Selection of Reference Genes for qRT-PCR
Normalization to study Hif1α and Hif2α Expression in Hypobaric Hypoxia Susceptible and Tolerant Rats Lung

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Abstract
Acute hypobaric hypoxia may damage brain and lung tissues due to the development of disorders such as High Altitude Cerebral Edema (HACE) and High Altitude Pulmonary Edema (HAPE). Rats, like humans, exhibit susceptibility and tolerance in the same population to such extreme conditions. We selected reference genes to normalize the qRT-PCR study in order to evaluate whether a change in expression of Hif1α and Hif2α occurs in lungs of such rats exposed to acute hypobaric hypoxia. The hypobaric hypoxia susceptible (HHS) and tolerant (HHT) Sprague-Dawley rat groups, formed on the basis of their gasping time, were exposed to a simulated altitude of 9144 m at 24°C in a decompression chamber for a short duration of one hour. A set of reference genes including Gapdh, Actb, Rpl11, Rpl10a, Rps15 and Ppia was examined for normalization in qRT-PCR study to analyze expression of target genes Hif1α and Hif2α in the lungs of these groups as compared to the normoxic control. Rpl11, Actb and Rps15 genes in combination represented the most suitable reference genes based upon GeNorm, NormFinder and BestKeeper analyses. Expression of Hif1α and Hif2α genes was reduced in both HHS and HHT rat lungs. However, enhanced protein expression of HIF-1 in HHS group, and weak expression of HIF-2 protein in both groups was recorded. Our findings suggest that HIF-1 may play a significant role in mediating early responses towards acute hypobaric hypoxia in lung samples of HHS as well as HHT rats, with its profound protein expression in the former.

Key Words: Hypobaric hypoxia, Gene expression, Tolerance to high altitude, Acute hypoxia

Introduction
Hypobaric hypoxia is characterized by oxygen deficiency in the body due to a progressive decline in atmospheric pressure with ascending altitude. It may lead to acute altitude illness that begins after a few hours to days of continued exposure to hypoxia and is categorized into Acute Mountain Sickness (AMS), High Altitude Cerebral Edema (HACE), and High Altitude Pulmonary Edema (HAPE). AMS begins with headache and progresses with neurological dysfunction that may cause brain swelling as a consequence of fluid accumulation due to increased blood brain barrier permeability and intra-cranial pressure. In severe condition, it leads to HACE, which may cause death due to brain herniation (1). Onset of HAPE has been postulated to result from different events like pulmonary capillary stress failure depicting the hydrostatic mechanism (2), uneven vasoconstriction leading to regional capillary over
perfusion (3), and calcium influx with closed voltage gated potassium channels (4). A further increase in lung damage advances to death if proper medication and treatment is not provided on time.

Some populations and, in fact, some individuals within a population having augmented arterial systolic pressure show more predisposition towards development of pulmonary edema than others depicting variable response to hypoxia adaptation (5). Females appear to be more resistant towards it suggesting a natural gender bias (6). Some genome wide association and proteome based studies have been done on high altitude adaptation on blood samples obtained from different populations (7, 8). Although, some polymorphisms have been found associated with susceptible and resistant hypobaric hypoxia in populations (9), however, the genetic and molecular pathways of disease progression in hypobaric hypoxia remain unexplored.

It is well known that HIF1 transcription factor binds to the hypoxia responsive element (HRE) and regulates the expression of different genes involved in angiogenesis, glycolysis and NO production contributing to various adaptation mechanisms operating during exposure to hypoxia. HIF2 regulates expression of genes such as Epo, Oct4 and those involved in cell cycle progression, NOTCH signaling and inhibition of NO production (10). HIF2 has recently been linked to long term adaptation in Tibetans to high altitudes (11). Owing to the involvement of HIF 1α and HIF2α subunits in these adaptive responses to hypobaric hypoxia, we hypothesized that these master regulators might be differentially expressed in rats, which are either susceptible or tolerant to extreme hypobaric hypoxia. For that purpose, rats were screened and segregated into hypobaric hypoxia susceptible and tolerant rats on the basis of their gasping time by exposing them to a simulated altitude of 9754 m (12). After a week of acclimatization, these two groups were later subjected to a simulated altitude of 9144 m for one hour to study their response to acute hypobaric hypoxia stress. The gene and protein expression of HIF-1 and HIF-2 were examined in lung samples obtained from these groups.

