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Equine Hyperbaric Oxygen Therapy

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Abstract

Hyperbaric oxygen therapy (HBOT) could induce oxidative stress over time that may cause lung inflammation and alter the mRNA expression of inflammatory cytokines in lung cells of horses.

Identifying stable reference genes is necessary to obtain reliable relative quantitative polymerase chain reaction (QPCR). We hypothesized that (1) HBOT induces lung inflammation in healthy horses and (2) hyperbaric oxygen (HBO) induces an increase in arterial oxygen levels with no other effect on arterial blood parameters.

Eight horses were used in a randomized controlled cross-over design. Treated horses were exposed to 100% oxygen at 3 atmosphere absolute (ATA) for 20 minutes for 10 days whereas the chamber was not pressurized for control horses. A bronchoalveolar lavage (BAL) was performed at baseline and on day 10 for total and differential cell counts as well as for the mRNA expression. Groups of pre- and post-HBOT and control were compared. IL-1β, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p35, IFN-γ, TNF-α and Eotaxin-2 were measured by QPCR in BAL fluid. Genes' expression was measured by QPCR after efficiency correction using relative expression software tool (REST) software analysis. The expression stability of four candidate reference genes, GAPDH, HPRT, SDHA, RPL-32, were determined using NormFinder and Genorm^{plus}. Arterial blood parameters were measured before and right after HBOT on day 1 and 10. Four additional horses were used to measure arterial blood gases collected through an arterial line during HBOT at baseline, 3 ATA (for 0, 10 and 20 minutes), during (2 ATA) and 0 and 10 minutes after decompression.

Results show that HBOT induced a significant decrease in total and neutrophilic cell counts for the HBOT pre vs. post groups. The mRNA expression of cytokines was significantly down- regulated with HBOT for Eotaxin-2 (HBO post vs. control post) and IL-4 (HBO pre vs. post). GAPDH was found to be the most stable reference gene. The number of reference genes used for optimal normalisation included GAPDH and HPRT or RPL-32. Arterial blood parameters during HBOT showed a rapid increase of PaO_2 (>800mmHg), which decreased to baseline values within 10 minutes after HBO.

These results suggest that HBO reaches extremely high blood oxygenation levels very transiently and does not induce inflammation in the lungs of horses.

Contents

Introduction

No data are available on the effects of hyperbaric oxygen therapy (HBOT) on lung physiology in equines. Research was performed to get to know the effects of Hypobaric Oxygen Therapy on the respiratory tract of horses.

Hyperbaric oxygen medicine was first developed in human medicine. However, equipment and applications for equine medicine have been initially developed more than 10 years ago. The list of proposed applications of HBOT in horses is long, including conditions, which involve wound healing, infections, anaemia, neurological damage and bone deficits. The purposes of HBOT in these cases are used for minimalizing the ischemic injury and killing bacteria[1]. Unfortunately, there is a complete lack of research to support most of the proposed applications in equine medicine and the basis and potentially negative effects of hyperbaric oxygen therapy on the equine athlete have never been studied.

Because the availability of the equipment is extremely limited in North America, the expertise and research in the field has unfortunately been very limited. Currently, there are three publications about hyperbaric oxygen therapy in horses, one is on skin grafts[2], one is about endotoxemia[3] and the last one is about using stem cells in combination with HBOT[4]. The first two publications showed slight improvements on their therapy such as slightly better wound healing (histologic examination of skin grafts showed less granulation tissue, edema and neovascularization but more inflammation[2]) and less endotoxemia (it significantly ameliorated the effect of LPS but did not improve other abnormalities associated with endotoxemia). The last publication, Dhar et al.[4], found significant improvement in stem cell concentrations during HBOT.[3]

Background
HBOT is an inhalation therapy that is achieved by having the patient breathe 100% oxygen inside a pressurized chamber[5-9]. Pressure at sea level is created by the atmosphere and is similar to 760 mmHg, 14,7 lb/in (psi) or 1 atmosphere absolute (ATA), whereas HBOT is defined as a pressure higher than 1 ATA by the Undersea and Hyperbaric Medical Society[1,9].

Oxygen is delivered to the tissues through respiration and lungs are the only organ that is, together with the skin, directly exposed to HBOT[10-12]. The benefits of HBO, such as improved wound healing and preventing ischemic damage, are delivered from the physiological and pharmacological effects of high-dose oxygen as well as from the mechanical effects of pressure. HBOT is explained in several physical laws[13].

Dalton's law describes the pressure of an individual gas in a mixture. Whereas ambient air normally consists of 21% oxygen, HBOT provides a supply of 100% oxygen. Because of Boyles law, which describes the relationship between pressure and concentration of a gas, the application of hyperbaric therapy increases the oxygen molecule density in each alveolus. This presents more oxygen molecules for diffusion across the alveolar-capillary interface and therefore into the blood. Graham's law, together with Fick's law describes the relationship of the pressure (concentration) of a gas and how it moves from one area to another. Oxygen diffusion from the alveolus to the capillaries increases, as the difference in concentration between both areas is bigger due to the pressure gradient. The same law of diffusion applies at tissue level and therefore makes it possible to let oxygen molecules diffuse much more further from the capillaries than normal[14-16]. At last, Henry's law relates the pressure of a gas

to how much of that gas can be dissolved in a liquid. 97% of a patient's hemoglobin will be saturated when breathing air at sea level with a very small amount of oxygen dissolved in the plasma. Breathing 100% oxygen at 1 ATA will completely saturate hemoglobin, usually around a paO2 of 200mmHg, and increase the amount of oxygen dissolved in the plasma. For each increase in absolute pressure more oxygen will dissolve in the plasma[1,6,13]. HBOT can reach arterial oxygen pressures of more than 2000 mmHg[6,17]. This increase in arterial oxygen tension following HBOT has been documented in several species but not yet in horses[18,19].

Therapeutical applications for use in humans
Based on the physics of hyperbaric oxygen therapy many therapeutic applications have been described in human medicine: HBOT is commonly used for decompression sickness, air embolism, tissue infection, impaired wound healing (such as diabetic feet, thermal burns, skin grafts and flaps), ischemia and reperfusion disease[5,7,9]. Another list of less frequent indications are; neurologic disease and head trauma, bone repair and refractory osteomyelitis, crush injury, radiation necrosis, blood loss and carbon monoxide toxicity[5,7,9,14,17,20,21].

Proven physiological and pharmological effects
Hyperbaric oxygen (HBO) has been shown to have effects on immunity, oxygen and cellular metabolism. A higher oxygen content in the cell causes a pathway of actions which can be subdivided in better wound healing and post-ischaemic tissue survival [22].

Therefore it is stated that HBOT mainly acts through the decrease of hypoperfusion and ischemia (hypoxia) [23,24]. Both processes are contributed to decreased wound healing by decreasing fibroblastic proliferation, lowering collagen production and impairing capillary angiogenesis. HBO has been proved to modify cytokines and therefore stimulates the production of growth factors, i.e. vascular endothelial growth factor (VEGF). It thus stimulates the development of capillary bedding within wound tissues, promotes cellular and fibroblast proliferation and accelerates collagen deposition[25-28].

HBOT also modulates the immune system response by reducing the neutrophil-endothelial adherence. The lowering of chemokine production by monocyte-macrophages prevents the tissues for creating more local inflammation and ischemia. Besides that, HBOT gives already present neutrophils an oxidative bust prohibiting more productions of reactive oxygen radicals (ROS) in damaged tissue[13,22].

It also reduces oedema caused by vasoconstriction, which contributes to the treatment of crush injuries and compartment syndromes. However, due to the hyperoxygenation of tissues HBOT does not cause a lack of oxygenation in the cells because of reduced blood flow[29].

On the other hand, HBO fights infections through a couple other pathways. In most cases HBO affects the bacteria because of the production of ROS, which can not be cleared by the bacteria because of a lack of production of antioxidants. This is mostly concerning anaerobic bacteria because they are not used being in a highly oxygenated area[30]. Second, it causes improvement of the oxygen-dependent transport across the bacterial membrane, which again leads to production of ROS in the bacteria[31]. Hyperbaric oxygen also maintains a better effect of antibiotics due to enhancing the inhibitory effects of growth of bacteria. HBO by itself could be bacteriostatic and in combination with antibiotics it causes a synergistic effect [32].

Side effects of Hyperbaric Oxygen Therapy

Hyperbaric oxygen and can generate either positive or negative effects depending on it's concentration and intracellular localisation[22]. Side effects occur due to an abnormal proportion of pressure and oxygen content. In the diving industry these effects are often categorized into direct and indirect affects. The direct effects mainly occur because of pressure and the indirect effects because of oxygen toxicity[33].

