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Prophylactic Administration of Curcumin Abates the Incidence of Hypobaric Hypoxia Induced Pulmonary Edema in Rats: A Molecular Approach

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Abstract

Background and purpose: High Altitude Pulmonary Edema (HAPE) is a severe high altitude illness with serious pulmonary manifestations. The present study reports benefits of prophylactic administration of curcumin in prevention of hypoxia induced pulmonary edema.

Experimental approach: Male Sprague Dawley rats (n=6 per group) were exposed to a stimulated hypobaric hypoxia at 7620 m for 6 h. The groups studied were (I) Normoxia, (II) Hypoxia (6 h), (III) Normoxia+curcumin (50 mg/kg BW) and (IV) Hypoxia+curcumin (50 mg/kg BW). Curcumin at 50 mg/kg BW, given orally 1 h prior to hypoxia exposure was considered from dose dependent studies as the optimum dose, due to significant reduction in the level of lung water content and lung transvascular leakage ($p<0.001$) as compared to control (6 h hypoxia). Biochemical analysis, vascular leakage studies, differential expression of proteins were determined by ELISA, Western Blotting and Immuno-histochemistry. Changes in lung parenchyma were evaluated by histopathology.

Results: Curcumin administration (50 mg/kg BW) to rats, 1 h prior to hypoxic exposure showed a significant decrease in lactate dehydrogenase (LDH), albumin extravasation in broncho-alveolar lavage (BAL) fluid, oxidative stress (ROS and MDA) levels along with concomitant increase in antioxidant status (GSH, GPx and SOD) in lungs of rats compared to control. Curcumin significantly attenuated the IKKαβ , IKBβ there by leading to down regulation of NFκB protein levels and their downstream regulatory genes (pro-inflammatory cytokines and cell adhesion molecules). Further, hypoxia enhanced HIF-1α and VEGF levels in lungs were significantly down regulated by curcumin leading to reduction in vascular leakage in lungs of rats under hypoxia over control (Hypoxia). The histopathological observations provide substantial evidence in reduction of edema and inflammation by curcumin treatment.

Conclusion: These results indicate that, curcumin to be a potent drug against HAPE as it effectively attenuates inflammation as well as fluid influx in the lungs of rats under hypoxia.

Keywords: Hypoxia; Curcumin; Inflammation; HAPE; NFkB; HIF-1α; Oxidative stress; Acclimatization

Abbreviations: ANOVA: Analysis of Variance; ARDS: Adult Respiratory Distress Syndrome; BAL: Bronchoalveolar Lavage; BW: Body Weight; Cur: Curcumin; DMSO: Dimethyl sulphoxide; DNA: Deoxyribonucleic acid; EDTA: Ethylene diamine tetra acetic acid; ELISA: Enzyme Linked Immuno Sorbsnt Assay; GSH: Glutathione Reduced GPx: Glutathione Peroxidases; HAPE: High Altitude Pulmonary Edema; Hif1-α: Hypoxia Inducible Factor 1 alpha; Hg: Mercury; HRP: Horse Radish Peroxidases; ICAM: Intercellular Adhesion *Molecule; IKB:* Ikappa B; IKK: Ikappa kinase; IL: Interleukin; LDH: Lactate dehydrogenase; LPS: Lipopolysaccharide; MDA: Malondialdehyde; MW: Molecular Weight; NASID: Non steroidal anti-inflammatory drugs*; NF-kB:* Nuclear Factor-Kappa B; OFR: Oxygen Free Radicals*; PBS:* Phosphate *Buffered* Saline; r.f.u: Relative Fluorescence Unit; ROS: Reactive Oxygen Species; RT: Room Temperature; SOD: Superoxide Dismutase; *TNF*-α: Tumor necrosis factor; UFAW: Universities of Federation for Animal Welfare; *VCAM-I: Vascular Cell Adhesion Molecule; VEGF: Vascular Endothelial Growth Factor*

Introduction

High Altitude Pulmonary Edema (HAPE) is defined as acute respiratory failure following exposure to high altitude hypoxia, developing in a normal subject with no pre-existing pulmonary or cardiac disease. HAPE is the result of fluid accumulation in the lungs. It occurs generally an altitude above 2450 m; but some HAPE cases have been reported at altitudes as low as 1400 m in apparently normal individuals [1,2]. Symptoms occur within 2-3 days. Severe instances can lead to death if not treated quickly. Almost one mountain trekker or climber out of two develops several symptoms of HAPE above 2500 m [3]. The hall mark of HAPE however is an excessively elevated pulmonary artery pressure, which precedes the development of pulmonary edema [4]. Although the exact pathophysiology of HAPE is unclear, but most of the studies concur that increased sympathetic tone, exaggerated hypoxic pulmonary vasoconstriction, uneven hypoxic pulmonary vasoconstriction with over perfusion of some regions of the pulmonary vascular bed, increased pulmonary capillary pressure and alveolar fluid leak across capillary endothelium resulting in interstitial and alveolar edema [3,5]. The treatment includes-immediate descent to lower altitude, administration of O2, portable hyperbaric chamber and/or prophylactic administration of different pharmacological drugs. The portable hyperbaric chamber works by increasing the pressure inside the chamber using a foot pump. At high altitudes this increased pressure delivers therapeutically significant amount of extra oxygen to the victim, which stimulates a descent. Therefore the person's body chemistry will soon get into the lower altitudes. With regard to prophylactic methods, inhibition of altitude induced pulmonary hypertension has

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been the primary criteria in pharmacological prevention of HAPE. It seems very clear that the different drugs that are recommended against HAPE (nifedipine, dexamethasone, salmeterol, tadalafil and sildenafil, etc.) not only reduce pulmonary artery pressure [3,6-10] but also decreases inflammation [6,11-14] and therefore, leads to reduction in incidence of high altitude pulmonary edema. Acetazolamide (Diamox) is an already well accepted drug for prevention and treatment of Acute Mountain Sickness (AMS) and High Altitude Cerebral Edema (HACE) works by the mechanism of inhibiting the renal carbonic anhydrase, therefore creates a mild metabolic acidosis that stimulates ventilation; but showed its efficacy in preventing HAPE-like alveolar protein leak in rats exposed to hypobaric hypoxia most likely via mitigation of Hypoxic Pulmonary Vasoconstriction (HPV) [15]. Even though these preventive medicines for HAPE are currently being used but they have limitations in their applications, due to their adverse side effects. For example nifedipine can cause reflex tachycardia, peripheral edema and hypotension; salmeterol causes tachycardia and it is not recommended for heart problem patients; tadalafil is associated with headache and dyspepsia problems, acetazolamide treatment leads to significant red cell carbonic anhydrase inhibition which can impair carbon dioxide excretion, worsens dyspnoea and/or respiratory failure and also contraindicated to the patients suffering from sulpha energy problems [16,17], whereas dexamethasone is associated with mood disturbances, insomnia, and hyperglycemia and may show rebound effects once discontinued [5] . Over the years, there has been a flurry of interest in other drugs, remedies/adjunct for HAPE prevention such as diuretics, intravenous hydralazine, phentolamine, Ginko biloba or Rhodiola crenulata extracts etc.; but their mechanism of actions are unclear and their benefits have been unconvincing [18,19]. Therefore at present these drugs are not being considered under high altitude drug regime. Thus, identification of a potent prophylactic agent which can prevent or reduce hypobaric hypoxia-induced oxidative stress, as well as inflammation, might also reduce high altitude pulmonary edema. The best illustrated of such molecule is curcumin, a derivative of turmeric used for centuries to treat a wide variety of inflammatory conditions.

Curcumin (1,7-bis (4 hydroxy-3 methoxy phenyl)-1,6-heptadiene-3,5-dione) is a yellow pigment found in the rhizomes of the perennial herb *Curcuma longa* Linn. It is well known that turmeric (*Curcuma longa* L.) has a long history of use in India as an Ayurvedic medicine for the treatment of inflammatory conditions. Curcumin has been claimed to represent a potential antioxidant and anti-inflammatory agent with phytonutrient and bioprotective properties [20]. Several studies have revealed that curcumin can modulate several transcription molecules, cytokines, growth factors and kinases [21,22]. Nowadays, the most widely used anti-inflammatory drugs are steroids, but there are numerous side effects associated with them. In addition to steroids, numerous non-steroidal anti-inflammatory drugs (NSAIDs) (Salicylate, Ibuprofen, Diclofenac and Coxibs etc.) are also used but they are again associated with a constellation of side effects [23]. Extensive research over the last 50 years [20-22] has indicated that, curcumin is useful for the prevention and treatment of cancer. It is reported that curcumin is known to inhibit the activation of NFκB [20,24]. However, there is lack of information regarding the molecular mechanisms to decipher the role of oxidative stress and NF-kB in HAPE; therefore, in our previous studies, [25] we have reported that oxidative stress driven increase in lung NFkB content can contribute for the formation of pulmonary edema in rats under hypoxia. We have also reported that prophylactic administration of curcumin to rats and exposed to hypobaric hypoxia significantly reduced the transvascular leakage therefore leads to reduction in the incidence of HAPE. Therefore, in the present study, we reasoned (i) if curcumin is able to reduce the incidence of transvascular leakage by down regulating the NFkB activity, then at what level? Is it at downstream or at upstream of NFkB activation in cytoplasm of the cell? (ii) In addition to this, we were interested in finding out the association of NFkB with another most important transcriptional factor i.e. hypoxia inducible factor (HIF-1α) in contributing fluid influx in to the lungs. (iii) Hence our study was extended to evaluate the effect of curcumin on HIF-1α and the genes regulated by it (VEGF). Further, we explored to see how this natural drug will show its effect on lung injury and albumin extravasation in BAL of rats exposed to hypoxia? The histopathological observations provide substantial evidence in reduction of edema and inflammation by curcumin treatment.