**Quantitative Real Time Reverse Transcription Polymerase Chain Reaction**

Quantitative Real Time Reverse Transcription Polymerase Chain Reaction qRT-PCR study was used to measure the mRNA expression of target genes Hif1α and Hif2α. It is a highly efficient technique to study expression of individual genes and for validating high throughput gene expression profiling studies. However, before undertaking any qRT-PCR experiments, it is a prerequisite to select optimized normalization factors or reference genes that could serve as ideal controls for evaluating the target gene expression. This is done in order to minimize non-biological variations in a comparative gene expression analysis (13).

Six reference genes namely Gapdh, Actb, Rpl10a, Rpl11, Ppia and Rps15 were considered to validate and select suitable reference genes for normalization in our qRT-PCR study. Most studies use Gapdh and Actb as reference genes or so-called house-keeping genes. However, their use is limited owing to their differential mRNA expression under different experimental conditions. Three online softwares available for normalizing the gene expression were employed in the study: GeNorm (14), NormFinder (15) and BestKeeper (16). The outputs of these softwares were then compared and used to evaluate transcript expression of Hif1α and Hif2α in the rats kept under normoxic condition as control (Con), hypobaric hypoxia susceptible (HHS) and hypobaric hypoxia tolerant (HHT) groups. In order to verify whether the mRNA expression correlates with the protein expression, western blotting for HIF-1 and HIF-2 proteins was also carried out with Con, HHS and HHT lung samples. Our study is an important effort in the direction of understanding response to hypobaric hypoxia stress and subsequently devising strategies to develop more effective medicines and therapy to benefit travelers, visitors, sport persons and soldiers exposed to acute hypobaric hypoxia during their ascent to high altitude.
Methodology

Experimental Animal: Sprague Dawley male albino rats of weight 185 ± 10 g were maintained on a daily standard pelletized diet and sterile water with temperature 24±2°C maintained inside the animal house facility of DIPAS, DRDO, New Delhi. The rearing conditions of animals and all the below mentioned protocols of acute hypobaric hypoxia exposure were approved by Institutes Animal Ethical Committee of Defence Institute of Physiology and Allied Sciences (DIPAS), DRDO and all the guidelines were in compliance with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India.

Group Segregation into Hypobaric Hypoxia Susceptible and Tolerant Rats: The rats were selected on random basis and exposed to acute hypobaric hypoxia by gradually decreasing the atmospheric pressure to obtain a simulated altitude of 9,754 m and barometric pressure of 205.8 mm Hg at 32°C in an animal decompression chamber (Decibel Instruments, Delhi, India) attached to a mercury barometer. The desired altitude equivalent was achieved by incrementing simulated altitude at a rate of 3,000 ft per min (914 m/min). The relative humidity was maintained at 40-50% with airflow around 2 L/min in the chamber. The time taken for the appearance of first sign of gasping was recorded as gasping time using an electronic stopwatch. Rats were segregated into two groups HHS (gasping time less than 5 minutes) and HHT (gasping time greater than 45 minutes) on the basis of gasping time. A third group of normoxia labelled as Con (rats never exposed to hypoxic stress) was kept as control and maintained at standard conditions as mentioned above. All the three groups were kept for acclimatization under normal conditions at 24±2°C for a week.

Final Hypobaric Hypoxia Exposure and Tissue Collection: All the three groups (n=6 in each case) were then exposed to a simulated altitude of 9,144 m and atmospheric pressure of 225.6 mm Hg maintained inside a decompression chamber at 24°C for 1 hour. Same conditions of relative humidity and airflow were maintained as stated above. The rats were anaesthetized using ketamine (80 mg kg⁻¹ body weight, i.p.) and xylazine (20 mg kg⁻¹ body weight, i.p.) and sacrificed to collect lung tissue for the qRT-PCR expression study. These samples were stored at -80°C.