While HBOT does improve healing in vivo, is has been known for several decades to cause a toxic systemic reaction due to oxidative stress[33,34]. These indirect side effects are associated with high levels of oxygen, which give formation of reactive oxygen species (ROS) and thereby associated tissue reactions such as lipid peroxidation, protein and DNA oxidation and enzyme inactivation[35-37]. Oxygen toxicity is often manifested in either the central nervous system or the pulmonary system[1] and occasionally causes retinal detachment[13]. Central nervous system toxicity can occur in human at levels of 3 ATA for 2 hours in just one session and causes convulsions, nausea, dizziness, muscle twitching, anxiety and confusion due to grand mal seizures[1,13]. On the contrary, pulmonary toxicity often occurs after prolonged sessions of exposure to HBOT[1]. High levels of oxygen causes diffuse damage such as thickening of the alveolar membrane, interstitial and intra-alveolar oedema, impaired gas exchange and extensive infiltration by inflammatory cells causing coughing and dyspnoea[1,38,39]. Recent studies showed that HBOT induces lung damage secondary to an inflammatory response in rats[39]. Exposure to intermittent episodes of air would decrease the inflammatory response[40].

Direct side effects are due to barotrauma. In that case the body is not able to equalize the pressure. Side effects due to barotrauma are squeezes and blocks on the tympanic membrane, sinuses, intestines and dental fillings[1,13,33]. The direct side effects which cause pulmonary trauma occur when gas in the lungs is not able to escape adequately from the interstitium to the alveoli and vice versa due to (de)compression. This potentially causes subcutaneous emphysema, pneumothorax, pneumomediastium or air gas embolisms.

Aim of the study

The aim of our project is to determine the effects of hyperbaric oxygen therapy and to look for inflammatory aspects in the lungs of the normal equine athlete. With reports of hyperbaric oxygen (side) effects in humans and laboratory animals, research needs to be done in horses to evaluate the actual damage of its effects on lung function before its therapeutical indications can be recommended. The goal of this study was to assess the effects of hyperbaric oxygen therapy on lungs of healthy horses treated with HBOT and to measure arterial blood oxygen levels, to actual confirm hyperoxygenation of the blood.

Our hypotheses are; (1) Hyperbaric oxygen therapy induces a transient increase in arterial oxygen levels with no other effect on arterial blood parameters and (2) hyperbaric oxygen induces lung inflammation in healthy horses.

Therefore, the objectives of this study were 1) to measure actual arterial oxygen blood gas levels and other parameters, 2) to determine total and differential cell counts in bronchoalveolar lavage fluid (BALF), 3) to identify the most stable reference genes in the bronchoalveolar lavage fluid cells of horses treated with HBOT and 4) to evaluate if Th1 and Th2 inflammatory cytokines expression were increased in the BALF of the horses treated with HBOT.

Material and methods

This study was approved by the Animal Care Committee of the Health Science Centre at the University of Calgary. The authors used the REFLECT statement guidelines to report this study[41].

Horses
Both parts of the study included only thoroughbreds, from the same sex and age (age: 5-19, SD = 4,75) years of age. All horses were mares used for breeding.

Study design
The design for this study is a randomized controlled cross-over clinical trail. The sample size for the study was calculated to be eight horses (BAL neutrophils mean percentage is 3% in normal horses and 13% in mild inflammation cases, standard deviation of 7%, power of 80% and $p<0,05$). Inclusion factors for this study were; healthy horses evaluated from the physical examination, their recent history, normal cell blood count, chemistry analysis and baseline BAL cytology[42]. The horses were randomly (Microsoft ® Excel) split into two groups; HBOT (treatment) vs. control both including four animals.

Horses in the HBOT group were treated with hyperbaric oxygen at 3 ATA for 20 minutes in a horizontal hyperbaric chamber[a] for ten consecutive days, while the control group was only exposed to ambient air at atmospheric pressure into the chamber. After a wash out period of two months the study was repeated using the same horses and protocol. Treated horses were used for the control group and vice versa.

Sample collection
A bronchoalveolar lavage was performed at baseline, day 0, and at the end of the trail, day 10. The BAL was performed by using a standard protocol as described in a previous performed field study by Wasko et al., 2011[43]. Horses were sedated (0,6-1,0mg/kg xylazine (Rompun[b]) and 20-30ug/kg butorphanol (Torbugesic[c]) i.v.). The BAL was performed using a nine mm video-endoscope with 0,5% lidocaine (Lidocaine Neat[d]) which was instilled during progression through the airway. Two boluses of 250 ml sterile 0,9% sterile sodium chloride were instilled under vacuum pressure of 15 kPA into the bronchus when the endoscope was wedged. Bronchoalveolar lavage fluid (BALF) was collected and its volume was recorded using a 2 times 250 ml plastic Nalgene jar[e].

5 ml of BALF was placed in a Vacutainer EDTA tube[f] and used for analysis regarding the total and differential cell counts. The BALF was stored at ice for transport and analysed at the laboratory.

Samples collected for PCR analysis were directly prepared on-site. Two aliquots of 50ml of fluid were centrifuged, 3450rpm for 10 minutes, and 1,5 ml of cell pellet was resuspended in collecting tubes with RNA protect[g]. Samples were stored them at -80 degrees Celcius.

Total and differential cell counts
We compared total and differential cell counts of the BALF of both HBOT horses and control horses. Total cell counts were counted manually by one person using a haemocytometer. All samples were blinded before counting. Differential cell counts were counted under a microscope at 100x magnification counting up to 400 nucleated

cells where results were read double blind. This was done after preparing microscope slides using a Cytospin 2[h] at 100 x 10 rpm for four minutes including 100 and 200µl of BAL fluid and an automatic stainer with a modified Wright Giemsa staining (Hema-tek 2000[e]).

RNA extraction and cDNA synthesis
To prepare the samples for PCR we first accessed RNA extraction and cDNA synthesis. The BALF samples were thawed on ice at room temperature. Followed by centrifuging for 5 minutes at 13,2 rpm whereas the supernatant was aspirated. The cells from the pellet were homogenized using a tissue rupture[i] (Omni Tissue Homogenizer TH).

RNA extraction was done using the RNeasy Mini Kit[g] followed by a cDNA synthesis which retro-transcribed the RNA samples into cDNA with OmniscriptRT[g] in combination with RNase-OUT[g] and Oligo(dT) primers[j]. This was all done according to a previous described study on performing quantitative real-time PCR (QPCR) of BALF of equine[44,45]. Both products were checked on quality using the Nanodrop ND-1000 spectrophotometer[k], by measuring the yield (ug/ul) and purity (optical density (OD):A260/A280 nm) of the extracted RNA and cDNA. We used a calculated amount of RNA of 500ng, which resulted in an approximal amount of 1000ug/ul cDNA and an OD of +/- 1,80.

Relative Quantitative Reverse Transcriptase PCR
The QPCR was performed using the Stratagene MX3005P[l]. Reactions had a final volume of 25ul and consisted 13ul PerfeCta TM SYBR®Green Supermix[m], 40nM forward and reverse primer[j], 2ul of cDNA and 7ul nuclease free water[j]. The PCR reaction performed an initial denaturation of 95 degrees for 5 minutes, than 45 cycles of denaturation (95 degrees for 1 minute), followed by an annealing time for 30 seconds, extension time of 30 seconds at 70 degrees finished by the melting curve from 60 to 95 degrees. Reactions were executed in triplicate and each cytokine was run on a separate plate with negative controls (2ul of sterile water[g]).

Specificity of the products was verified for each reaction with a dissociation (melting) curve analysis, which resulted in single product specific melting temperatures. In addition, a gel electrophoresis was done too. This resulted in a single product with the desired length.

Primerdesign
Primerdesign was done according to the same previous project as mentioned earlier[44,45]. The primer sequence of TNF-α was described in another project and done according to Giguere et al., 1999[46]. According to those previous projects, we assessed the expression of 4 reference genes and 10 target inflammatory genes (*table 1*). Reference genes were glyceraldehyde-3p-dehydrogenase (GAPDH), ribosomal protein L32 (RPL-32), hypoxanthine ribosyltransferase (HPRT) and succinate dehydrogenase complex subunit A (SDHA). The target genes response included a cytokine list of most interest, which were interleukin (IL)-12p35, TNF-α, IFN-ϒ, IL-4, IL-5, IL-6, IL-1B, IL-10 and chemo attractants Eotaxin-2 and IL-8. All sequences were designed by Primer3 software and were based on the equine species from the Ensembl database[44,45].

