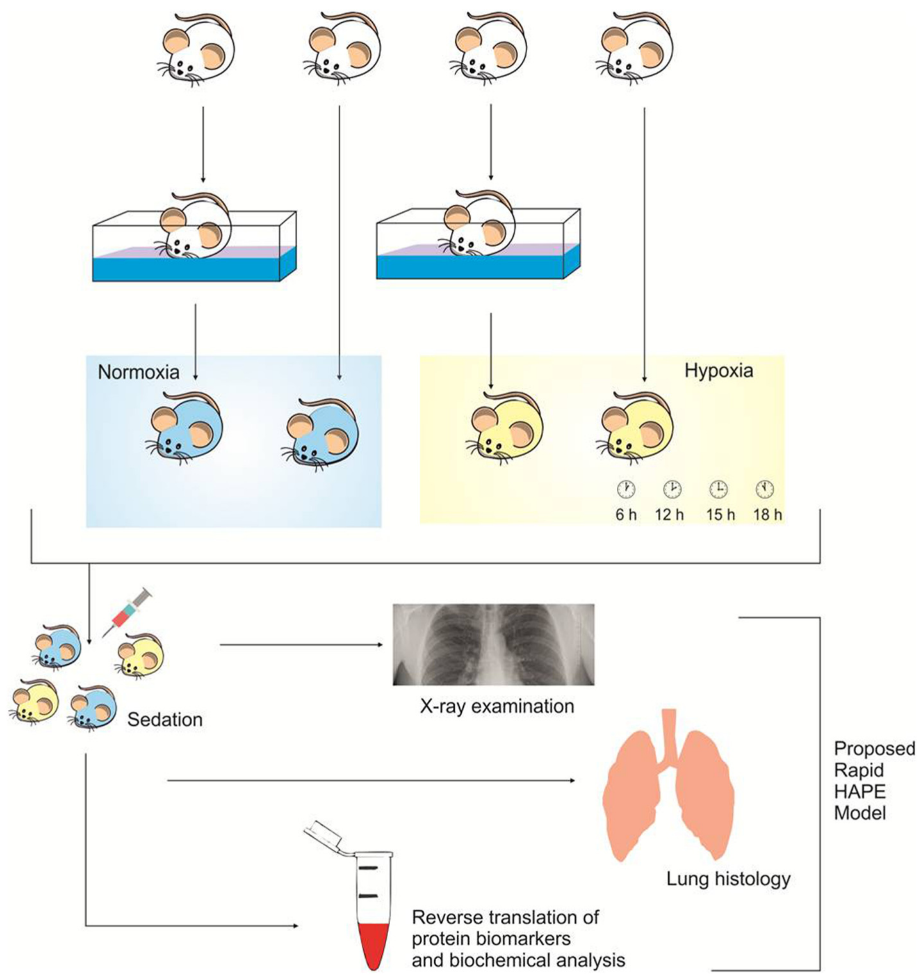

Fig. 1. Experimental design. Male SD rats weighing 220–250 g (n = 10 per group) were divided into four groups randomly, namely Normoxia (N), Normoxia +Swimming (N + S), Hypoxia (H) and Hypoxia +Swimming (H + S). Normoxic group were exposed to normoxic conditions at room temperature. Hypoxic group was exposed to simulated altitude of 25,000 ft (7620 m) for 18 h at 25 °C. H + S and N + S groups performed exhaustive swimming in a circular tank with water temperature maintained at 20–25 °C. H + S group was immediately exposed to simulated altitude of 25,000 ft after exhaustive swimming. The duration of simulated altitude exposure lasted for 6 h, 12 h, 15 h and 18 h with pulmonary edema being roentgenographically confirmed in H + S group after 18 h of simulated altitude exposure. Finally, lung histology, protein markers and biochemical stress assays were analyzed in N, N + S, H and H + S (18 h) groups. Based on the roentgenographic data and associated experimental results, we propose a model for rapid induction of HAPE in otherwise normal healthy SD rats.