RNA Extraction and cDNA Synthesis: Total RNA was extracted using TriReagent (MRC Inc.) from lung samples of all the three groups. These RNA samples were subjected to quality analysis and concentration estimation using Nanodrop and samples having 260/280 absorbance ratio between 1.9 and 2.0 were treated with DNase1 (Fermentas) to remove any traces of genomic DNA. RNA was then purified using Chloroform: Isopronanol precipitation and dissolved in nuclease free DEPC treated water after ethanol wash. These samples were again tested for RNA integrity using UV-Vis spectrophotometer. Good quality RNA samples were subjected to first strand cDNA synthesis using ThermoScientific Revert Aid First strand cDNA synthesis Kit.

Primer Designing and qRT-PCR Optimization: The cDNA sequences of Gapdh, Actb, Rpl11, Rpl10a, Rps15, Ppia, Hif1α and Hif2α were downloaded from NCBI Entrez Nucleotide database. These sequences were used as templates for primer designing using PrimerBLAST database of NCBI (17) using default parameters, except that the length of the amplicon was kept between 70 to 150 bp taking mRNA RefSeq database of Rattus norvegicus as reference. OligoCalc software (18) was used online to check any non-specific template binding, hairpin loop and dimer formation in designed primers (Table1).

The cDNA samples were used as qRT-PCR templates after primer optimization and optimum template dilution using RT-PCR and gel electrophoresis in order to standardize the conditions for amplification of the desired reference and target genes. Quantitative RT-PCR was carried out using Syber Green dye for labelling any double stranded amplicon in the reaction mixture in a 96 well Real Time thermal
The initial denaturation was performed at 95°C for 3 min followed by 40 cycles of three step RT-PCR: 95°C for 20 sec, annealing temperature of 55°C for 20 sec and extension at 72°C for 15 sec in each cycle. Efficiency of each primer pair was checked by serial dilutions to obtain a standard curve. Reactions were carried out in triplicates including those for standard curve and No Template Controls (NTC) in each run of the experiment. The melting curve analysis was simultaneously done to check and rule out the formation of non-specific products and primer dimers.

**qRT-PCR Data Analysis:**

**qRT-PCR results were analysed using these softwares: GeNorm (14), NormFinder (15), and BestKeeper (16).**

**GeNorm analysis:** GeNorm calculates the pair wise variation ratios of \( \Delta C_q \) values of one candidate reference gene with that of other which represents the standard deviation obtained from the log transformed expression ratios. A reference stability value called 'M value' is calculated as average pairwise variation of one candidate reference gene from all others. All the six reference genes were then studied in the present study according to their M values.

**NormFinder analysis:** NormFinder calculates the geometric mean calculation following the pairwise correlation analysis of all candidate normalization gene pairs. The software also carried out regression analysis and the genes having the standard deviation \( \leq 1 \) from the calculated BestKeeper index were excluded leaving behind the suitable reference genes.

**BestKeeper analysis:** Raw Cq values were taken as direct input by BestKeeper software for geometric mean calculation along with the inter- and intra-group variations. The software also excluded the specific products and primer dimers.