Gene	Oligo	Sequence	PCR products Size (bp)	Sequence Accession Number (s)
GAPDH[44]	Forward	GGTGAAGGTCGGAGTAAACG	106	AF157626
	Reverse	AATGAAGGGGTCATTGATGG		
HPRT[44]	Forward	AATTATGGACAGGACTGAACGG	121	AY372182
	Reverse	ATAATCCAGCAGGTCAGCAAAG		
RPL-32[44]	Forward	GGGAGCAATAAGAAAACGAAGC	138	CX594263
	Reverse	CTTGGAGGAGACATTGTGAGC		
SDHA[44]	Forward	GAGGAATGGTCTGGAATACTG	91	DQ402987
	Reverse	GCCTCTGCTCCATAAATCG		
IL-1B[45]	Forward	ACCATAAATCCCTGGTGCTG	179	D42147; U92481; D42165
	Reverse	CGTCCCACAAGACAGGTACA		
IL-4[45]	Forward	CCGAAGAACACAGATGGAAAGGA	151	L06010; AF035404
	Reverse	TCACAGTACAGCAGGTCCCGTTT		
IL-5[45]	Forward	AAACTGTCCAAGGGGATGCT	169	U91947
	Reverse	TCCGTTGTCCACTCAGTGTT		
IL-6[45]	Forward	AGCAAGGAGGTACTGGCAGA	173	U64794; AF005227; AF041975
	Reverse	CCTTTTCACCCTTGAACTCG		
IL-8[45]	Forward	CGCACTCCAAACCTTTCAAT	165	AY184956; AF062377
	Reverse	TCAAAAACGCCTGCACAATA		
IL-10[45]	Forward	ATCGATTTCTGCCCTGTGAA	174	U38200
	Reverse	CGTTCCCTAGGATGCTTCAG		
IL-12p35[45]	Forward	CATGAATGCCAAGCTGTTGA	185	Y11130
	Reverse	AGGCATGAAGAAGGATGCAG		
TNF-α[46]	Forward	CTTGTGCCTCAGCCTCTTCTCCTTC	215	M64087
	Reverse	TCTTGATGGCAGAGAGGAGGTTGAC		
IFN-Y[45]	Forward	CTATTACTGCCAGGCCGCGTT	q	U04050; D28520
	Reverse	TCCTCTTCCGCTTCCTCAGGTT		
Eotaxin-2[45]	Forward	CCTGAGAGCCGAGTGGTAAG	152	ENSECAT00000023737
	Reverse	TTCTTGGCAGCCAGATTCTT		

Table 1: *Primers information for candidate reference genes and target genes. Primer sequences were determined using primer3 software and Ensembl Genome Browser (EMBL accession numbers are indicated) according to previous projects (Beekman et al.,2011[44], Beekman et al., 2012[45], Giguere et al., 1999[46]). The sign 'q' indicates no specific information on this item from previous project.*

Lin-RegPCR and ΔCt-method

Before using the actual data analysis software data needed to be corrected for differences in efficiency between PCR reactions. Therefore a method was used which was previously described in a project of Beekman et al., 2011[44]. The raw RT-QPCR amplification data were exported from the MxPro® software (Stratagene) to Microsoft® Excel. Lin-RegPCR, the software used for correction in efficiency, is a freeware that calculates a straight line though the PCR data set by correcting the non-baseline data to an equal baseline for each sample. This is determined by a linear regression analysis whereas the efficiency of each PCR sample is calculated by the slope of the line[47]. The data of each triplicate were imported to Microsoft® Excel again for a ΔCt-method to convert the data to a linear scale. The average of each individual efficiency sample, out of the triplicates, was used for the gene expression level analysis (REST) and the comparison of candidate reference genes (NormFinder and Genorm^{plus}).

Genorm^{plus} and NormFinder for valdidation of reference gene

A data analysis for the validation of the reference genes was done with the use of two programs: NormFinder (Excel-sheet imported program) and Genorm^{plus}, a program integrated in qbase^{plus} (a software program designed for real-time PCR data-analysis).

NormFinder is based on a Microsoft® Excel imported ANOVA model, the software calculates a stability value for all reference genes tested. The stability value is calculated on the combination of estimate intra- and inter-group expression variations of the candidate genes. A high expression stability is determined trough a low stability value which indicates a low combine intra- and inter-group variation[48].

Genorm[plus] calculates the stability using a pairwise comparison model. The software calculates two values for optimal stability en normalisation of the data set. The parameter M is ranks the candidate reference genes according to their stability. A low M-value represents high expression stability. On the other hand, the calculated V-value indicates the optimal number of reference genes required for accurate normalisation. This part of the software calculates the pairwise variation between sequential normalization factors containing an increasing number of reference genes. If an added gene gives a larger variation it means that it has a significant effect and should be included for calculation of a reliable normalization factor. A V-value smaller than 0,15 is required for an accurate normalisation[49].

Relative expression software tool for cytokine gene expression
For the data-analysis on cytokine gene expression we used the relative expression software tool (REST), which is also described in a previous project[45]. REST calculates an expression ratio of the choses target genes compared to the expression of the choses reference genes. It uses a statistical randomization algorithm to calculate a significant difference in expression ratio in between two groups (control vs. HBO or HBO pre vs. HBO post).

Arterial blood gas parameters
In second part of the study, we investigated the arterial blood parameters before, during and after HBOT using four horses. Carotid arterial blood samples were taken for blood gas analysis at baseline and at day 10, right after treatment. Samples were taken with the use of ultrasound guidance. The blood gas analysis was done on-site right away with the portable blood gas analyser (iStat[n]. This analyser has been validated for research purposes[50].

For measuring blood parameters during HBO a new protocol was set up according to previous research in human[51]. The protocol was set up as followed; an arterial catheter was bought in the right arteria transversa faciei and blood was taken for a baseline level at normal atmospheric pressure. The horse was put in the hyperbaric oxygen chamber for a treatment session while blood samples where taken with the use of a long infusion line plugged trough the wall of the hyperbaric oxygen chamber. The chamber was checked for leaks before using this technique. Blood gas parameters were measured again with the help of the handheld iStat[n] blood gas analyser. Measurements were done at baseline, at 29,5 psi (3 ATA) and at 5, 10 and 20 minutes after this compression level was reached. Measurements during decompression started were taken at 15 (2 ATA) and 0 (1 ATA) psi. The final measurement was done ten minutes after the horse had been taken out of the chamber.

Statistical analysis
Statistical analysis (ANOVA) was done to test for significant differences in bronchoalveolar lavage fluid cytology parameter within groups (pre/post HBO), as well as between groups (control vs. HBO) at the baseline and day 10. Normality of distribution of data was tested by a Shapiro-Wilk normality test. Followed by parametric, Kruskal-Wallis All-Pairwise Comparisons Test, ANOVA for the total cell counts and a nonparametric analysis of variance (one-way ANOVA followed by a Bonferroni All-Pairwise Comparisons Test) to assess differences in differential cell counts.

The blood gas parameters variation over time was also tested using a one-way ANOVA for non-parametric data followed by a Bonferroni All-Pairwise Comparisons Test.

Both Genorm^{plus} and NormFinder are provided with their own statistical analysis for ranking a stability of reference genes. Also REST software uses it's own statistical analysis. All three software are mentioned before.

P-values <0,05 were considered significant throughout the study.

Results

Total and differential cell counts

BALF total cell counts were found to be significant different for pre- versus post treatment (p=0,0053, S.E.=18,976). In the HBO post group there was a decrease in total cell counts compared to the HBO pre and control group who did not received hyperbaric oxygen (*figure 1)*. Differential cell counts on BALF neutrophils showed a significant decrease for HBO pre horses versus HBO post and control (p= 0,0033, S.E.=1,1659) (*figure 2a*). However, BALF differential cell counts of all other types of inflammatory cells, such as lymphocytes, macrophages, mast cells and eosinophils (*figure 2b, 2c, 2d, 2e*), were not found to be significant different comparing HBO horses pre and post to the control pre and post.

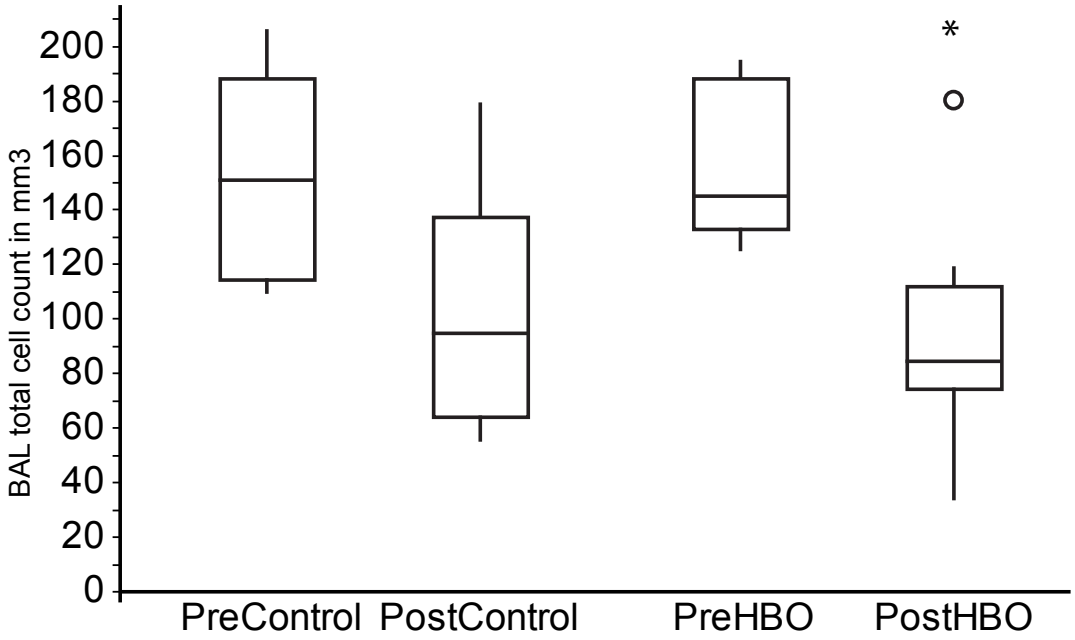

Figure 1: *Results for total cell counts in bronchoalveolar lavage fluid: Comparison of pre control and post control vs. pre HBOT and post HBOT. Amounts are given in cells per mm^3. The star dot indicates the group which is significant different from the others, the normal dot indicates a mild extreme value and the middle line indicates the median.*