Materials and Methods

Animals

The experiments were conducted using adult male Sprague Dawley rats as an animal model weighing 150-200 gm Body Weight (BW). Rats were maintained at 25 ± 1°C, humidity 55% ± 2% with day and night cycles of 12 h each and given food and water *ad libitum*. All rats were maintained in the institute animal house, kept in groups of 3 in polypropylene cages (32 in. × 24 in. × 16 in.). Paddy husk was used as bedding material and was changed every day. All animal procedures and experimental protocols were approved by Institutional Animal Ethics Committee (Authorization number: 27/1999/CPCSEA) and followed the standards set forth in the Guide for the Care and Use of Laboratory Animals (National Academy of Science, Washington, D.C.). All efforts were made to minimize the animal suffering and to reduce the number of the animals used.

Administration of curcumin

The drug curcumin was purchased from Sigma Aldrich (St. Louis, MO, USA). The drug was freshly dissolved in Dimethyl sulphoxide (DMSO) (0.5%) and administered orally to rats, 1 h prior to hypoxia exposure.

Safety profile of curcumin: No long term adverse effects from the use of curcumin are noted in the medical literature [26]. Phase I clinical trials have shown that curcumin is safe even at high doses (12 g/day) in humans [27]. The average intake of turmeric in the diet in India is approximately 2-2.5 g in a 60 kg individual which corresponds to an intake of approximately 60-100 mg of curcumin daily [28]. In various animal studies, a dose range of curcumin 100-200 mg/kg BW exhibited good anti-inflammatory activity and also seemed to have negligible adverse effects in rats and human systems as well [29]. In sub-acute toxicity experiments, no significant toxic side effects were observed in rats when the extract was administered for 4 weeks at a dose level of 1-2 g/kg and [29] and even for 90 days at a dose level of 1.8 g/kg per day [30]. A recent report had also demonstrated an anti-inflammatory activity of curcumin in acute and chronic models of inflammation in rats and mice [29]. Curcumin is lipophelic in nature and it tightly binds to serum albumin [31] and several other molecules in the body. Regarding curcumin bioavailability, Ravindranath and Chendrashekara [32,33] have reported that, about 90% of curcumin was found in stomach and small intestine in 30 min up on oral administration of 400 mg of curcumin, which later reduced to 1% at 24 h. These authors further reported that 60-66% of curcumin absorbed was remained constant regardless of the dose, indicating that administration of higher concentration of curcumin to rats does not result in higher absorption. Research reveal that the poor bioavailability of curcumin can be overcome by combination of curcumin with piperine (a component from black pepper), liposomal or phospholid complexes or development of nanocurcumin or curcumin analogues

etc. [34] which bring significant delay in its elimination. In the present study, we have administered the different concentrations of curcumin to rats from 25-200 mg/kg BW and exposed them to 6 h of hypobaric hypoxia. Since these rats were exposed to hypobaric hypoxia only for 6 h after administration of different concentrations of curcumin, we did not find any adverse effects in any one of the experimental rats among the different doses tested under hypoxia.

Experimental set up

The experiment was carried out in two phases:

Phase I a: To find out the effect of different hours of hypoxia on Lactate Dehydrogenase (LDH) and albumin leakage in to the Bronchoalveolar Fluid (BALF), a total of 36 rats were exposed to different hours of hypoxia. The rats were divided into 6 groups and each group containing 6 rats. Group 1 served as control or normoxia (0 h); Group 2 was exposed to hypoxia for a 1 h duration; Group 3 was exposed to hypoxia for 3 h duration; Group 4 was exposed to hypoxia for 6 h duration; and Group 5 was exposed to hypoxia for a 12 h duration and group 6 was exposed to 24 h duration.

Phase 1 b: Dose dependent studies of the effect of curcumin on lung transvascular leakage in rats were carried out in 36 rats. The rats were divided into 6 groups and each group contains 6 rats. Group 1 served as control (normoxia), received only vehicle; group 2 served as hypoxia and exposed to hypoxia for 6 h, received only vehicle (hypoxia exposed rats without curcumin supplementation); group 3 animals were supplemented with curcumin at 25 mg/kg Body Weight (BW) 1 h prior to hypoxia exposure; group 4 animals were supplemented with curcumin at 50 mg/kg BW 1 h prior to hypoxia exposure; group 5 animals were supplemented with curcumin at 100 mg/kg BW 1 h prior to hypoxia exposure; and group 6 animals were supplemented with curcumin at 200 mg/kg BW 1 h prior to hypoxia exposure.

Phase II: The dose dependent studies of curcumin (from phase I b studies) revealed that, curcumin dose at 50 mg/kg BW showed significantly reduced edema index and transvascular leakage, therefore considered as optimum dose compared to other doses tested (Figures 1a and b). Therefore the rest of the experiments were carried out using 50 mg cur/kg BW, in 24 rats. Under the Phase II experiment, the animals were divided into 4 groups, each group containing 6 rats. Group 1 served as control (normoxia), received only vehicle; group 2 animals received only vehicle and exposed to hypoxia for 6 h duration; group 3 animals were supplemented with curcumin at 50 mg/kg BW without any hypoxic stress; and group 4 animals were supplemented with curcumin at 50 mg/kg BW 1 h prior to hypoxia exposure.

Exposure to high altitude hypoxia

The rats were exposed to a simulated altitude of 7620 m (280 mmHg) in a hypobaric chamber (Decibel Instruments, India) for 6 h. The reason for exposing the rats to hypobaric hypoxia for 6 h is based on our previous studies [25]. The temperature of the hypobaric chamber was maintained at 25 ± 1°C with an air flow rate of 4 l/h, humidity 55% and barometric pressure of 280 mmHg. The partial pressure of arterial oxygen in control rats was found to be 95 ± 2 mm Hg, and in hypobaric rats it was found to be 38 ± 2 mm Hg, indicating that the rats were exposed to reduced levels of partial pressure of oxygen in the hypobaric chamber. We have exposed the rats to above mentioned hypobaric hypoxia because the smaller animals have higher capillary density in tissues, making them more resistant to hypoxia than man [35]. The animals were provided with adequate quantities of food and water during exposure to hypoxia. It should also be noted that, we could not measure the pulmonary pressure and urine output in rats during the study, owing to the limitations of simulated hypobaric hypoxia chamber.

Determination of pulmonary edema

Determination of vascular permeability: The vascular permeability of lungs was determined following the method of Baba et al. [36], with some modifications. In brief, half an hour before the completion of hypoxic exposure, the rats were taken out of the hypobaric chamber, and 200 µl of sodium fluorescein dye (5 mg/kg BW in PBS; Sigma Chemical Co., St. Louis, MO) was injected through the tail vein. Later the rats were placed back in the hypoxia chamber and exposed again to hypoxia for additional 30 m. Later the animals were taken out of the hypoxia chamber, anesthetized, and perfused with Phosphate Buffered Saline (PBS) through the left heart ventricle to remove the fluorescent tracer from the vascular bed. The lungs were removed, washed with