### Table I: Details of the candidate reference genes and the target genes studied.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>NCBI Accession No.</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Tm (°C)</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>Glyceraldehyde</td>
<td>NM_017008.4</td>
<td>AGGCTGGGGCTCACCTGAAG</td>
<td>GCAATGGTGATGCAAGGAGG</td>
<td>57±0.2</td>
<td>149</td>
</tr>
<tr>
<td>Rps15</td>
<td>Ribosomal protein S15</td>
<td>NM_017151.2</td>
<td>CCAAGGTGGAATCAACCCCGCC</td>
<td>TCCTCTACAGGGTATGGG</td>
<td>54±0.3</td>
<td>75</td>
</tr>
<tr>
<td>ActB</td>
<td>Actin, beta</td>
<td>NM_031144.3</td>
<td>CAGGGTGTGATGGGTGGG</td>
<td>ATGTTGCTGACATCCGGT</td>
<td>55±0.2</td>
<td>115</td>
</tr>
<tr>
<td>PPIA</td>
<td>Peptidylprolyl isomerase A</td>
<td>NM_017101.1</td>
<td>GCTACCCACCGGTGTCCCT</td>
<td>ATGTTGCTGACATCCGGT</td>
<td>55±1.6</td>
<td>133</td>
</tr>
<tr>
<td>Rpl11</td>
<td>Ribosomal protein L11</td>
<td>NM_001025739.1</td>
<td>GTCTCCTGCAACGCTACGAG</td>
<td>ATGTTGCTGACATCCGGT</td>
<td>54±1.3</td>
<td>103</td>
</tr>
<tr>
<td>Rpl10a</td>
<td>Ribosomal protein L10A</td>
<td>NM_031065.1</td>
<td>CCGCAGCGCTGACGAGGAGAG</td>
<td>ATGTTGCTGACATCCGGT</td>
<td>59±0.4</td>
<td>113</td>
</tr>
<tr>
<td>Hif1α</td>
<td>Hypoxia inducible factor 1</td>
<td>NM_024359.1</td>
<td>ATGTTGCTGACATCCGGT</td>
<td>ATGTTGCTGACATCCGGT</td>
<td>58±0.3</td>
<td>151</td>
</tr>
<tr>
<td>Hif2α</td>
<td>Hypoxia inducible factor 2</td>
<td>NM_023090.1</td>
<td>ATGTTGCTGACATCCGGT</td>
<td>ATGTTGCTGACATCCGGT</td>
<td>57±0.7</td>
<td>111</td>
</tr>
</tbody>
</table>
Target Gene Expression Analysis: The reference genes found to be stable in terms of expression in all the three software were taken for further analysis. Their geometric mean was calculated and Livak method (19) was used subsequently to analyze the differential expression pattern of Hif1α and Hif2α in HHS and HHT samples as compared to Con sample utilizing value 2^ΔΔCq as approximate fold change or the relative quantification value. The Cq denotes the amplification cycle or threshold at which the real time curve crosses baseline and the superscript ΔCq (Con-HHS (or HHT) is the deviation of ΔCq value of sample (either from group HHS or HHT) versus Cq value of Control.

Protein Expression Analysis: The protein expression of the target genes Hif1α and Hif2α in the lung tissue samples from Con, HHS and HHT groups was determined by developing western blots. An ice cold buffer A (0.5 M sucrose, 10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 10% glycerol, 1 mM EDTA, 1 mM DTT, 1 mM phenyl-methyl sulphonyl fluoride) containing protease inhibitors cocktail (Sigma) was added four times the amount of tissue sample to homogenize the lung tissue. After 15 minutes of incubation, NP-40 (Nonyl phenoxypoly-ethoxylethanol) was added to the homogenate to make up 0.6% of the total volume of the solution and centrifuged at 2000 g for 10 min at 4°C. For nuclear fraction, the pellet obtained after removing the supernatant was re-dissolved in equal volumes of ice cold buffer B (20 mM HEPES, pH 7.9, 0.3 mM NaCl, 1.5 mM MgCl₂, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM phenyl-methyl sulphonyl fluoride) containing the same protease inhibitors cocktail as mentioned above. This solution was incubated on ice for about half an hour and centrifuged at 20,000 g for 15 min at 4°C. The supernatant aliquots containing nuclear fraction were stored at -80°C for immunoblotting and protein estimation. Lowry's method was used to estimate concentration of total protein in each sample aliquot. The proteins in each sample were separated using SDS-PAGE by loading 50 μg of each sample. These gels were electro-blotted on to a nitro-cellulose membrane (Millipore, USA). This nitro-cellulose membrane bearing protein bands was blocked with 3% BSA for 2 hours and then washed with Tris buffer saline with Tween 20 (0.1%) to decrease non-specificity. The blocked membranes were probed with their respective primary monoclonal antibody of HIF-1 obtained from mouse (1:1000 dilution) and polyclonal antibody of HIF-2 from rabbit in the ratio of 1:500 dilution (Santacruz, CA, USA) for 3 hours. After washing with TBST, the membranes were incubated with secondary antimouse and antirabbit-IgG-HRP conjugate (each with 1:25,000 dilution) sequentially for 2 hours. These membranes were then incubated with chemiluminescent substrate (Sigma) to visualize the specific protein bands. Bands were developed on X-ray films (Kodak, USA) in dark.