3. Results

3.1. Induction and roentgenographic evidence of HAPE

The rats had no changes in their light/day cycles, temperature and humidity levels as well as dietary factors prior to or during the protocol for HAPE induction. After swimming till exhaustion in a circular tank with no wave action or extreme temperature, the rats were dried off gently and subjected to a very high ascent rate of > 550 m/min till a simulated altitude of 25,000 ft (282 mmHg) was achieved. Upon completion of 18 h at the stated altitude, the rats were kept in normoxic conditions for 30 min (Fig. 1). Then, they were anesthetized and roentgenography was performed. The rats were kept in supine anteroposterior position during roentgenographical assessment of HAPE. As per Vock's criteria for roentgenographically assessing HAPE in humans [26], the lungs were divided into 4 quadrants where normal parenchyma was given a score of zero. Areas of the lung with questionable pathologic areas were scored as 1; clearly visible interstitial disease < 50% of any lung quadrant was scored as 2. Non-confluent interstitial disease > 50% of any lung quadrant was scored as 3; and partly confluent alveolar disease was scored as 4. A roentgenogram with at least one lung quadrant having a minimum score of 2 was considered positive for HAPE. This was in addition to current clinical practice wherein chest roentgenograms showing decreased pulmonary transmittance, increased or obscure lung markings, ground glass-like changes or patchy shadows in the lung are accepted as symptoms of HAPE. We observed that all rats exposed to 18 h of simulated hypobaric hypoxia corresponding to 25,000 ft subsequent to exhaustive swim had some degree of edema in lungs. Rales were also observed in this group of rats during anterolateral auscultation. Out of the total group of 16 rats in H + S (18 h), mortality occurred in 3 rats only. We did not observe any phlegm or frothy pink sputum. H + S groups with hypobaric hypoxia exposures of 6 h, 12 h and 15 h did not show any edema in lungs as per roentgenographic evidence. H, N and N + S groups did not show any signs of pulmonary edema (Fig. 2).

3.2. Reverse translation of SULT1A1

In a previous article, we had shown that in human HAPE patients, sulfotransferase 1A1 levels are higher than controls [13]. We intended to reverse-translate our findings for this model of HAPE. We performed an ELISA where it was observed that as compared to controls, the H + S group rats undergoing 18 h of hypobaric hypoxia exposure had the highest levels of SULT1A1 (11.57-fold increase vs control) (Fig. 3a). Animals exposed to 18 h of hypobaric hypoxia (H group) also demonstrated increased levels of SULT1A1 (8.76-fold vs control) in accordance with previous results [13]. But this was still lower than H + S group. As observed in previous section, H + S group also demonstrated roentgenographic evidence of HAPE.

3.3. Inflammation as a feature of HAPE

Levels of Il-1b, TNF-a and IFN-g were evaluated using ELISA. We observed that levels of IL-1b increased significantly only in H + S group (1.77-fold vs control) (Fig. 3b). Levels of TNF-a did increase significantly in H + S (18 h) group (50-fold vs control)and in H (18 h) group (3.3-fold vs control) but the increase in H + S (18 h) group was extremely high (Fig. 3c). Interestingly, levels of IFN-g were observed to