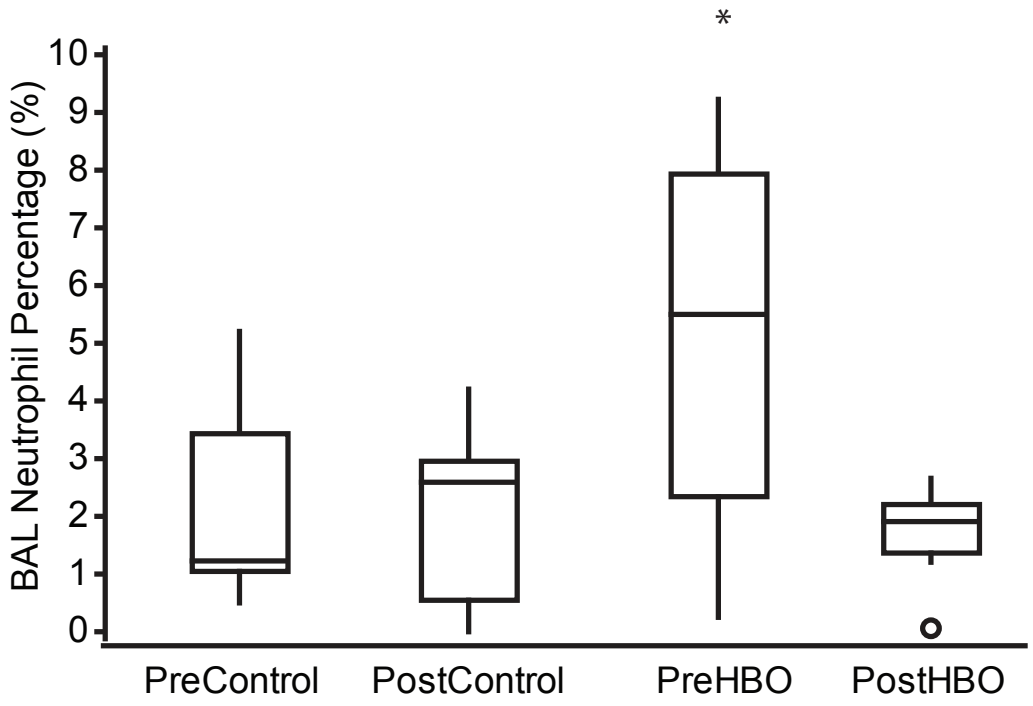

Figure 2a: *Results for differential cell counts of neutrophils in bronchoalveolar lavage fluid: Comparison of pre control and post control vs. pre HBOT and post HBOT. Amounts are given in percentages of the total amount of nucleated cells. The star dot indicates the group which is significant different from the others, the normal dot indicates a mild extreme value and the middle line indicates the median.*

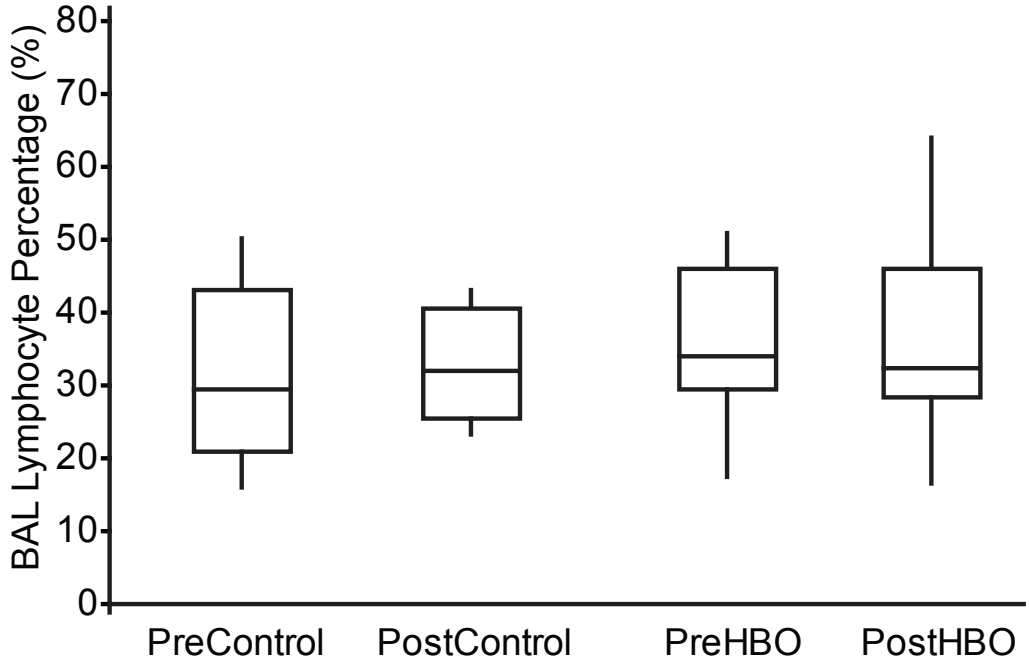

Figure 2b: *Results for differential cell counts of lymfocytes in bronchoalveolar lavage fluid: Comparison of pre control and post control vs. pre HBOT and post HBOT. Amounts are given in percentages of the total amount of nucleated cells. The middle line indicates the median.*

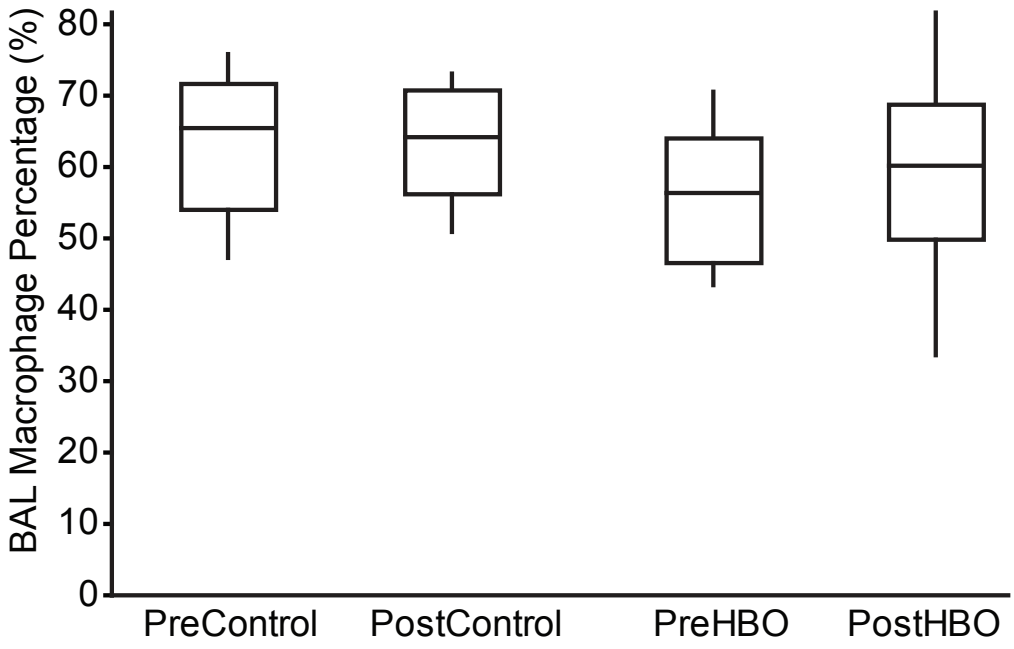

Figure 2c: *Results for differential cell counts of macrophages in bronchoalveolar lavage fluid: Comparison of pre control and post control vs. pre HBOT and post HBOT. Amounts are given in percentages of the total amount of nucleated cells. The middle line indicates the median.*