Results

qRT-PCR Expression Analysis in Control, Susceptible and Tolerant Groups: For performing qRT-PCR, cDNAs from the three groups, i.e., Con, HHS and HHT were synthesized as described in methodology. The PCR efficiency predicted on the basis of the slope of standard curves in case of all the primers was found to be in the range 90-95%, which is within the acceptable limits. Single peak obtained in the melting curve analysis in each case and Cq values above 35 cycles observed in the no template control (NTC) indicated that there was no contamination in the reactions and PCR products were primer specific.

qRT-PCR Data Analysis for Optimum Reference Gene Selection: Three softwares that work on different algorithms and principles were employed to determine the most stable genes suitable for normalization in qRT-PCR. These softwares have been used in recent studies (20, 21). GeNorm calculates the log transformed values itself to calculate stability value, while NormFinder uses the log transformed Cq values or ΔCq values (base 2) and converts it into natural base e log values to calculate intra and inter group variations along
with a different stability values. BestKeeper directly takes Cq value irrespective of group to which they belong and calculates the standard deviation with respect to BestKeeper index along with pairwise correlation coefficient and power fold change indicating the difference in the expression of the gene under test condition as compared to the control.

Three sets of data were used for comparison of gene expression during hypobaric hypoxia (no treatment vs hypoxia treatment) and included: Con vs HHS, Con vs HHT, and Con vs combined (HHS and HHT). The GeNorm analysis indicated that in all the three groups, the order of the ‘M’ stability value and pattern of graph remains similar that is \textit{Gapdh} > \textit{Ppia} > \textit{Rps15} > \textit{Actb} > \textit{Rpl10a} > \textit{Rpl11} (Figure 1). This finding indicates that \textit{Rpl11} and \textit{Actb} with M values less than 0.6 are highly stable genes whose expression does not vary much as compared to other genes under study. This observation is also evident while comparing a normalization factor from the $V_n/V_{n+1}$ values that remained less than 0.15 for the 2 or 3 most stable targets. \textit{Gapdh} having highest ‘M’ value (always above 0.9) in each case seemed to be the most unsuitable candidate having highest variation in terms of expression. The analysis suggested that the geometric mean of reference targets \textit{Rpl11} and \textit{Gapdh} will serve well as optimum factor for normalization.

When NormFinder algorithm was used, the order of stability value remained \textit{Gapdh} > \textit{Ppia} > \textit{Rps15} > \textit{Actb} > \textit{Rpl10a} > \textit{Rpl11} for Con vs HHS, \textit{Gapdh} > \textit{Ppia} > \textit{Rpl10a} > \textit{Rps15} > \textit{Actb} > \textit{Rpl11} for Con vs HHT, and \textit{Rpl10a} > \textit{Gapdh} > \textit{Ppia} > \textit{Rps15} > \textit{Actb} > \textit{Rpl11} for Con vs both (HHS and HHT). \textit{Rpl11} and \textit{Actb} with the lower stability values than others were found to be the most suitable reference gene in Con vs HHS, Con vs HHT and Con vs both (HHS and HHT) (Figure 2). The resultant inter-and intra-group variation in combined comparison (Con vs HHS and HHT) calculated from the log transformed values of expression revealed that \textit{Rps15} and \textit{Rpl11} had least intra-group variation as visible through error bars in Con vs HHS, Con vs HHT and Con vs both (HHS and HHT) groups in the graph, whereas \textit{Rpl11} and \textit{Actb} were found to have least inter-group variation in the same group (Figure 3). Thus, it was concluded from the present results that \textit{Rpl11} and \textit{Actb} were most stable reference genes having minimum stability values and variation within and across all sample groups.