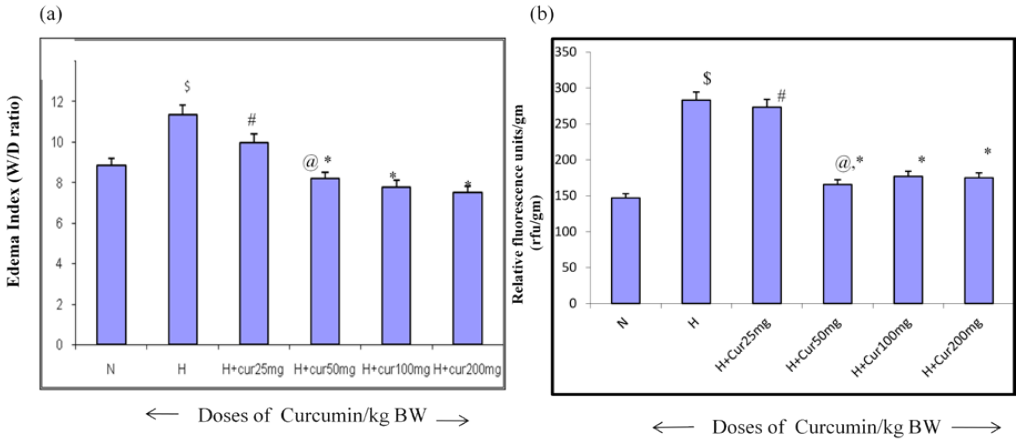

Figure 1: Optimization of curcumin dose in hypoxia (7260 m for 6 h) exposed rats. (a) Edema index (b) Transvascular leakage. Curcumin was administered as prophylactic drug against HAPE. Rats were pretreated with curcumin with different doses viz. 25, 50, 100 and 200 mg/kg BW orally, 1 h prior to hypoxic exposure at 7620 m for 6 h. The optimum dose of curcumin was found to be 50 mg/kg BW, where the minimum lung vascular leakage was obtained and this dose was found to be significantly different from other doses tested. This indicates that the observed reduction in fluid efflux might be caused by the anti-inflammatory activity of curcumin. Values are mean ± SD (n=6). Significant difference between groups was determined by ANOVA followed by Student-Newman-Keul's test. $P<0.001 compared with Normoxia group (0 h). *P<0.001 compared with Hypoxia group (6 h). #P<0.05 compared with hypoxia group (6 h), @P<0.01 compared with hypoxia (6 h)+25 mg Cur. N=Normoxia, H=Hypoxia, Cur=curcumin.

cold saline, and divided into two equal parts. One part of the lung was kept in 3% formamide for about 18 h at Room Temperature (RT). Later, the tissues were centrifuged for 10 m at 3,000 rpm, and the fluorescence in the supernatant was measured using a spectrofluorimeter (Varian) with 485 nm excitation and emissions at 530 nm. The other part of the lung was weighed and kept in an oven at 80°C for 72 h. Later, the dry tissues were collected and weighed again to determine the dry weight. The results were presented as relative fluorescence units per gram (rfu/g) dry weight.

Determination of lung water content: To quantify the lung water content in lungs from normoxic and hypoxic animals, the wet weight of the lungs was determined immediately after removal. The samples were rinsed with the cold PBS and dried at 80˚C for 72 h, and the edema index was expressed as wet-to-dry weight ratio (W/D ratio) [37].

Biochemical parameters

Broncho-alveolar Lavage (BAL) was performed on normoxia and hypoxia exposed rats. Rats were anaesthetized and a trimmed sterile 18-guaze micro-cannula (Major surgical India ltd, India) was inserted into the lumen of the exposed trachea. The lungs were lavaged *in situ* with two separate 1 ml washes of sterile normal saline. The BAL fluid was centrifuged at 3000 × g at 4°C for 10 min. The supernatant was stored at -80°C until further use. Total protein content in the BAL and in lungs of rats was estimated by Lowry's method [38]. Lactate dehydrogenase activity in cell free BAL was measured spectrophotometrically using kit (RANDOX, UK) as an indicator of cell damage. Total albumin content in cell free BAL was measured using a commercially available Kit (ICL, Portland, USA) as per manufacturer's instructions.

After hypoxic exposure, animals were sacrificed and lung was perfused with cold PBS. The lung tissue was collected washed with cold saline (0.9%NaCl) and stored at -80°C for further biochemical analysis. Reactive Oxygen Species (ROS) in lung homogenate was estimated as described earlier [39]. Malondialdehyde (MDA) in lungs was estimated by the method of Okhawa et al. [40]. Reduced glutathione (GSH) in lungs was determined as described elsewhere [41]. Glutathione peroxidase (GPx) and Superoxide dismutase (SOD) in lungs was measured kinetically by using commercially available kits (RANDOX, Crumlin, UK) as per manufacturer's instructions.

Analysis of inflammatory cytokines by enzyme-linked immunosorbent assay (ELISA) (TNF-α and IL-6) from BALF: ELISA was used to measure IL-6 and TNF-α (BD Bioscience, PA, USA) protein levels from BAL fluid obtained from both normoxia and hypoxia exposed animals. The assay was performed according to the protocols given by the manufacturer. Sample observations were read with an ELISA plate reader (BMG lab tech, Germany) adjusted to 450 nm and the concentrations were determined based on rat IL-6 and TNF-α standards provided by the manufacturer.

Protein expression studies

Sample preparation: To confirm the inhibiting action of curcumin on the expression of the genes up regulated by NFκB, we determined the expression of NFκB in the lung homogenates of rats exposed to hypoxia by western blotting. The lung tissue of rats were homogenized to extract pure nuclear and cytoplasmic fractions by using the Nuclear/Cytosol fractionation Kit (Biovision, Mountain View, CA) according to manufacturer's instructions.

The protein (50 µg) in the samples were separated on 10% SDS PAGE (NFκB p65, IKKαβ, IKBβ, IL-1, IL-18, TNF-α, VEGF and β-actin) and 8% (ICAM-1, VCAM-1, P-selectin, E-selectin, and HIF1-α) (Bio-Rad, Hercules, CA). The proteins were then electro blotted onto nitrocellulose membranes (Millipore, USA). The membranes were then incubated with respective primary antibodies (Santa Cruz Biotechnology) and later incubated with its respective secondary antibodies conjugated with HRP at Room Temperature (RT). Membranes were developed using kit (Chemiluminescence substrate; Sigma) and bands were visualized on X-ray film (Kodak). Densitometric analysis was carried out by using Gel Doc System (UVP, Bio Imaging system, U.K.).

Transcription factor (NFκB) activation studies using electrophoretic mobility shift assay (EMSA): The EMSA for NFκB was carried out using a commercials kit (Pierce, USA) as per manufacturer's instructions. The NFκB oligonucleotide probe was supplied by Operon, the sequence being NFκB, F 5'-AGT TGA GGG GAC TTT CCC AGG C-3', NFκB R 5'-GCC TGG GAA AGT CCC CTC AAC T-3', NFκB mutant F 5'AGT TGA GGC GAC TTT CCC AGG C 3', NFκB mutant R 5'GCC TGG GAA AGT CGC CTC AAC T3' (the underlining indicates DNA binding site for NFκB).

Immunohistochemistry

Immunohistochemistry was performed in normoxia or hypoxia exposed rat lung tissue as described by Beytut et al. [42] with some modification. Briefly, thin (20 µm) cryosections (Leica CM 1950, microtome, Leica Wtzlar, Germany) of alveolar tissue were prepared from normoxia/hypoxic rat lungs perfused with saline to clear of the blood before removal from the animal. Thin sections were taken and fixed with paraformaldehyde. Endogenous peroxidase activity was blocked with hydrogen peroxide (3%) in distilled water for 30 min. The sections were incubated with PBS, (pH 7.2) for 5 min and subsequently placed into 0.05% Trypsin EDTA for 20 min for antigen retrieval. After washing with PBS for 5 min, the sections were incubated with 5% normal goat serum for 60 min at RT. The sections were then incubated with each of the primary antibodies (NFkB, TNF-α and VEGF from Santa Cruz Biotechnology; HIF 1α from Abcam, UK) for overnight at 4°C. Following 4-5 times washing with PBST, the sections were incubated with HRP conjugated goat anti-rabbit IgG in PBST for 60 min at RT. Secondary antibodies were supplied by Sigma MO, USA. Labelling was "visualized" with 3,3'-diaminobenzidine (DAB) or 3-amino-9-ethylcarbazole as the chromogen. The images were captured by using Olympus BX51TF (Centre valley, PA, USA).

Histopathological analysis: Each of the lung tissues of all the four groups of phase II experimental animals were fixed in 10% formalin solution in PBS at room temperature overnight. Tissues were embedded in paraffin wax. The paraffin-embedded tissue sections (5 µm) were stained with haematoxylin and eosin (H&E) using standard techniques. The slide images were captured with a digital camera (Nikon, Tokyo, Japan) which is attached to a light microscope (Olympus BX50).

Statistical analysis

Statistical analysis was performed using SPSS for windows (15.0) software (SPSS Inc., Chicago, IL). Comparison between experimental groups and curcumin treated groups were made by using one way ANOVA with student Newman-Keul's test for comparison between groups. Differences were considered statistically significant for $P<0.05$. Results are expressed as mean ± SD.