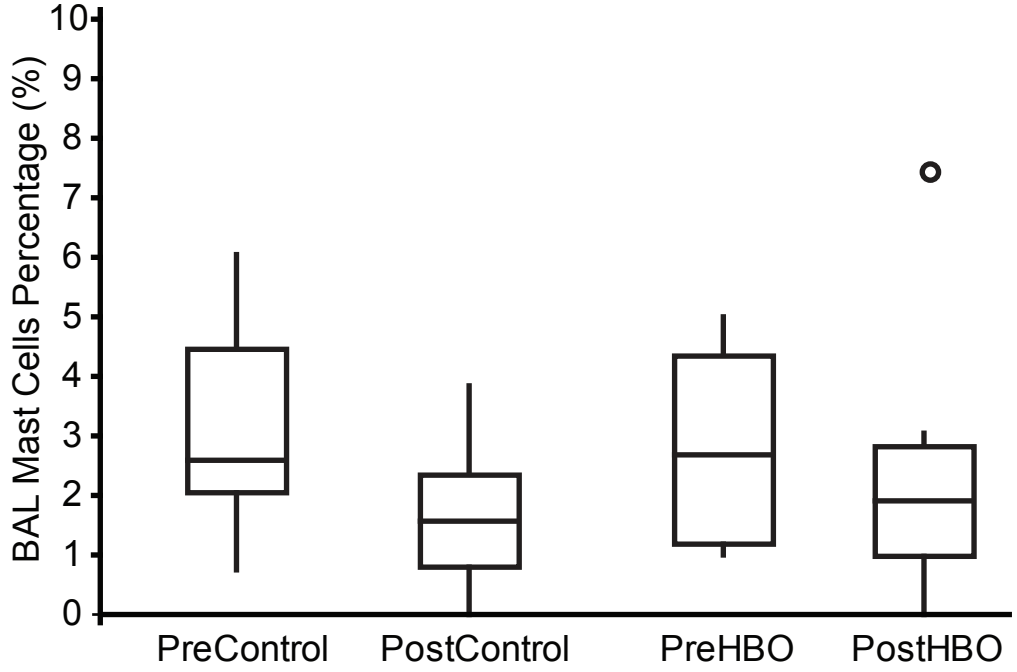

Figure 2d: *Results for differential cell counts of mast cells in bronchoalveolar lavage fluid: Comparison of pre control and post control vs. pre HBOT and post HBOT. Amounts are given in percentages of the total amount of nucleated cells. The normal dot indicates a mild extreme value and the middle line indicates the median.*

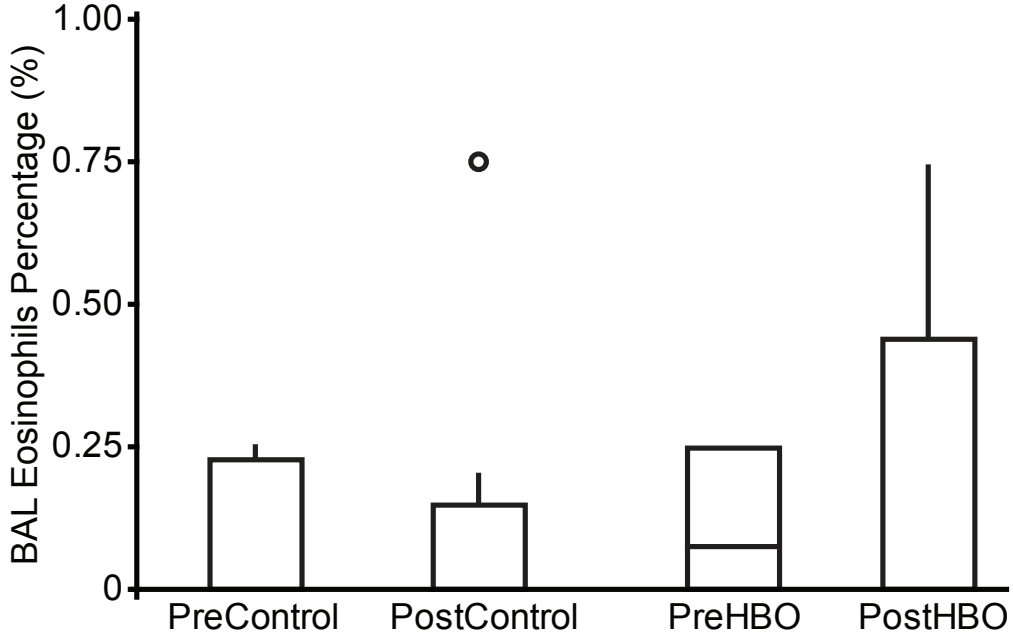

Figure 2e: *Results for differential cell counts of eosinophils in bronchoalveolar lavage fluid: Comparison of pre control and post control vs. pre HBOT and post HBOT. Amounts are given in percentages of the total amount of nucleated cells. The normal dot indicates a mild extreme value and the middle line indicates the median.*

Genorm^{plus} and NormFinder for valdidation of reference gene

As for the reference genes, GAPDH was found to be the most stable reference gene both using NormFinder and Genorm^{plus}. NormFinder calculated stability factors for GAPDH (stability value 1: 0,036), RPL-32 (stability value 1: 0,044), HPRT (stability value 1: 0,114) and SDHA (stability value 1: 0,198). With exclusion of SDHA in the NormFinder software we found a lower stability value for GAPDH (stability value 2: 0,032) and a much more equal value for HPRT (stability value 2: 0,081) and RPL-32 (stability value 2: 0,082) (*table 2*). Genorm^{plus} uses the same technique as NormFinder; a high stability value (M-value) refers to a low stability. Again GAPDH (M-value: 0,307) points out to be the best, whereas HPRT (M-value: 0,344) and RPL-32 (M-value: 0,361) are almost the same. SDHA (M-value: 0,547) is the least stable reference gene used in this calculation (*figure 3*).

Gene name	**Stability value 1:**	**Stability value 2:***
GAPDH	0,036	0,032
RPL-32	0,044	0,082
HPRT	0,114	0,081
SDHA	0,198	

Table 2: *NormFinder ranks the genes based on a calculated stability value. The lower the stability value, the higher the expression stability. Stability value 1 represents a calculation including all reference genes and Stability value 2, with the star dot, indicates a calculation with exclusion of SDHA.*

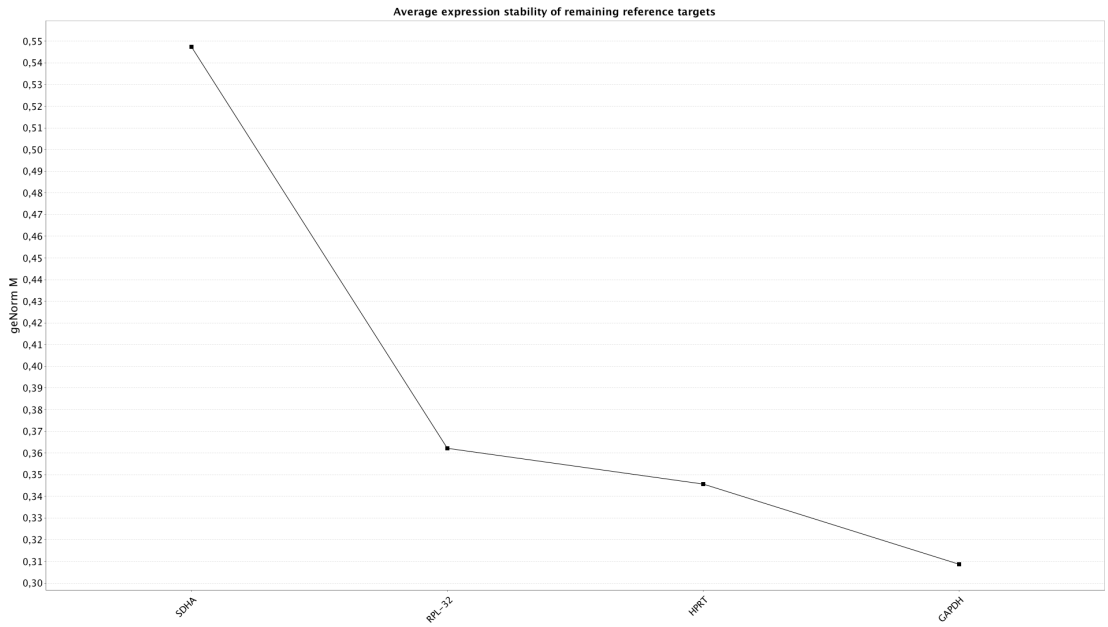

Figure 3: *Genorm^{plus} ranks candidate reference genes according to their M-value. The lower the M-value the more stable the reference gene.*

The reference genes used for optimal normalisation included GAPDH and HPRT according to Genorm^{plus}. Which means that the optimal number of reference targets in this experimental situation is two (GAPDH & HPRT) out of the three best selected reference genes (GAPDH, HPRT, RPL-32). This indicates a V-value of: 0,124. A good V-value is smaller than 0,15 when comparing a normalization factor based on the two or four most stable targets. As such, the optimal normalization factor can be calculated out of the geometric mean of reference genes. If we calculated a comparison where three (GAPDH, HPRT & RPL-32) out of four reference genes (GAPDH, RPL-32, HPRT, SDHA) were used for analysis we found a V-value of: 0,176 (*figure 4*).

For NormFinder the number of reference genes used for optimal normalisation included GAPDH and RPL-32 (stability value 3: 0,028). GAPDH and HPRT (stability value 3*: 0,044) were recommended as the best combination of two genes when SDHA was excluded from the software (*table 3*).