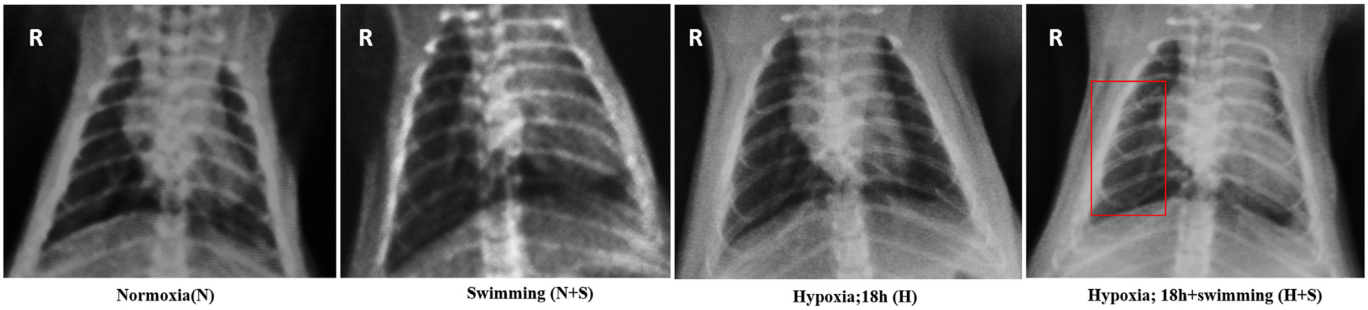

Fig. 2. Roentgenograms confirming HAPE. Representative chest roentgenograms of male SD rats for the experimental groups: Normoxia (N), Swimming (N + S), Hypoxia (H) 18 h and Hypoxia + Swimming (H + S)18 h. Animals in H and H + S groups were kept for 30 min in normoxic conditions before x-ray exposure. The right lung in H + S group shows signs of edema (region shown within rectangle). All animals were sedated and kept in supine anteroposterior position during x-ray exposure.

decrease significantly in H + S group (0.22-fold vs control) after increasing slightly in H group (1.44-fold vs control) (Fig. 3d). Lung tissue H&E staining (Fig. 4) also revealed a higher degree of inflammation along with damaged lung tissue in HAPE afflicted rats. However, lung sections of hypoxia control group (H) as well as N + S group did not show such high degree of inflammation. Thus, HAPE is associated with inflammation as per our data as there is no evidence of very high pro-inflammatory signaling in hypoxic controls' plasma and lung tissue, respectively. But mild increases in inflammatory markers like TNF-a (in plasma) and immune cells' infiltration (in lung tissue) were observed. An obvious inference from our data is that HAPE and inflammation progress in tandem and are synergistic to each other.

4. Discussion

Of the ailments that can be termed the curse of high-altitude exploration, HAPE remains the most commonly lethal malady. Its incidence and etiology have been noted in multiple regions. Its physiological and underlying molecular context has also been investigated in multiple studies [4,5,8–11,17,24,26]. Yet, we are barely scratching the surface when it comes to molecular markers of HAPE and HAPE specific therapeutics/prophylactics. Two major drawbacks in HAPE investigations have been lack of a huge cohort of patients to be tested as per a planned study and a reliable model of HAPE. More particularly, a model of HAPE that can be confirmed using more direct analytical tools like roentgenography instead of gravimetric analysis of wet:dry weight ratios. The models envisaged previously rely on fairly complex logistics like a treadmill set up in a hypoxia chamber for forced walk/run of rats [21]. This complex set up had to be kept working for fairly long time periods (48 h) with human monitoring involved. The larger animals considered for modeling HAPE like pigs, primates and cattle require notable human expertise and logistical costs apart from ethical issues. Thus, the focus of the present study was creation of a quick, reliable and easy-to-use model of HAPE using SD rats. We induced HAPE in otherwise healthy male SD rats within 20 h via exhaustive swim followed by 18 h of simulated hypobaric hypoxia exposure. The point of exhaustion was considered to be the time point at which a swimming rat was unable to keep its snout above water for 10 s. This is without the use of any toxins or pharmacological agents as reported in some studies [19]. Again, the model is reliable for at least 30 min after the completion of exposure, as evidenced for the first time by edema in the lungs of an animal HAPE model using roentgenography. Sedation with barbiturates and lignocaine was performed prior to roentgenography. Barbiturates are known to induce hypotension and cardiorespiratory depression in rats [27]. This helped in maintaining a status quo in terms of the edema buildup in lungs remaining static throughout the roentgenography and sacrifice as breathing rate and depth were blunted. These do slow down the breathing rates in animals. Auscultation also confirmed rales in the rat model. But we failed to replicate intrinsic human HAPE symptoms like pink frothy sputum in our model. This could be due to the possibility that lower airways were not irritated as humidity levels were constant at ~55% throughout the experiment in a setting devoid of airway irritants. Sputum is produced in the lower respiratory tract to keep the airways moist while also trapping unwanted foreign particles which are then expulsed from the airways. Excess pink frothy sputum may be there in actual cases of HAPE as high altitudes have very low ambient humidity and potential airway irritants like dust and pollen. Since dry air irritates the airways, there is increased sputum production which then becomes pink colored and frothy due to the vascular leakage depositing transudate into the respiratory system. However, in the rat model exposure to dry air and airway irritants was negated. This led to excess sputum not being produced in lower airways and thus pink frothy sputum was not observed. Resonance can be found in a study which reported that HAPE-susceptible individuals had poor nasal transepithelial transport [28].