Results

Determination of pulmonary edema

Lung water content and vascular permeability: The lung water content was determined by calculating the wet to dry weight ratio of lung tissue of rats supplemented with different doses of curcumin i.e. from 25 to 200 mg/kg BW (Figure 1a). Hypoxic exposure for 6 h (11.36

± 1.5 W/D ratio) resulted into significant increase (P<0.001) in edema index compared to normoxia (8.6 ± 0.24 W/D ratio). The lung water content was reduced significantly and dose dependently compared to hypoxia exposed rats. Although the curcumin dose at 25 mg/kg BW showed a significant reduction in edema index compared to hypoxia, but the maximum significant reduction in edema index (P<0.001) started appearing at 50 mg/kg BW (8.2 ± 0.62 W/D ratio) and it was found to be more or less same upon increasing higher doses (i.e. 100 and 200 mg/kg BW) compared to hypoxia. Moreover, this dose was found to be more or less similar to that of normoxia control as well. Hence it was considered as the best optimum dose. Thereafter, further experiments were carried out using 50 mg/kg BW as the optimum dose. Similar findings were obtained in vascular permeability studies also (Figure 1b). Rats supplemented with 50 mg/kg BW during hypoxia showed a significant reduction (P<0.001) in vascular permeability (169.23 ± 12 rfu/gm tissue) as compared to hypoxia exposed rats for 6 h (286.73 ± 34.67 rfu/gm tissue). However, the same dose under normoxia did not alter the lung water content and vascular permeability (data not shown) compared to normoxia.

Changes in biochemical parameters

Reactive Oxygen Species (ROS) generation and lipid peroxidation (MDA): There was a significant increase in ROS levels (37% ↑) in lung tissue of animals exposed to hypoxia. Supplementation of curcumin under normoxia did not alter the ROS levels. However, the same dose under hypoxia showed a significant reduction in ROS (41% ↓) levels in lung homogenate of rats compared to hypoxia (6 h) (Table 1).

Parameter	N	H	N+Cur	H+Cur
ROS nmol/min/mg/tissue	72.5 ± 12	198 ± 6[a]	76.2 ± 13	81.6 ± 12[b]
MDA nmol/gm/tissue	10.52 ± 2	50.63 ± 2[a]	10.23 ± 2	9.28 ± 1.3[b]
GSH µmol/gm tissue	4.2 ± 0.6	2.8 ± 0.4[a]	3.5 ± 0.84	3.94 ± 0.34[b]
GPx U/mg protein	0.132 ± 0.03	0.086 ± 0.1[a]	0.138 ± 0.06	0.140 ± 0.05[b]
SOD U/mg protein	0.51 ± 0.09	0.35 ± 0.07[a]	0.57 ± 0.1	0.54 ± 0.1[b]

Values are mean ± SD (n=6).
Significant difference between groups was determined by one way ANOVA followed by student-Newman-Keuls test.
[a]P<0.001 compared with Normoxia (Oh) group.
[b]P<0.001 compared with hypoxia (6h) group.
N=Normoxia, H=Hypoxia, Cur=Curcumin.

Table 1: Effect of curcumin on lung Reactive Oxygen Species (ROS) generation, lipid peroxidation (MDA) and antioxidant status (GSH, GPx, and SOD) in lungs of rats exposed to hypoxia at 7620 m for 6 h.

There was an increase in the malondialdehyde (MDA) levels (21% ↑), in the lungs of rats exposed to hypoxia. Animals receiving curcumin under normoxia did not alter the MDA levels in lung tissue. Whereas curcumin significantly reduced the MDA levels (18% ↓) in lung tissue compared to hypoxia (6 h) (Table 1).

Antioxidant levels: The reduced glutathione (GSH--γ-L-glutamyl-L-cysteinyl-glycine) levels in the lung tissues of animals exposed to hypoxia were reduced significantly (P<0.001) (67% ↓) compared to control. However, curcumin supplementation during hypoxia increased GSH levels (72% ↑) more or less similar to that of normoxia values (Table 1). Upon exposure to hypoxia a significant fall in tissue GPx levels (65%↓) were observed as compared to normoxia. Curcumin supplementation resulted in higher GPx levels (P<0.05) during normoxia and it completely arrested the fall in GPx levels (61%↑) during hypoxia (Table 1). Similarly, SOD levels in lungs of rats exposed to hypoxia was reduced significantly (69% ↓) as compared to normoxia. Prior administration of curcumin to rats significantly enhanced the lung SOD levels during normoxia and hypoxia (65% ↑) (Table 1).

Albumin and LDH content in Bronchoalveolar Lavage (BAL) fluid: To confirm further, increased transvascular leakage leads to increased BAL protein which is an indicator of changes in alveolar membrane permeability, bronchoalveolar lavage was performed for the determination of total protein (albumin) content. The increase in total protein concentration is suggestive of transudation of plasma proteins (i.e. alternations in alveoli and the capillary barrier).

There was a gradual and significant increase (P<0.001) in albumin content was observed in BALF of rats right from the 3 h (40.08 ± 1.59 ηg/ml) of hypoxic exposure to 24 h of hypoxia (83.07 ± 3.71 ηg/ml) compared to control (22.06 ± 0.84 ηg/ml) (Figure 2a). Whereas, prior treatment of rats with curcumin significantly reduced the albumin extravasation in BALF (25.42 ± 3.82 ηg/ml) compared to hypoxia (6 h) (58.16 ± 8.63 ηg/ml) (Figure 3a). Since noncardiogenic pulmonary edema arises from damage to the alveolar and micro vascular compartments, we measured the LDH levels in BAL fluid of rats exposed to different hours of hypoxia. A significant increase in LDH activity (P<0.001) was observed in rats under hypoxia from 1 h (16.2 ± 1.6 U/I) to 24 h exposure (124. 07 ± 18.69 U/I) over normoxia (13.01 ± 1.1 U/I) (Figure 2b). However, prophylactic administration of curcumin significantly reduced the LDH activity (25 ± 2.4 U/I) in BAL fluid of rats compared to hypoxia (6 h) (53.46 ± 9.31 U/I) (Figure 3b). No significant difference was found in albumin and LDH activity in BALF of curcumin administered rats under normoxic conditions compared to control. Although we could not able to measure the

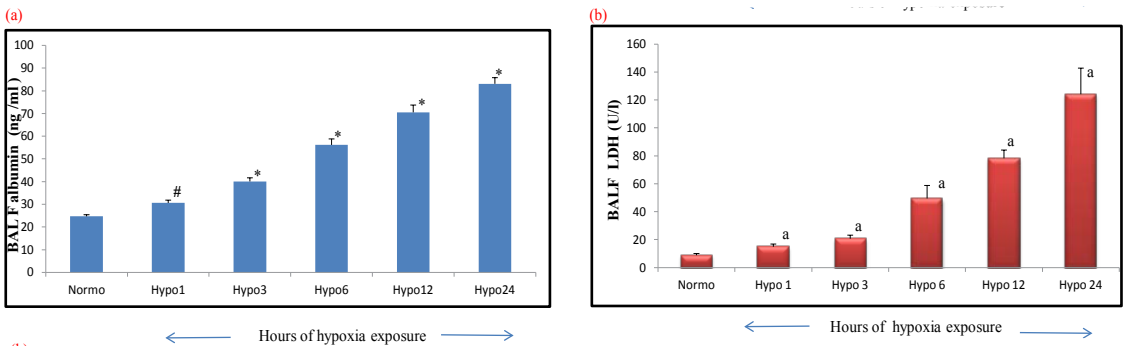

Figure 2: Effect of hypoxia in (a) Albumin and (b) LDH content in BALF of rats, exposed to hypobaric hypoxia at 7620 m for different durations (0 h, 1 h, 3 h, 6 h, 12 h and 24 h). It is noticed that, hypoxia resulted into significant increase in total protein content in BALF as well as causing lung injury (as indicated by ↑ LDH levels in BALF) facilitating increased vascular leakage. Values are mean ± SD (n=6). *P<0.001 compared with Normoxia (0 h) group. #P<0.05 compared with normoxia. Nor=Normoxia, Hypo=Hypoxia.