Best combination of reference genes:	**Stability value 3:**
GAPDH and RLP-32	0,028
GAPDH and HPRT*	0,044

Table 3 results: *NormFinder calculates the best combination of two genes for a two gene normalization factor. Stability value 3 represents a calculation out of all reference genes whereas the calculation with the star dot indicates an exclusion of SDHA.*

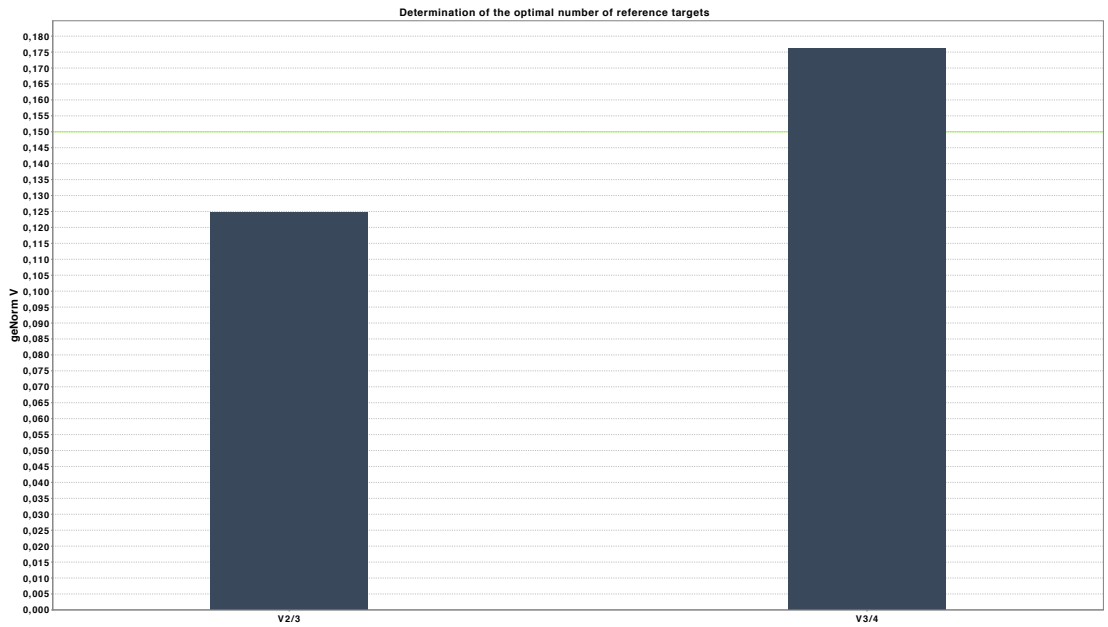

Figure 4 results: *Genorm[plus] calculates the normalization factor from an increasing number of genes (starting with at least two) for which the variable V defines the pairwaise variations between two sequential normalizations factors. V-values below 0.15 are suitable for a good data-analysis according to the Genorm[plus] software.*

Relative expression software tool for cytokine gene expression

We did a data-analysis on both GAPDH/RPL-32 and GAPDH/HPRT. The mRNA expression of almost all cytokines was not significantly up- or down- regulated with HBOT, however there was a significant down-regulation for Eotaxin-2 between the HBOT post and the control group post, both using GAPDH/RLP-32 and GAPDH/HPRT as reference genes. Eotaxin-2 is significantly down-regulated in the HBO post group in comparisons to the control post group (using GAPDH and HPRT as reference genes) by a mean factor of 0,873 (S.E. range is 0,743-1,029) and with a p-value of 0,033 (*figure 5*). However, when we used GAPDH and RPL-32 as reference genes we found a significant down-regulation of eotaxin-2 in the HBO post group compared to the control post group with a p-value of 0,024 by a mean factor of 0,868 (S.E. range is 0,743-1,006) (*figure 6*). Where GADPH and RPL-32 were used as reference genes there was also a significant down regulation of IL-4 with a mean factor of 0,891 between the HBO pre group and after the HBO post group (p= 0,046, S.E. range= 0,781-1,015) (*figure 7*).

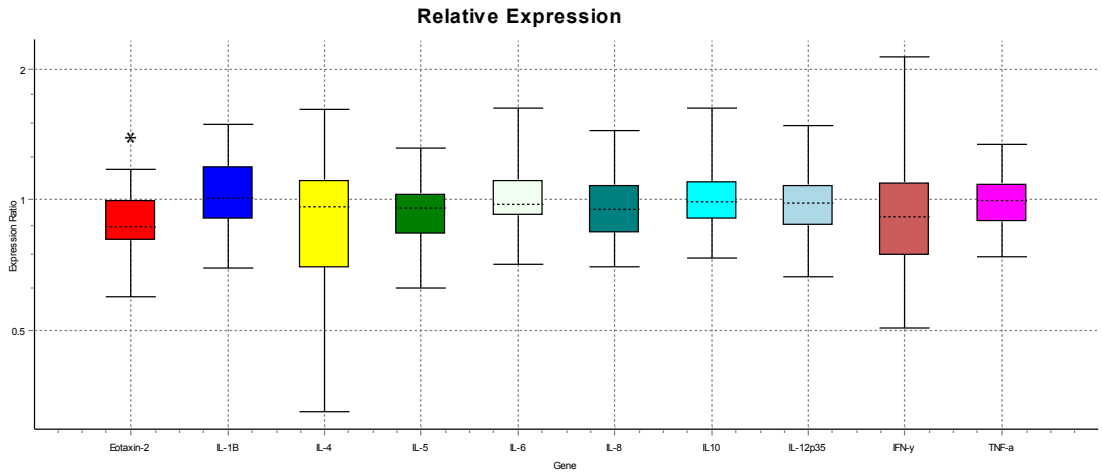

Figure 5: *REST calculates the difference in expression between HBOT post vs. control post compared to reference genes (GAPDH/HPRT) for inflammatory target genes. 1 means no change in expression, <1 means a down-regulation and >1 an up-regulation. Star dots mean significant difference.*

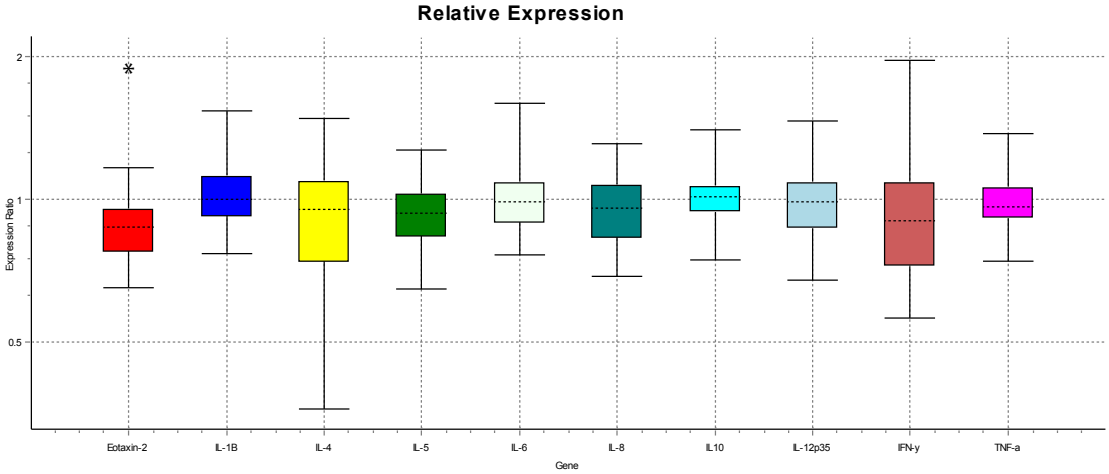

Figure 6: *Results for inflammatory target genes: REST calculates the difference in expression between HBOT post vs. control post compared to reference genes (GAPDH/RPL-32) for the inflammatory target genes. 1 means no change in expression, <1 means a down-regulation and >1 an up-regulation. Star dots mean significant difference.*

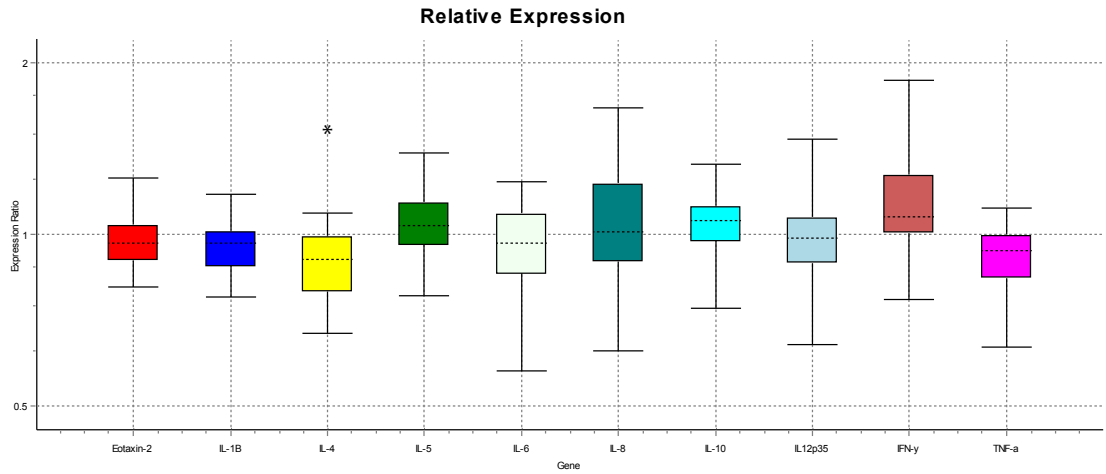

Figure 7: *Results for inflammatory target genes: REST calculates the difference in expression between HBOT pre vs. HBOT post compared to reference genes (GAPDH/RPL-32) for the inflammatory target genes. 1 means no change in expression, <1 means a down-regulation and >1 an up-regulation. Star dots mean significant difference.*