Further, we focused on the immediate uses of this model. One such avenue was the reverse translation of previously published translated HAPE marker in human plasma [13]. Another was analyzing the rat model for association between HAPE and inflammation, as has been conclusively observed in human cases [1,3–5]. We investigated whether SULT1A1, a previously translated marker of HAPE and negative control in acclimatized individuals (under review Scientific Reports), showed similar increases in the rat model. As per our data, SULT1A1 is a faithful marker of HAPE, as its levels are immensely high only in cases of extreme hypobaric hypoxia and more particularly in cases of HAPE. Secondly, there was a marked association between HAPE and systemic inflammatory signaling. The levels of IL-1b and TNF-showed notable increases predominantly in H + S (18 h) group, roentgenographically proven to be suffering from HAPE. This is in line with our previous reports where similar simulated hypobaric hypoxia alone failed to elicit any significant increases in inflammatory cytokines levels [29]. Also, we failed to observe any inflammatory signals in the hypoxic controls plasma and tissues. Thus, it can be stated that HAPE and inflammation are not cause and effect but tandem processes exerting synergistic actions in the affected rats. This has been further corroborated by the histopathological observation of lung tissue (Fig. 3d). We observed that exudate consisting primarily of eosinophilic cells accumulated in H + S group only. Interestingly, the significantly lower levels of IFN-g in the backdrop of notably higher levels of both TNF-a and IL-1b points towards a unique possibility of either augmenting IFN-g levels or depleting TNF-a and IL-1b levels to better prevent or treat HAPE. IFN-g is known to activate IL-1Ra [30]. IL-1Ra suppresses the inflammatory effects of IL-1b and TNF-a by inhibiting their release from phagocytes [31,32]. The relative inhibition of both IL-1b and TNF-a in H group coupled with nearly significant increase in IFN-g despite similar hypobaric hypoxic exposure as H + S group is evidence of the same. This model can be used for specifically identifying translation worthy protein markers of HAPE, in line with the findings of our previous study on