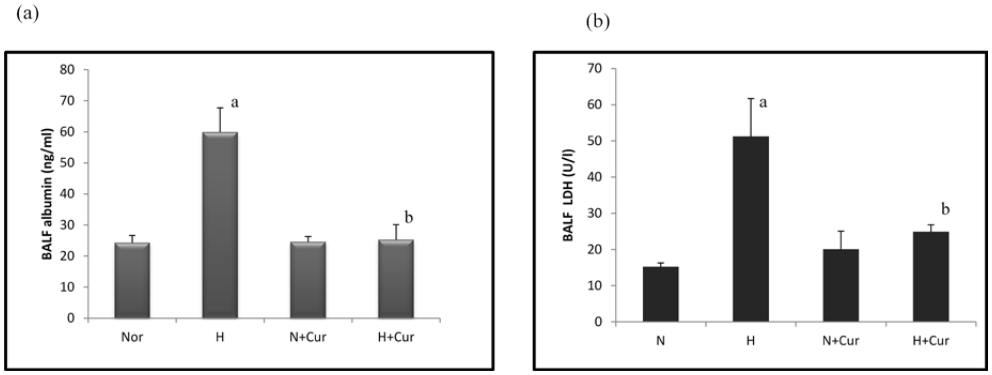

Figure 3: Effect of curcumin in (a) Albumin content and (b) LDH content in BALF of rats, exposed to hypobaric hypoxia at 7620 m for 6 h. It is observed that, Hypoxia has resulted into significant increase in total protein content in BALF, indicating the increased vascular leakage. However, prior treatment of rats with Curcumin and exposed to hypoxia resulted into significant decrease in fluid efflux in lungs. It is speculated that curcumin has ability to maintain the vascular membrane integrity tightly. Values are mean ± SD (n=6). aP<0.001 compared with Normoxia (0 h) group. bP<0.05 compared with hypoxia (6 h) group. N=Normoxia, H=Hypoxia, Cur=curcumin.

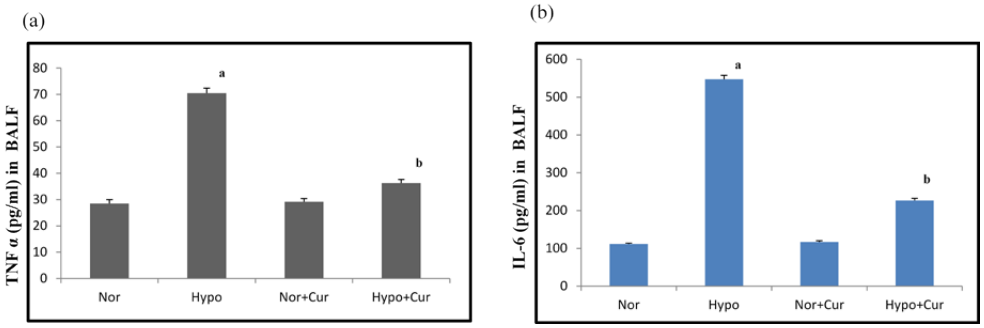

Figure 4: Protective efficacy of curcumin on (a) TNFα and (b) IL-6 content in BALF of rats exposed to hypobaric hypoxia at 7620 m for 6 h. It is noted that, hypoxia resulted into significant increase in proinflammatory cytokine molecules TNF-α and IL-6 in BALF, an indicative of the increased pulmonary inflammation. However, prior treatment of rats with curcumin and exposed to hypoxia resulted into significant decline in pulmonary inflammation in lungs. Values are mean ± SD (n=6). [a]P<0.001 compared with Normoxia (0 h) group. [b]P<0.05 compared with hypoxia (6 h) group. Nor=Normoxia, Hypo=Hypoxia, Cur=curcumin.

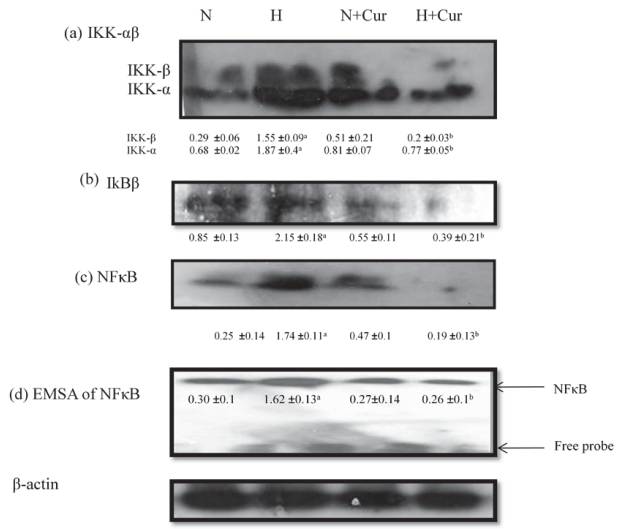

Figure 5: Effect of curcumin on expression of NFκB protein in the lungs of rats exposed to hypoxia Western blot analysis of (a) upstream activating kinases IKKαβ (b) inhibitory proteins IKBβ (c) NFκB protein expression and (d) NFκB–DNA binding activity. The observed increase in NFκB expression levels (nearly 7 fold) and NFκB –DNA binding activity (5.4 fold increase) under hypoxia was significantly down regulated by curcumin treatment. The arrow indicates position of NFκB and free probe. Up regulated protein expression of both IKK-αβ and IKB β levels under hypoxia were significantly down regulated by curcumin treatment. The densitometry analyses were represented below their respective analyses. Values are mean ± SD (n=6). [a]P<0.001 compared with Normoxia (0 h) group. [b]P<0.001 compared with hypoxia (6 h) group. N=Normoxia, H=Hypoxia, Cur=curcumin.

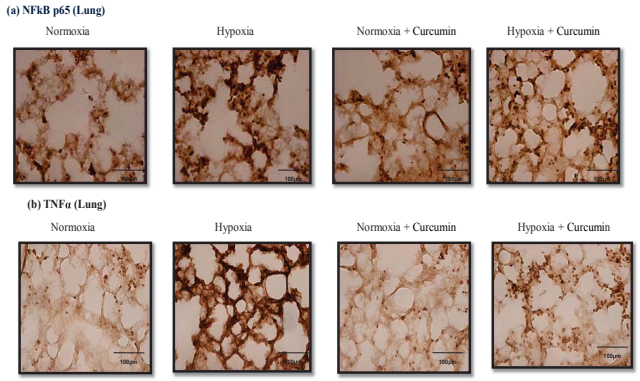

Figure 6: Immunohistochemical staining for NFκB and TNFα distribution in rats exposed to hypobaric hypoxia (6 h, 7620 m) or control (without hypobaric hypoxia) with and without curcumin administration (50 mg/Kg BW). It is noticed that, Hypoxia resulted into significant increase in inflammatory transcriptional factor NFkB and its effector proinflammatory cytokine molecule TNF-α in rat pulmonary tissue upon hypoxia exposure, indicating the increased pulmonary inflammation. However, prior treatment of rats with curcumin and exposed to hypoxia abolished the increased expression of NFκB and TNFα expressions in pulmonary tissues leading to a significant decrease in pulmonary inflammation in lungs. The figure is representative of at least 4-5 animals from each group. The signals were detected by DAB staining. Magnification: 40X.

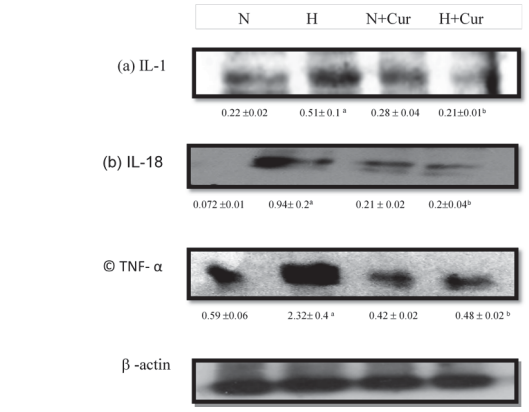

Figure 7: Western blot analysis showing the effect of curcumin on the expression of pro-inflammatory cytokines (a) IL-1 (b) IL-18 and (c) TNF-α in the lungs of rats exposed to hypobaric hypoxia at 7620 m for 6 h. The enhanced levels of these pro-inflammatory cytokines under hypoxia were significantly down regulated by curcumin treatment indicating the potent anti-inflamatory activity of curcumin under hypobaric hypoxia conditions. The densitometry analyses were represented below their respective western blot analysis. Values are mean ± SD (n=6). [a]P<0.001 compared with Normoxia (0 h) group. [b]P<0.001 compared with hypoxia (6 h) group. N=Normoxia, H=Hypoxia, Cur=Curcumin.

different cells present in BALF, but we noticed that BAL fluid appeared pinkish-red in colour giving an indication of traces of hemoglobin and RBC in hypoxia exposed rats.

Curcumin modulates the hypoxic expression of redox sensitive transcriptional factor-NFκB in lung

Hypoxic exposure resulted into a significant up regulation of NF-κB p65 expression (nearly 7 folds↑) compared to normoxia, whereas rats supplemented with curcumin during hypoxia showed reduction in expression of NFκB ($p<0.001$) in nuclear extract isolated from lung of rats compared to hypoxia (Figure 5c). The IKK complex is the convergence point for many diverse NFκB activating stimuli including TNF-α, LPS, and IL-1 [43]. We investigated whether hypoxia dependent NFκB activation involves this pathway and how curcumin can modulate this pathway? Rats exposed to hypoxia showed a significant increase in IKKαβ and phosphorylated IKBβ in cytoplasmic fraction of lung homogenate (Figures 5a and 5b). However, it was observed that prophylactic administration of curcumin significantly reduced the IKKαβ, IKBβ expression levels in lungs of rats compared to hypoxia.