Arterial blood gas parameters

The oxygen pressure (paO_2) on arterial blood gases, measured right before and after HBO, was not found to be significant different. By the time the horse was out of the hyperbaric chamber and the arterial sample was taken, PaO_2 was back to baseline values. However, arterial oxygen levels measured during treatment were increased significantly. After 10 minutes, when the chamber was fully pressurized at 3 ATA, arterial oxygen pressure of all horses went over 800mmHg/l (above maximal range of the iStat analyser). Although within 10 minutes after treatment oxygen concentrations were already at baseline level (*figure 8a*). Another remarkable outcome of these arterial blood parameters was a rise in pH (*figure 8b*), a transient alkalosis. $PaCO_2$ (*figure 8c*) and HBO_3- (*figure 8d*) did not show any prominent changes despite a small increase.

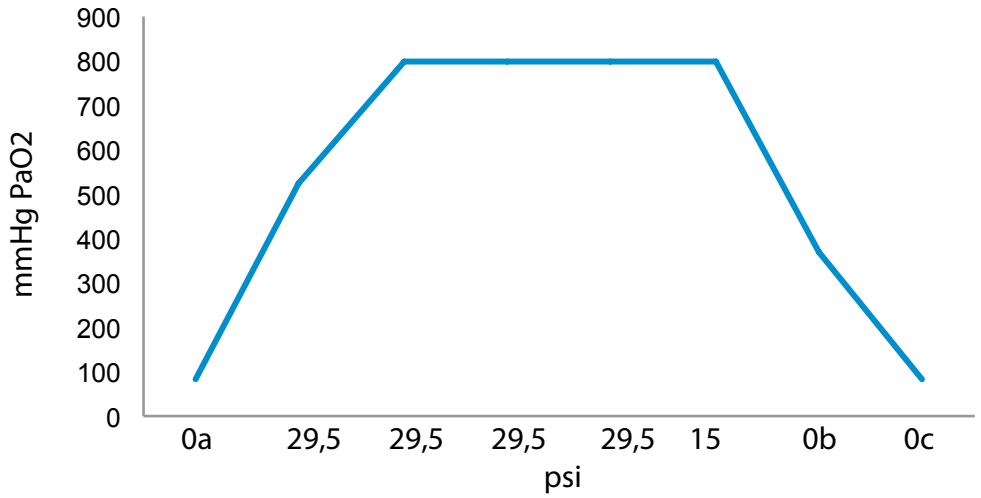

Figure 8a: *Results for arterial blood gas parameters during treatment: paO_2 given in mmHg. 0a baseline, 0b: baseline after decompression, 0c: 10 minutes after decompression.*

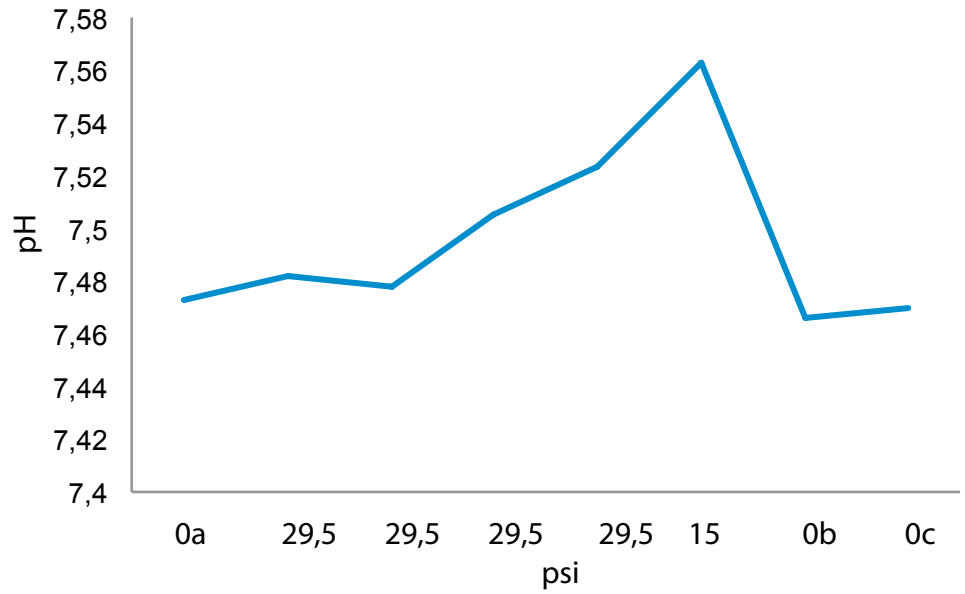

Figure 8c: *Results for arterial blood gas parameters during treatment: pH. 0a baseline, 0b: baseline after decompression, 0c: 10 minutes after decompression.*

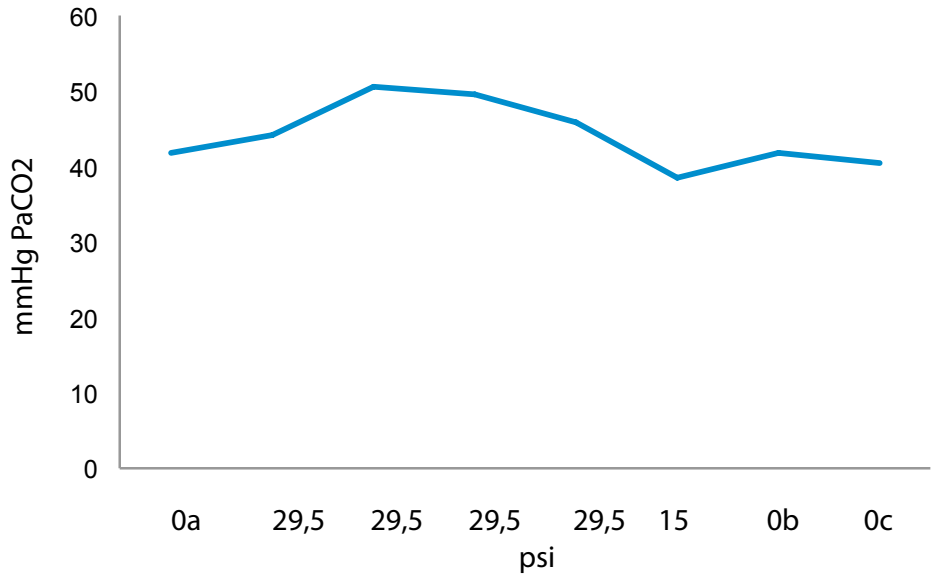

Figure 8b: *Results for arterial blood gas parameters during treatment: $paCO_2$ given in mmHg. 0a baseline, 0b: baseline after decompression, 0c: 10 minutes after decompression.*

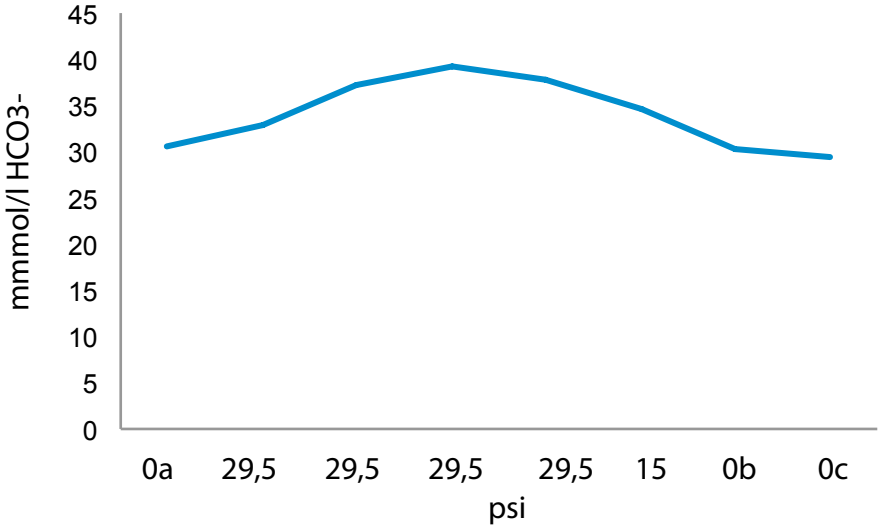

Figure 8d: *Results for arterial blood gas parameters during treatment: HCO_3^- given in mmol/l. 0a baseline, 0b: baseline after decompression, 0c: 10 minutes after decompression.*

Discussion

The aim of this study was to determine arterial oxygen levels and look for their possible toxic effect on the healthy equine lungs. We wanted to evaluate if total and differential cell counts and cytokine gene expression increase in BALF of horses after HBOT due to oxidative stress. Therefore, we also wanted to identify suitable reference genes in the bronchoalveolar lavage fluid of horses treated with HBOT. On the other hand, if there would be any signs of inflammation we also wanted to consider if there was an actual increase of paO_2 in the arterial blood gas parameters and see if we could link those findings. Several studies have showed that oxygen toxicity occurs in rats, mice and humans and gives lung damage secondary to an inflammatory response[10-12,33,39,40,52-54]. However, referring to our results, HBOT (as supplied to our protocol) does not induce any inflammatory reactions in the bronchoalveolar lavage fluids in healthy horses. In our study we found a decrease of total and differential (neutrophil) cell counts in the BALF of horses. In general HBOT did not influence cytokine gene expression in healthy horses in BALF samples except the down regulation of IL-4 and Eotaxin-2. This despite of a significant arise of PaO_2 arterial blood gas levels.