![Graph representing curves obtained by plotting stability values ‘M’ of GeNorm for Con vs HHS, Con vs HHT and Con vs both (HHS and HHT) group. Most suitable reference gene Rpl11 has least M value, while Gapdh is least suitable reference gene with highest M value in all three comparisons](image_url)

Sharma et al
Fig. 2. Plot obtained by plotting stability values calculated by NormFinder software for Con vs HHS, Con vs HHT and Con vs both (HHS and HHT). Rpl11 and Actb were found to possess lower stability values than others and therefore, most suitable as normalizer genes according to NormFinder.

Fig. 3. Bar graph with rectangular bars representing intergroup variation and error bars intra-group variation respectively in combined analysis (Con vs Both (HHS and HHT)), for the stability values obtained using NormFinder. As depicted by NormFinder stability values, Actb and Rpl11 are most suitable reference genes with lower inter-group variability than others. However, Rps15 and Rpl11 show lowest intra-group variation.

Gene expression in acute hypobaric hypoxia
BestKeeper results suggested that Rps15, Actb and Rpl11 had standard deviation (S.D.) <1 for comparison set Con vs HHS, while S.D.<1 was observed in Rps15 and Rpl11 for Con vs HHT and combined (Con vs both (HHS and HHT)) with all results having significant p-value <0.05 and coefficient of correlation close to 1 (Table 2). Therefore, Rps15 and Rpl11 were suggested to be the desired reference genes.

In our study, data analysis by GeNorm and BestKeeper showed that Gapdh has expression variability and is therefore, unfit for normalization. It was decided to take more than two reference genes namely, Rpl11, Rps15 and Actb in compliance with the softwares result, as inclusion of more than one reference gene makes the result statistically more reliable. When the expression of these genes was studied along with other candidates, it was found that Rpl11 and Rps15 were showing least variation in their Cq values. Besides that, as explained in below mentioned results, Rpl11, Actb and Rps15 were found to be suitable normalization factors for acute hypobaric hypoxia irrespective of the susceptible and tolerant rat samples under study.

Differential Expression analysis of HIF-1 and HIF-2.

Geometric mean of Cq values of the genes Rpl11, Actb and Rps15 was used to calculate the ΔΔCq values for obtaining the fold change in target genes Hif1α and Hif2α. The average Cq values obtained in each group were first taken as input to obtain average ΔCq values by calculating the mean difference of Cq observed in target and reference genes. Thus, the fold change values obtained after calculating ΔΔCq in each case for Hif1α were 0.0208±0.0067 and 0.0189±0.0062 in HHS and HHT rat samples, respectively and similarly, for Hif2α the values were 0.833±0.009 in HHS and 0.205±0.017 in HHT rat samples (Figure 4). Thus, level of mRNA expression in both Hif1α and Hif2α decreased with little or no change in the transcript expression of Hif2α in HHS with respect to Con group.

In the present experiment, the protein expression of HIF-1 was observed to be higher in case of HHS and HHT, when compared to normoxic group. In fact, more intense bands of
HIF-1 represents more accumulation of protein in HHS than HHT rats and probably points towards its substantial requirement to overcome the worsening pathological condition in HHS than HHT rats (Figure 5).

**Discussion**

Hypobaric hypoxia resulting from oxygen deficiency at higher altitudes may cause various high altitude maladies such as High Altitude Cerebral Edema (HACE) and High Altitude Pulmonary Edema (HAPE) in people who ascend to such regions. If not treated early or the person is not taken immediately to lower altitudes, the

**Table 2:** BestKeeper software output showing genes selected to be best suitable candidates for normalization with standard deviations <1. Lower standard deviation and higher correlation coefficient values indicate suitability of genes (Rpl11, Rps15 and Actb).