Lung NFκB-DNA binding assay: To further confirm whether increased translocation of NFκB also results in increased DNA binding activity, Electro Mobility Shift Assays (EMSA) were performed using highly specific biotinylated-oligonucleotide probe. The results revealed, nearly 5.4 fold increases in NFκB-DNA binding activity in the lungs of rats exposed to hypoxia over normoxia animals. The rats receiving curcumin showed reduced DNA binding activity (nearly 6.2 fold↓) under hypoxia compared to hypoxia (hypoxia exposed rats without curcumin supplementation) (Figure 5d).

Expression of some genes reflected to be involved in inflammation

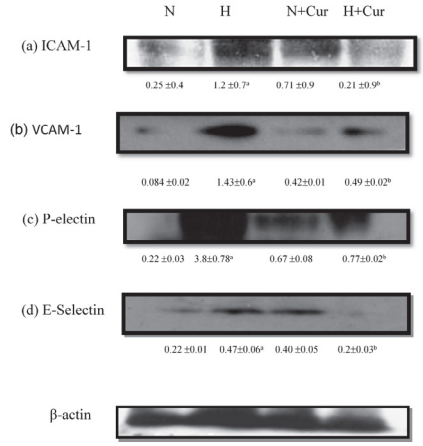

Figure 8: Western blot analysis showing the effect of curcumin on the expression of cell adhesion molecules. (a) ICAM-I (b) VCAM-1 (c) P-Selectin and (d) E-Selectin in the lungs of rats exposed to hypobaric hypoxia at 7620 m for 6 hours. The results showed significant increase in the cell adhesion molecules under hypoxia compared to control. Whereas prior treatment of rats with curcumin and exposed to hypoxia resulted into significant reduction in the expression of these cell adhesion molecules compared to control (Hypoxia, 6 h).The densitometry analyses were represented below their respective western blot analyses. Values are mean ± SD (n=6). [a]P<0.001 compared with Normoxia (0 h) group. [b]P<0.001 compared with hypoxia (6 h) group. N=Normoxia, H=Hypoxia, Cur=curcumin.

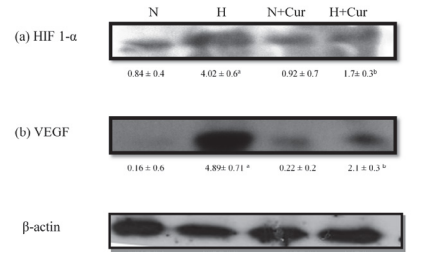

Figure 9: Western blot analysis showing the effect of curcumin on HIF-1α expression in the lungs of rats exposed to hypoxia (a) HIF-1α and (b) VEGF. Rats treated with curcumin and exposed to hypoxia showed stabilized HIF-1 α expression and reduced levels of VEGF expression compared to control. The densitometry analyses were represented below their respective analyses. Values are mean ± SD (n=6). [a]P<0.001 compared with Normoxia (0 h) group. [b]P<0.05 compared with hypoxia (6 h) group. N=Normoxia, H=Hypoxia, Cur=curcumin.

(IL-1, IL-18, TNF-α, ICAM-I, VCAM-1, E-Selectin and P-Selectin): From western blot analysis it was confirmed that hypoxic exposure resulted into enhanced levels of cytokines (IL-1, IL-18 and TNF-α) (fold increase 2.3↑, 13↑, and 4↑ respectively) (Figure 7) and cell adhesion molecules (ICAM-1, VCAM-1 and Selectins like P-Selectin and E-Selectin) (fold increase 5 ↑, 17↑, 17.3↑, and 2 ↑respectively) (Figure 8), in the lungs of rats compared to normoxia. However, administration of curcumin at 50 mg/kg BW prior to hypoxia exposure resulted into reduced (fold reduction) expression of these cytokines IL-1 (2.42↓), IL-18 (4.5↓), and TNF-α (5↓) (Figure 7) and cell adhesion molecules ICAM I (5.7↓) VCAM-I (3↓) P-Selectin (5↓) and E-Selectin (2.35↓) (Figure 8) as compared to hypoxia.

Expression of two potent pro-inflammatory cytokines (IL-6 and TNF-**α) from BALF: modulation by curcumin preconditioning:** Besides these, we observed that, there was a significant increase in TNF-α (70.82 ± 7.3 pg/ml) and IL-6 levels (545.92 ± 38.4 pg/ml) ($p<0.001$) in BAL fluid of rats exposed to hypoxia compared to their corresponding normoxia control (28.36 ± 4.5 pg/ml and 112.28 ± 12.82 pg/ml respectively). Preconditioning with curcumin completely abrogate these changes under hypoxia (hypoxia vs. hypoxia +curcumin 70.82 ± 7.2 vs. 38.36 ± 2.8 pg/ml for TNF-**α** and 545.92 ± 38.4 pg/ml vs. 222.68 ± 18.68 pg/ml for IL-6) (Figures 4a and 4b respectively). However, animals receiving curcumin under normoxia did not alter these two cytokines levels in BALF.

Effect of curcumin in maintaining oxygen homeostasis through HIF-1α stabilization and VEGF under hypoxia

One of the important transcriptional factors involved in hypoxia acclimatizing effect, i.e. hypoxia induced factor (HIF1-α) expression during exposure to hypoxia was studied in the present study. Our western blot analysis revealed that HIF1-α expression was significantly increased (nearly 5 fold ↑) under hypoxia. Animals receiving curcumin under normoxia did not alter the HIF-1α levels significantly in lung tissue (Figure 9a) indicating that curcumin (at this concentration) in normoxic conditions does not alter the body's oxygen homeostasis mechanism. Whereas, administration of curcumin prior to hypoxia exposure maintained the higher HIF1-α expression levels in a stabilized manner (Figure 9a). One of the genes regulated by HIF1-α, i.e., VEGF was found to be significantly enhanced (nearly 30 fold↑) upon exposure to hypoxia over normoxia (Figure 9b). Whereas preconditioning with curcumin and exposed to hypoxia showed reduced expression of VEGF levels (nearly 2.3 fold ↓) in lugs of rats compared to hypoxia (hypoxia exposed rats without curcumin treatment). To ensure the equal concentration of protein had been loaded, β-actin protein expression was determined in the lung homogenate by western blotting.

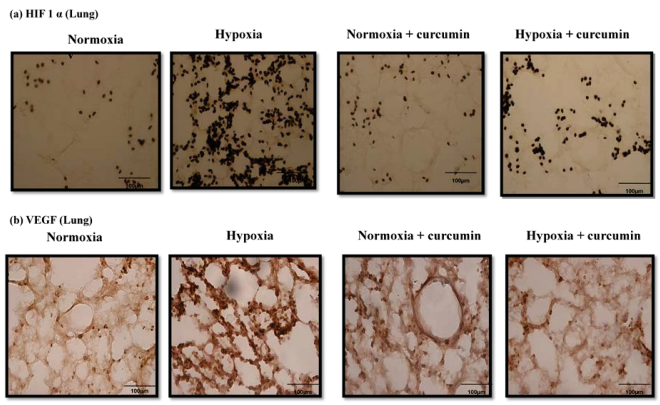

Figure 10: Immunohistochemical expression of (a) HIF1-α and (b) VEGF in lungs of rats, exposed to hypobaric hypoxia at 7620 m for 6 h. It is noticed that, hypoxia exposure (6 h) resulted into significant increased in transcriptional factor HIF1-α and its effecter vascular dilator VEGF in rat pulmonary tissue upon hypoxia exposure, indicating the increased permeability pulmonary edema. However, prior treatment of rats with curcumin and exposed to hypoxia resulted into stabilized HIF 1-α and reduced VEGF levels which might have led to a significant decrease in pulmonary edema in lungs. The figure is representative of at least 3-5 animals from each group. The signals were detected by DAB staining. Magnification: 40X.