We were unable to ascribe specific consequences to most of the changes in total cell counts, neutrophil cell counts and cytokine expression reported here. However referring to our results, HBOT, as supplied to our protocol, led to a significant decrease of total and neutrophil cell counts and a down regulation of IL-4 and Eotaxin-2. This indicated no specific signs of inflammation, which is also found in other studies, showing attenuation of cytokines due to HBOT[55-58]. Several treatment protocols were made on the use of equine HBOT and their supply to several diseases (Hagyard Equine Medical Institute). Unfortunately there is a lack of information on protocols and therefore a treatment period of 20 minutes for 10 days consecutive was, if there would be any inflammatory reaction, likely to be long enough. Other studies showed pulmonary and central nervous system toxicity at 1,5 ATA[59] and severe lung oedema due to oxygen toxicity at 200 to 400 minutes exposure at 4 ATA[60]. Lung damage is due to oxygen toxicity; pure high pressure has no significant effect on lung damage. Although recent studies showed that oxidative parameters appear to be directly proportional to the extent of HBO exposure, indicating pressure has influence in combination with high oxygen levels[54]. Demchenko et al. suggests that pulmonary damage is much worse at higher pressure in a shorter period of time but pulmonary inflammatory response is less[39], which means lung damage is an accumulation of damage according to the duration and the amount of HBOT sessions consecutive. On the contrary, cerebral toxicity is due to long term exposure to oxygen and pressure in just one session[1]. Plafkli et al. assured that oxygen toxicity of the lung is either due to very intense or prolonged courses[33]. Since our study was a continued exposure for 10 days consecutive and most of the human and rodent studies show oxygen toxicity after already several days of treatment results are likely to be accurate.

We used the QPCR method with relative quantification and chose reference genes according to previous studies performed[44]. Multiple studies have performed validation on reference genes for normal equine skin and equine sarcoids, peripheral blood mononuclear cells or BALF in horses with either AID or RAO but never on the use of HBOT[44,61-64]. If we want to measure gene expression again in BALF of horses treated with hyperbaric oxygen, a good combination of references genes would be either GAPDH and RPL-32 or GAPDH and HPRT. Stability of used reference genes have to be validated because variations in expression level between experimental conditions can alter results[65]. Results of Normfinder and Genorm^{plus} are not identical,

as the algorithms are completely different. However, this demonstrates that the results are almost similar, meaning that both algorithms label the worst genes as unstable, and the best genes as stable.

In addition, as another important methodological precaution, we used efficiency correction of the PCR reactions (LinregPCR) [47]. Reaction efficiency varies among samples and therefore small differences in PCR efficiency could have affected conclusions of this study[66]. The software for determining cytokine gene expression, REST, has been proposed for the ratio of expression. This is seen as a great advantage because of it's ability to access the significant of these changes due to a statistical analysis[6768].

Since direct quantification of the equine cytokines is not currently possible with existing commercially available reagents, we elected to access gene expression of these cytokines indirectly by measurement of mRNA using real-time quantitative PCR. One of the limitations of our approach is the assumption that mRNA levels directly reflect those of the biologically active cytokine. Until monoclonal antibodies that enable quantification of equine cytokines become available and allow for correlations to be made between mRNA concentrations and protein levels, caution should be used as to not over-interpret results.

Measuring antioxidant status, reactive oxygen species (ROS), would also have been an option to determine our hypotheses, since a lot of research is already performed on human and laboratory animals[8,10,39,40,52,54,69-71]. Measuring those parameters is not very common in equine research. A lack of information and validation of tests kept us from this option. Future options on this should point out significant changes in antioxidant status and ROS concentrations and therefore oxidative stress after prolonged periods of HBOT. This after accurate validation and normalisation of equine antioxidant status and ROS concentration measured in BALF.

Another option would have been measuring gene expression of growth factors as showed in a previous study performed by Kang et al. [28]. Growth factor are seen as important mediators in tissue repair and could be of influence in decreased inflammation[22]. However, assays were made from human skin so design and validation of tests on equine bronchoalveolar lavage cells are future investigations.

While a better approach to measure lung inflammation in our study would also have been using immunohistochemistry instead of measuring total and differential cell counts in BALF[39,54], the fact that the horses are used for breeding and not for research precludes the use of the lung biopsy technique. In Plafki et al. pulmonary function tests were used on human determining effects of pulmonary oxygen toxicity although is was mainly used as a screening tool to detect patients at risk for pulmonary barotrauma[33]. In this study we used total cell counts as well as differential cell counts determining pulmonary inflammation because in equine medicine it is a well-appreciated and sensitive technique determining a possible inflammation reaction[43,72]. Other studies also used BALF for measuring results[39,70].

Although there was a significant rise of arterial oxygen levels, these were transient and dropped down quickly after treatment. The oxygenation of arterial blood did reach extremely high levels but no changes in concentrations were shown in horses comparing samples 10 minutes before and 10 minutes after treatment. This actually suggested that therapeutic effect of oxygen was only determined during the actual treatment session and therefore we should realize that each HBOT session is its own

treatment and after-therapeutical effects are unlikely to occur because oxygenation drops down so quickly. In comparison, Chavko et al. showed that intermittent air breaks are mechanisms of protection for pulmonary oxygen toxicity due to HBOT[40]. Unfortunately, damage made during treatment can occur a long period after the actual treatment session. Also the increase of pH in arterial blood gas parameters did not seem of clinical relevance since values are already decreased to baseline levels within a few minutes after decompression. This transient alkalosis could probably be due to a metabolic compensation of a short acting respiratory acidosis because of an increase of paCO2 due to an apnoea.

A new approach of this study could also be determining if HBOT would be useful in patients. Since there was a decrease of inflammation it would be a future option testing if equine recurrent airway obstruction and/or inflammatory airway disease would be manageable and therapeutical helpful with intermittent HBOT sessions. Up till now, human studies did not include any data on lung diseases linked to HBOT and since the equine is much more often a model for human lung diseases this study will be an ultimate option for human medicine too[73].

Conclusion

Concluding, hyperbaric oxygen treatment in equines, as supplied to our protocol, did not induce inflammation in the lungs. It even slightly decreased total cell counts and neutrophil differential cell counts on the BALF of healthy horses. Cytokine gene expression of IL-4 and Eotaxin-2 were also down regulated. For a reliable data-analysis, GAPD, HPRT and/or RPL-32 are suitable and stable reference genes used for a data set according to BALF in equine horses treated with HBOT. Arterial oxygen levels did increase rapidly during treatment but no high oxygen levels were measured within 10 minutes after treatment. It is likely to say there is no correlation between the transient increases of PaO_2 in arterial blood and the slight decrease of inflammation in bronchoalveolar lavage fluids of equine lungs. Future investigations should point out if high oxygen levels are of therapeutical effect on equine lungs.

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Footnotes

a Equineox Technologies Limited, Maple Rigde, Canada

b Hayer, Toronto, Canada

c Fort Dodge, Guelph, Canada

d Wyeth Animal Health, Guelph, Canada

e Nalgene, Rochester, New York, USA

f Beckton-Dickinson, Franklin Lakes, New Jersey, USA

g Qiagen, Mississauga, Ontario, Canada

h Thermo Scientific, Waltham, Massachusetts, USA

i Onmi International, Kennesaw, GA, USA

j Invitrogen, Burlington, Ontario, Canada

k Thermescientific, Wilmington, USA

l Stratagene, La Jolla, CA, USA

m Quanta BioSciences, Gaithersburg, MD, USA

n Heska coporation, Fort Collins, Colorado, USA

Abbreviations

ATA: atmosphere absolute

BAL(F): Bronchoalveolar Lavage (Fluid)

GAPDH: Glyceraldehyde-3p-dehydrogenase

HPRT: Hypoxanthine ribosyltransferase

HBO(T): Hyperbaric oxygen (therapy)

IAD: Inflammatory airway disease

IL-: Interleukin-

REST: relative expression software tool

RPL-32: Ribosomal protein L32

ROS: reactive oxygen species

(RT-)QPCR: (reverse transcriptase) quantitative PCR

SDHA: Succinate dehydrogenase complex subunit A