<table>
<thead>
<tr>
<th>Gene</th>
<th>S.D. [± CP]</th>
<th>p-value</th>
<th>Power [x-fold]</th>
<th>Coeff. of correlation [r]</th>
<th>Coeff. of correlation [r²]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gapdh</td>
<td>1.96</td>
<td>0.001</td>
<td>3.63</td>
<td>0.989</td>
<td>0.978</td>
</tr>
<tr>
<td>Rps15</td>
<td>0.31</td>
<td>0.001</td>
<td>1.22</td>
<td>0.981</td>
<td>0.962</td>
</tr>
<tr>
<td>Actb</td>
<td>0.63</td>
<td>0.008</td>
<td>1.52</td>
<td>0.928</td>
<td>0.861</td>
</tr>
<tr>
<td>Ppia</td>
<td>1.39</td>
<td>0.003</td>
<td>2.57</td>
<td>0.953</td>
<td>0.908</td>
</tr>
<tr>
<td>Rpl11</td>
<td>0.69</td>
<td>0.001</td>
<td>1.58</td>
<td>0.988</td>
<td>0.976</td>
</tr>
<tr>
<td>Rpl10a</td>
<td>1.16</td>
<td>0.003</td>
<td>2.11</td>
<td>0.954</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Gene expression in acute hypobaric hypoxia
condition can become life threatening. However, certain individuals show variable adaptation response to such diseases as evident from various studies involving human and animal subjects. The susceptibility and tolerance to hypobaric hypoxia in rats has long been studied on the basis of gasping time, measured after exposure of animals to simulated extreme altitude conditions (12). Since, the higher capillary density of rats renders them more tolerant to lower altitudes than humans (22) and in order to maintain the susceptibility and tolerance trait observed at 9754 m in the groups, a simulated altitude of 9144 m was adopted for acute hypobaric hypoxia exposure for a short duration of one hour to avoid unnecessary damage to animals. Since the expression of both Hif1α and Hif2α genes is associated with adaptation to high altitude, we examined whether Hif1α and Hif2α are differentially expressed in lungs of hypobaric hypoxia susceptible and tolerant rats during acute and extreme hypobaric hypoxia conditions as compared to normoxia. In order to study the expression of Hif1α and Hif2α, a set of reference genes Gapdh, Actb, Ppia, Rpl10a, Rpl11 and Rps15 were evaluated to select best suitable reference genes for normalization in qRT-PCR studies. A few studies on HAPE have used endogenous controls such as β-actin, 18S rRNA (normalization not done) and ASAH1 and MAN1A1 (chosen normalization factors) for qRT-PCR analysis of gene expression of HAPE affected and unaffected individuals (23, 24). However, to the best of our knowledge, there has been no normalization study till date for gene expression studies on lungs of rats exposed to acute hypobaric hypoxia.

Reference Genes Used for qRT-PCR Analysis: Gapdh (Glyceraldehyde phosphodehydrogenase) is the most popular gene used as a reference gene in qRT-PCR (25, 26, 27). However, its expression has been shown to vary in different experimental conditions. Since Peptidylprolyl isomerase A or Cyclophilin A (PPIA), is a highly conserved multifunctional enzyme in animals catalyzing cis-trans isomerisation of peptidyl-prolyl bond of proteins and participates in protein folding (28). It seems fit to be considered as reference target and has been found as a suitable reference gene in combination with others in many recently published experiments (27, 20). Actb or Beta Actin also has been used as reference gene in many studies such as in up regulation of haemoglobin in alveolar epithelial cell culture exposed to hypoxia (29) and measurement of leptin expression without prior normalization (30). Rpl10a, Rpl11 and Rps15 genes were chosen because of their essential and conserved roles in ribosome formation. For a functional 80S ribosome, Rpl10a is required to assemble 40S and 60S ribosomes and thought to be conserved amongst many eukaryotic species (31). Similarly, Rpl11 is also required for the production of mature 28S and 5.8S rRNA. In addition, Rpl11 has been found to be also associated with 5S rRNA during ribosomal biogenesis in yeast (32). Ribosomal protein Rps15 being highly conserved plays an important role by interacting with 16S rRNA for small subunit central domain assembly (33). Thus, these ribosomal genes seem to be highly promising reference targets and it was expected that they will show more consistent expression in response to acute hypobaric hypoxia.