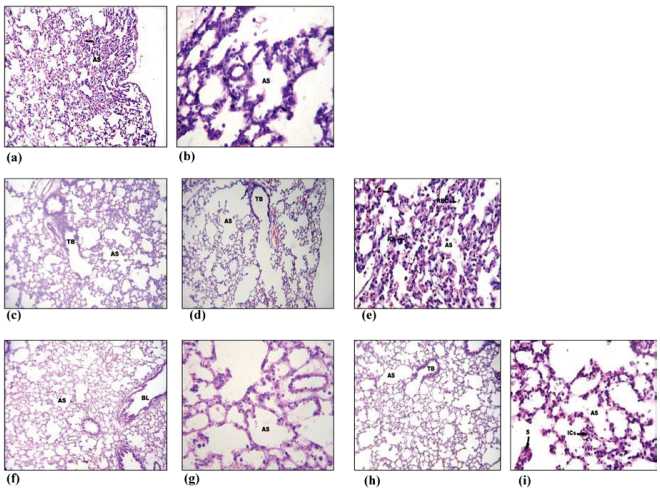

Figure 11: Histopathological images representing the infiltration of inflammatory cells and RBCs in lung of rats exposed to hypobaric hypoxia at 7620 m, for 6 h. (a) Low power photomicrograph (10X) of lung parenchyma of normoxia animal showing a terminal bronchiole (TB) and alveolar spaces (AS). (b) High power (40X) photomicrograph from the same section of lung showing the clear alveolar spaces (AS) with thin septae; (c). Low power (10X) photomicrograph of patchy area of collapse of lung parenchyma of hypoxia exposed animal. The alveolar spaces (AS) are occluded by the collapsed septae (arrow). (d). Low power (10X) photomicrograph of another area of lung parenchyma of hypoxia animal showing an emphysematous pattern. (e). High power (40X) photomicrograph of lung of hypoxia animal from the collapsed area showing alveolar spaces (AS) with thickened septae (S) and inflammatory cell infiltration (ICs). Red blood cells (RBC) are seen within some of the alveolar spaces; (f) Low power (10X) photomicrograph of normoxia animals administered with curcumin showing lung parenchyma with a medium bronchus (BL) and alveolar spaces (AS). (g). High power (40X) photomicrograph from the same section of lung showing the clear alveolar spaces (AS) with thin septae; (h). Low power photomicrograph of lung parenchyma of hypoxia animals administered with curcumin, showing a terminal bronchiole (TB) and alveolar spaces (AS). No area of collapse or emphysema was seen. (i). High power (40X) photomicrograph from the same section of lung showing the clear alveolar spaces (AS) with mild thickening of some septae with no inflammatory cells.

Immunohistochemistry

We, further confirmed our results by performing immunohistochemical studies of the two important transcription factors (NFkB, HIF1-α and the genes regulated by them i.e. TNF-α and VEGF respectively) under hypoxia. The immunohistochemistry studies have revealed that hypoxia exposure resulted into increased expression of transcription factors NFkB (Figure 6a) and one of its targeted potent pro-inflammatory cytokine i.e. TNF-α (Figure 6b) in lungs of animals compared to control. The prophylactic administration of curcumin attenuated steep increase in expression of these two molecules thereby controlled the pulmonary inflammation.

We further examined the activation of most important hypoxia regulated transcriptional factor i.e. HIF1-α. Our results revealed that, hypoxia has facilitated up regulation of HIF1-α (Figure 10a) in the lungs of rats. The activation of HIF1-α has triggered the increased expression of VEGF (Figure 10b) in lungs of rats under hypoxic conditions. It is

well established fact that the increased expression of VEGF will aid in increased permeability of vascular contents (edema) in to pulmonary tissues. The prophylactic administration of curcumin has assuaged the abrupt increase in VEGF activation (Figure 10b) but capable in stabilizing HIF1-α under hypoxia (Figure 10a).

Histological changes of lung tissue samples

Histological examinations of lung tissue samples of different groups of animals are depicted in Figure 11. Samples from the normoxic group (Figures 11a and 11b) and the normoxic group treated with curcumin (Figures 11f and 11g), both showed normal lung parenchyma. However, the lung sample from animals exposed to hypoxia showed ample evidence of damage to the lung parenchyma in the form of patchy collapse, compensatory emphysema, and inflammatory cell infiltration in the septae and red blood cells in the alveolar spaces (Figures 11c-11e). All these features were absent in the samples obtained from the animals exposed to hypoxia (6 h), administered with the drug curcumin orally, 1 h prior to hypoxic exposure (Figures 11h and 11i).

Discussion

In this study, we showed that prophylactic administration of curcumin significantly reduced the inflammation leading to reduction in fluid influx in lungs of rats under hypoxia by down regulating the pro-inflammatory cytokines and cell adhesion molecules through attenuation of NFkB activity at IKK αβ and IKB β and stabilizing HIF1-α leading to down regulation of angiogenic molecule (VEGF) followed by reduction in lung injury and albumin extravasation in BALF of rats. These studies have allowed us to identify the novel risk factors and underlying the molecular mechanism that may predispose the sustained hypoxic pulmonary edema and its prevention by curcumin.

Noncardiogenic Pulmonary Edema (HAPE) generally results when micro vascular and alveolar permeability to plasma proteins increase, one possible etiology being oxidant injury [44]. Exposure to hypoxic conditions favors the increase of ROS from mitochondria, as from NADPH oxidase, xanthine oxidase/reductase and nitric oxide synthase enzymes as well as establishing an inflammatory process leading to vasoconstriction and pulmonary edema [45,46]. Curcumin was found to be a very potent antioxidant [20]. It contains an unsaturated aliphatic chain and two aromatic rings, the electrons in curcumin are highly conjugated. Such structures generally act as antioxidants by forming stable radicals after receiving electrons [47]. In the present study the observed reduction in ROS and MDA levels in lungs of rats under hypoxia by curcumin may be due to its radical scavenging activity. Further, we observed that, reduced glutathione (GSH) levels were decreased in animals exposed to hypoxia, while curcumin administration resulted into enhanced GSH levels. The same dose of curcumin supplementation under normoxia showed unmodified GSH levels, indicating that during the stress (Hypoxia), GSH synthesis is increased to cope up with the oxidative stress which is an adaptive phenomenon. This is attributed to the fact that curcumin can modulate the key cell signaling pathways to robustly enhance the synthesis of antioxidant glutathione [48]. The observed decrease in GPx activity in animals exposed to hypoxia could be due to higher production of oxygen free radicals. The decrease in SOD levels in lungs of hypoxia exposed rats might be due to cope up with the free radicals generated in the body. Curcumin administration under hypoxic stress restored the SOD levels over control. It has been postulated that uneven pulmonary vasoconstriction enhances filtration pressure in non-vasoconstricted lung areas [49] leading to interstitial and alveolar edema. An interesting phenomenon observed in the present study was that, lung injury (measured in terms of LDH release in to BALF) started from 1 h of hypobaric hypoxia exposure, indicating that inflammation occurs preceding the fluid accumulation and might help and augment the vascular leakage. This is in association with the albumin (protein) influx in to the lungs within 1 h of hypoxic exposure onwards provides enough evidence to conclude that inflammation contribute in edema formation. Hypoxia enhanced albumin levels in BAL fluid was significantly decreased by curcumin treatment. The increased LDH activity in BAL fluid of rats under hypoxia indicated cellular injury, while curcumin treatment abridged the LDH levels.

Another interesting finding in this study was that curcumin significantly attenuated the up regulation of IKKαβ therefore leading to preventing the degradation of IKBα. This indicates that attenuation of NFkB activation by curcumin takes place at its upstream path in the cytoplasm of the cell under hypoxia. Obviously this explains the reason for prevention of IKB α phosphorylation and degradation leading to inhibiting the activation and translocation of NFkB in to the nucleus. This leads to inhibiting the production of pro-inflammatory cytokines (IL-1, IL-18 and TNF α) and cell adhesion molecules (ICAM-1, VCAM-1, E-Selectin and P-Selectin) which are regulated by NFkB. IL-18 originally referred as interferon √ inducing factor, is a pro-inflammatory cytokine that belongs to the IL-1 cytokine family [50] is required for the facilitation of neutrophil dependent injuries via the suppression of anti-inflammatory cytokine expression during hypoxia and reperfusion injury. Kim et al. [51] have reported that hypoxia induces transcription and secretion of IL-18, which subsequently induces the expression of HIF1-α through the activity of Rac1 dependent NFkB pathway. It seems reasonable to explain that, in the present study hypoxia enhanced IL-18 mediated increase in HIF1-α induction under hypoxia constitutes another link between inflammation and edema formation.