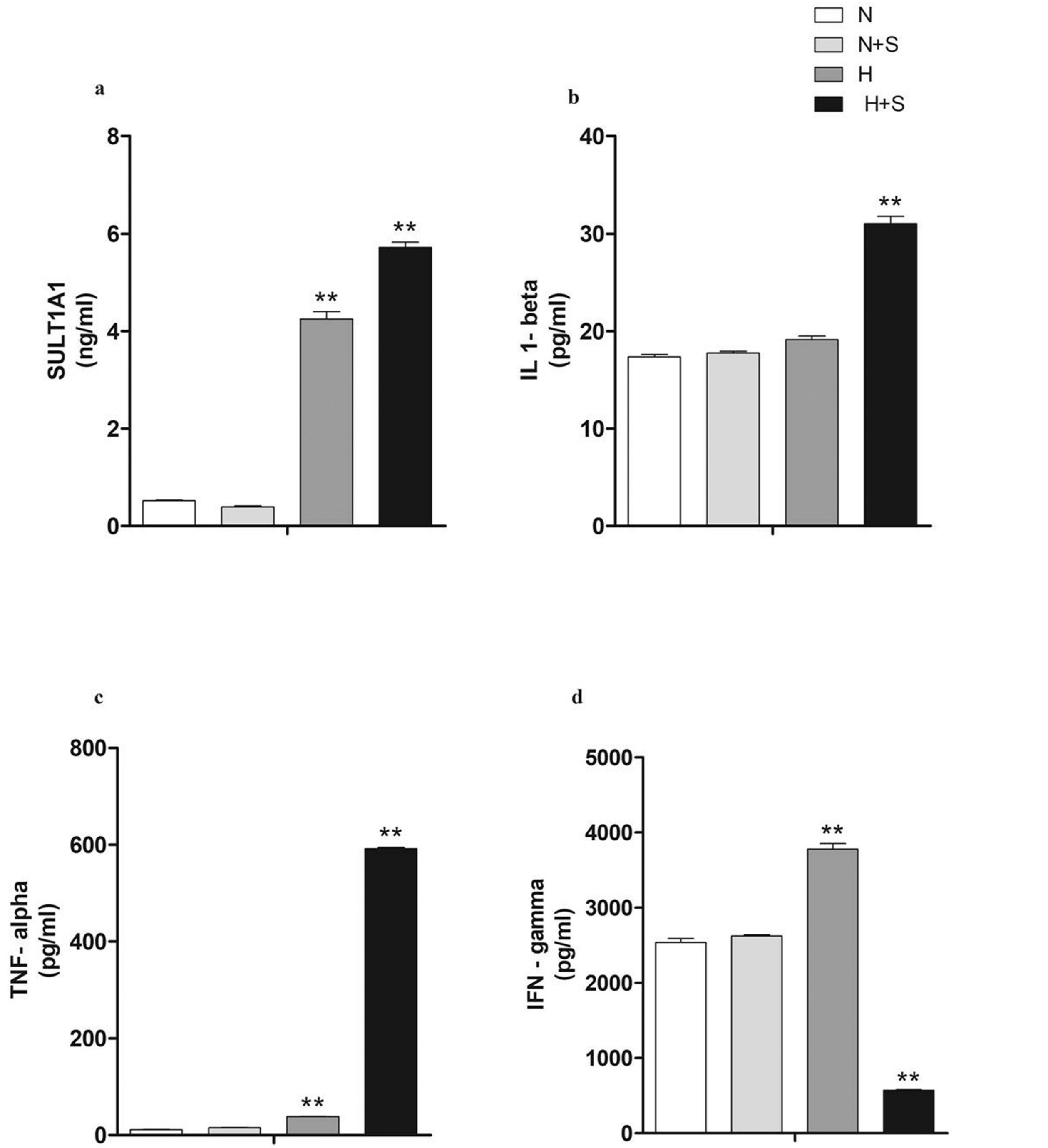

Fig. 3. Reverse translation of protein markers and inflammatory proteins in HAPE model. All protein concentrations presented as Mean ± SEM. Experimental groups: N is represented by the lightest colored bar closest to y-axis and N + S, H and H + S(18 h) are respectively represented by the progressively darker bars moving farther from y-axis. a. Sulfotransferase 1A1 (SULT1A1) levels were measured using ELISA. In line with previous result showing elevated levels in HAPE patients, we observed massively increased levels (11.57-fold vs N; 5.714 ± 0.1116 ng/ml vs 0.5173 ± 0.016450 ng/ml) in rat model of HAPE. Even in H group, levels were significantly higher (8.76-fold vs N; 4.246 ± 0.152 ng/ml). b. Interleukin 1-beta (IL 1-beta) was also assayed using ELISA. IL 1-beta was found to be close to Normoxic levels (17.36 ± 0.24 pg/ml) in all groups except in H + S group in which it was significantly up-regulated (1.77-fold vs N; 31.04 ± 0.7283 pg/ml). c. Tumor necrosis factor alpha (TNF-alpha) levels across groups is represented in a histogram. TNF-alpha showed 50-fold increase in H + S group (592.1 ± 2.556 pg/ml) as compared to normoxic group (11.53 ± 0.185 pg/ml). This was much greater than H group (3.3-fold vs N; 38.41 ± 0.518 pg/ml). d. Interferon-gamma (IFN-gamma) levels show slight increase in H group (1.44-fold vs N; 3777 ± 73.19 pg/ml vs 2536 ± 50.92 pg/ml). However, IFN-gamma levels are down-regulated in H + S group (0.22-fold vs N; 570.2 ± 9.957 pg/ml). Fold change values were normalized. Fold change value of 1.5 or more is considered up-regulation while fold change value of 0.66 or less is termed down-regulation. ***p*-Value < 0.0001.

an in-silico rat human hybrid [33]. This model may be further improved upon by physiologists to mimic human symptoms like pink frothy sputum etc. In the near future, HAPE specific therapeutics may also be used on this model which may prevent or alleviate HAPE without need for immediate descent and hospitalization.