A number of studies involving cancer cell lines also found altered expression of Gapdh (34, 35) and a plausible explanation for its altered and up-regulated expression in cancer cell lines has been provided as presence of hypoxia responsive elements in the upstream region of Gapdh (36). The unsuitability of Ppia as a reference gene in this experiment might be accounted by the fact that like Gapdh, Hif1α binding sites or hypoxia responsive elements were also found in its promoter and that, it has been shown to play important role in ROS mediated oxidative stress, inflammatory signalling and vasculo-smooth muscle cell proliferation (37), numerous cancers (38), in COPD pathogenesis (39), and in pathogenesis of various cardiovascular diseases (40). This justifies the significance of our study aimed to select the optimal reference genes in
order to measure gene expression of *Hif1α* and *Hif2α* in pulmonary tissue under the effect of acute hypobaric hypoxia.

In the presence of oxygen and other cofactors, HIF-alpha gets hydroxylated by *Prolyl Hydroxylase Domain-containing* proteins (PHDs) and Factor Inhibiting HIF-1 (FIH-1) at specific proline and asparagine N-803 residues, respectively. This hydroxylated protein subunit binds to pVHL to form E3 ubiquitin ligase VHL complex, which is then rapidly degraded by proteasomal complex. However, during hypoxia, it stabilizes and translocates into the nucleus from cytoplasm, where it binds to ARNT to form HIF complex. HIF then acts as a transcription factor and binds HREs in the regulatory elements of various genes, thereby inducing their gene expression (41).

While a decrease in mRNA expression of *Hif1α* in acute hypoxia has been previously seen and explained possibly due to chromatin modeling in a study involving cell lines exposed to a hypoxia mimetic (42). It was shown using ChIP assay that during hypoxia, the acetylation of histones H3 and H4 present in the proximal region of enhancer/promoter region near the transcription start site of *Hif1α* is decreased, thereby strongly reducing its transcription (42). We found protein expression of HIF-2 markedly decreased in HHT than HHS group when compared to the Con group, which is well correlated with its decreased mRNA expression pattern in hypobaric hypoxia. During its initial discovery, it was found that HIF-2 is markedly expressed in lungs during normoxia and is required for lung and vascular development (43). Mole *et al.* proved that HIF-2 may not contribute to acute hypoxia as it does not show much transcriptional activation of genes containing HREs, despite its high binding affinity for these sites (44). This may partly explain that HIF-1, but not HIF-2 seems to contribute significantly in any response to initial phases of hypobaric hypoxia due to marked decrease in its protein as well as mRNA expression. However, the precise reason of this pattern of expression can be found only by performing some epigenetic studies on HIF-1 and HIF-2. We also suggest that expression of some down targets of these genes and various other factors need to be examined, as these might directly or indirectly play a significant role in tissue protection or deterioration during initial exposure to acute and extreme hypobaric hypoxia.

In conclusion, *Rpl11, Actb* and *Rps15* were found to be the best suitable reference genes to analyse expression of target hypobaric hypoxia responsive genes in our study. Besides this, increased HIF-1 protein expression in HHS and HHT rats, and decreased HIF-2 expression signifies that HIF-1 may play a significant role in the cellular response to hypobaric hypoxia, though, the function of HIF-2 here is still not clear.

**Acknowledgements**

Authors thank the Director, DIPAS, DRDO for providing financial support and animal facilities for carrying out this work. We are also highly thankful to Mr. Bhagwat Singh, Mr. Varun Bhardwaj, Mr. Malhotra and Mr. Karan Pal for their technical support in animal handling, blot experiments and handling of instruments used in the experiment.

**Declaration of Interest**

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Gene expression in acute hypobaric hypoxia


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