Besides these, we have also determined the expression of another important transcriptional factor and one of its regulatory genes involved in mediating the acclimatizing effects under high altitude hypoxic conditions i.e. hypoxia inducible factor (HIF-1α) and vascular endothelial growth factor; two key components involved in hypoxia induced neovascularisation [52]. The increased VEGF levels in hypoxic tissues are thought to induce an angiogenic reaction which enables increased delivery of nutrients and oxygen to the hypoxic cells through newly formed vessels [53,54]. Curcumin administration stabilized the HIF1 α expression followed by down regulation of VEGF in lungs of rats under hypoxia which might be attributed to maintain the cellular homeostasis under hypoxic conditions. It is true that higher expression of HIF1-α and it's down regulatory gene expression of VEGF are necessary in maintaining the oxygen homeostasis in the cells during hypoxia, but excess is detrimental, as increased VEGF levels alter permeability, markedly exacerbating the high permeability pulmonary edema [55]. Earlier to these findings Kaner et al. [56] provided substantial evidence that mice pre-treated with intranasal administration of an adenovirus vector expressing a truncated soluble form of VEGF receptor *flt-1*(A*dsflt*) completely abrogated the increased wet/dry weight ratio caused by AdVEGF165 administration. Moreover, enhanced pro-inflammatory cytokines and cell adhesion molecules also further help in enhancing vascular leakage in to the lungs. The cumulative effect would be accumulation of excessive fluid in the lungs**.** These results demonstrate that curcumin inhibits hypoxia induced angiogenesis by stabilizing HIF-1α activity. At the molecular level curcumin has been shown to modulate a wide range of signaling molecules. The molecular targets of curcumin fall in to two categories: those to which curcumin binds directly; those activity to which curcumin modulates indirectly [57]. This is attributed to the fact that curcumin attenuated the NFkB up regulation is probably via the direct interaction of curcumin with IKKα and also stabilizing HIF-1α leading to attenuation of higher expression

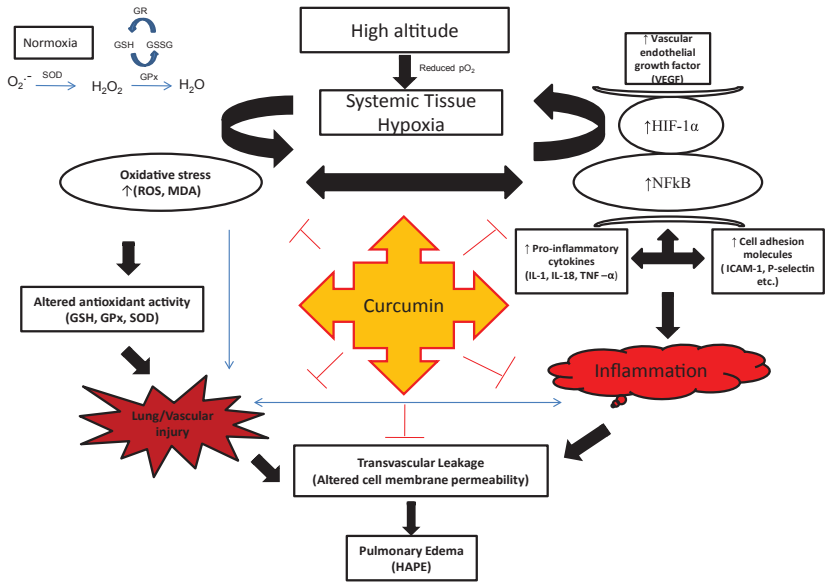

Figure 12: Schematic representation of possible mechanism of action of curcumin against HAPE as prophylactic drug. Exposure to hypoxia increased the oxidative stress (ROS, MDA) and concomitantly reduced the antioxidant status (GSH, GPX and SOD). This further caused enhanced transvascular leakage leading to fluid buildup in the lungs due to up regulated NFκB and HIF-1α and their downstream genes that play a major role in causing inflammation (pro-inflammatory cytokines and cell adhesion molecules) and vascular leakage (VEGF). Whereas prophylactic treatment of rats with curcumin reversed the changes brought by hypoxic treatment. ⊥ inhibition, ↑ up regulation, HAPE=High altitude pulmonary edema.

of VEGF levels. It seems very clear from the present data that curcumin supplementation reversed the trend. i.e. under hypoxia in presence of curcumin, there was a shift from altered state of oxidative stress to well balanced state of oxygen homeostasis through HIF-1α mediated mechanism. This perhaps helps in acclimatization of the animal. Our histopathological studies have also provided substantial evidence, as curcumin administration to hypoxia exposed animals resulted into a normal lung parenchyma showing without any involvement of edema and inflammation. Figure 12 represents the schematic representation of the possible mechanism of action of curcumin against HAPE as a prophylactic drug.

The extensive links between hypoxia and inflammation and the documented effects of ROS and inflammatory mediators to promote HIF-1α stabilization suggest that an inflammatory response could be involved in the development of acclimatization process [58]. It is not yet proved or understood whether inflammation is the cause of HAPE or consequence of HAPE? It is still a question of debate among no of research workers that, is the microcirculation responses of rodents are essentially same as those of Humans? Hypoxia in rats and mice causes increased ROS generation, leukocyte-endothelial interactions and increased vascular permeability in the lungs [59]. It is possible that, in rodents, the inflammatory responses may be an initial step in acclimatization process and perhaps help in improving O_2 supply to the tissues [58]. An inflammatory component in HAPE has been shown repeatedly in humans [60]. Inflammatory changes in pulmonary vasculature have been postulated as the primary process in HAPE [61], although a recent study disputed this [4]. According to these authors [4] inflammation is the consequences of HAPE. These authors did not measure the cytokine milieu during the course of development of HAPE in humans. Once HAPE develops inflammatory response to the hypoxia might resolves spontaneously and the microcirculation becomes resistant to inflammatory agents. In contrast to these findings humans subjected to a cicloergometric exercise at 3810 m showed elevated E-selecctin levels in BALF [62]. These authors further reported that humans with pulmonary edema at a moderate altitude (2600-3000 m) showed increased alveolar macrophages, lymphocytes, total proteins followed by increased IL-1β, IL-6, IL-8 and TNF-α, however these values were returned to normal after recovery. Earlier reports support these findings [61] in human subjects at similar heights. Similar finding was also noticed in the animals when exposed to hypoxia for several days, the inflammation resolves and exposure to lower PO_2 does not elicit further inflammation, suggesting that the vascular endothelium has acclimatized to hypoxia [63]. Numerous studies have reported that in the development of HAPE in addition to increased pulmonary artery pressure [64], impairment in ion channel function [65] and altered tight junction integrity also contribute for fluid accumulation in lungs. In our present study we tried to address the inflammatory changes that contribute for fluid accumulation in the lung during acute hypobaric hypoxic conditions. Madjdpour et al. [66] reported that decreased alveolar oxygen induces lung inflammation mainly through alveolar macrophages. In support to these findings Chao et al. [59] and Araneda and Tuesta [46] revealed that systemic inflammation of acute alveolar hypoxia observed in rats is not initiated by the low systemic tissue PO_2, but rather a chemokine - monocyte chemoattractant Protein-1 (MCP-1) released by alveolar macrophages stimulated by hypoxia and transported by circulation therefore leads to microvascular inflammatory cascade. Exposure of rats to 9,142 m for 5 h [46] and 48 h at 7619 m [67] favors the generation of pulmonary edema and promotes the release of several inflammatory mediators (TNF-α, INF-γ, IL-1, MCP-1 and TGF-β) and adhesion molecules like ICAM-I and VACM-I in BALF. Rats exposed to 10% O_2 increased NFkB, mRNA of ICAM-1, VCAM-1, HIF1-α [66] with increased ROS by alveolar macrophages were described earlier [68]. These studies are in accordance with in-vitro studies i.e. cell lines obtained from rat when exposed to 5% oxygen increased cell adhesion molecules [69]. It seems clear that the degree of inflammation generated under hypoxia is always depending up on several factors which account for the differences in findings among different studies. For example: concentration of oxygen, exposure of animals to either hypobaric hypoxia or normobaric hypoxia, duration of exposure and

also extent of reoxygenation might be of outmost important.

It seems rational to hypothesize that curcumin is able to inhibit fluid leakage in the lungs not only through previously demonstrated antioxidative and anti-inflammatory activity (NFkB) but also its suppressive effects of VEGF on neovascularization via stabilizing HIF 1α activity. This suggests that curcumin pre-treatment might be more effective in rat model of HAPE and also may provide similar protection in prevention of HAPE in human beings. Of course further studies are strongly warranted to evaluate the safety and beneficial prophylactic use of curcumin against HAPE in humans at high altitude. Additionally, the polyphenol curcumin, the active ingredient of *C. longa* having a long history of use in traditional medicine of India and China and other South East Asian countries has a favorable safe use. In addition the pharmacokinetics and the bioavailability of curcumin have been extensively investigated in patients with cancer [22,27]. All these can provide the potential evidence of curcumin for clinical applications.

Conclusion

These data provide information regarding the possible use of curcumin as a potential drug as it inhibited the oxidative stress, lung injury, pro-inflammatory cytokines and cell adhesion molecules together with suppression of HIF1-α and VEGF release, leading to reduction in inflammation, maintained oxygen homeostasis by facilitating acclimatization there by abridging the fluid influx in to the lungs of rats exposed to hypoxia under current experimental conditions. Therefore a conclusion can be drawn from this data that prophylactic administration of curcumin abates the incidence of hypobaric hypoxia induced pulmonary edema in rats.

Acknowledgement

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