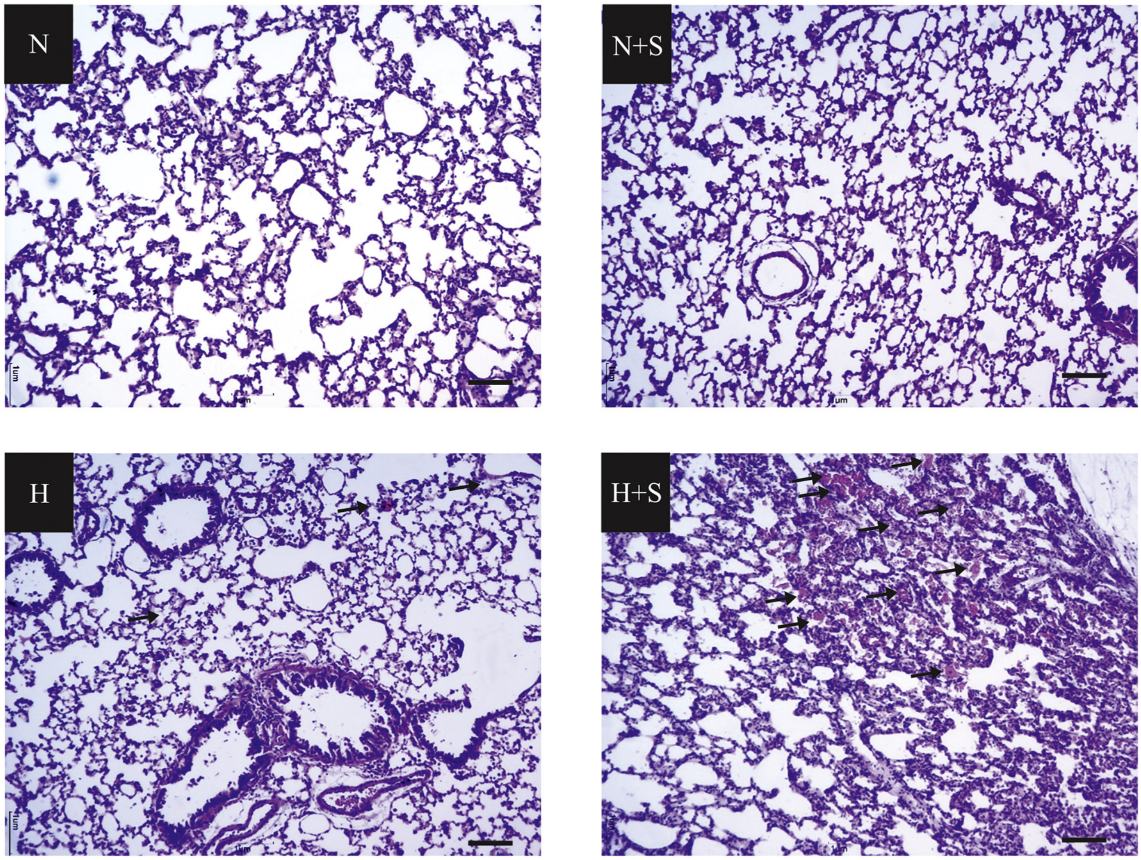

Fig. 4. Representative H&E stained lung tissues showing extensive damage in HAPE model lung tissues. H&E staining was performed to evaluate damage to lung tissue. Lung tissues in N and N + S groups show no signs of abnormal concentration of purple or red suggesting normal lung tissue. Lung tissues in H group show more regions with concentrated purple suggesting immune activation and inflammation in lung (some regions marked with arrows). Lung tissues in H + S group in addition to elevated purple color levels also show red patches suggesting inflammation and extravasation of fluid into lung tissue (shown with arrows). These are cardinal pathophysiological changes associated with HAPE afflicted lung tissues. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

5. Conclusion

In the present study, we have been able to provide a roentgenographically ascertained, quick, reliable, easy to use and cost-effective SD rat model of HAPE. It does not require any special apparatus or instrument to induce HAPE. There is no requirement of any pharmacological agents to induce HAPE. This method of induction also doesn't cause widespread mortality in the animal. We believe and state that this model will provide a viable, faithful replica of HAPE that can be used to find both molecular markers and therapeutics specifically for HAPE.

Declaration of Competing Interest

Authors declare no conflict of interest.

Acknowledgement

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Author contributions

YA conceptualized the study. SP and AG performed the experiments, analyzed the data and prepared the manuscript which was further edited by YA and KB. HP performed the blinded analysis of chest x-rays. All the authors read and approved of the manuscript.

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