Original Study

Journal of Veterinary Emergency and Critical Care **15**(1) 2005, pp 26–31

Determination of P_{50} for feline hemoglobin

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Abstract

Objective: The objective of this study was to determine the PO_2 at 50% hemoglobin oxygenation (P_{50}) of feline hemoglobin (Hb).
Design: Prospective *in vitro* laboratory study.
Setting: Research laboratory.
Animals: Blood from 10 healthy cats.
Interventions: Individual blood samples were equilibrated with calibrated gases of 95, 21, 8, 5, 4, and 2.5% oxygen for tonometric analysis.
Measurements: Partial pressure of oxygen (PO_2), oxygen content, oxyhemoglobin saturation, methemoglobin (MetHb), carboxyhemoglobin (COHb), Hb, packed cell volume, hydrogen ion concentration (pH), and partial pressure of carbon dioxide (PCO_2) were measured in duplicate for each blood sample by tonometry. The P_{50} was calculated from both PO_2/oxyhemoglobin saturation and PO_2/oxygen content (per gram of Hb) curves.
Main results: The P_{50} from the PO_2/oxyhemoglobin saturation curve was 35.6 mmHg and from the PO_2/oxygen content (per gram of Hb) curve was 36.2 mmHg.
Conclusions: The oxyhemoglobin dissociation curve for the cat is shifted to the right, and thus, feline Hb has lower oxygen affinity compared with human and canine Hb.

(J Vet Emerg Crit Care 2005; 15(1): 26–31)

Keywords: carboxyhemoglobin, hemoglobin dissociation, methemoglobin, oxyhemoglobin, oxygen content, tonometry

Introduction

The oxyhemoglobin dissociation curve is a graphic representation of the relationship between hemoglobin (Hb) oxygen saturation (SO_2) or oxygen content (Cont O_2), and the partial pressure of oxygen (PO_2). The normal curve is usually established under standard conditions of: hydrogen ion concentration (pH 7.4), carbon dioxide partial pressure (PCO_2 40 mmHg), temperature (37 °C), and normal 2,3-diphosphoglycerate (2,3-DPG) levels. The P_{50} is the PO_2 at which the Hb is 50% saturated and is a commonly used value to define whether the curve is shifted. A lower-than-normal P_{50} value is termed a left shift and represents an increase in Hb affinity for oxygen. A higher-than-normal P_{50} value is termed a right shift and represents a decrease in Hb affinity for oxygen.

Both SO_2 and PO_2 are not usually measured simultaneously, but rather one (usually PO_2) is measured and the other is derived, using a normal oxyhemoglobin dissociation curve. Many modern blood gas analyzers and oximeters calculate SO_2 and Cont O_2 from PO_2 utilizing a normal human oxyhemoglobin dissociation curve. Accurate derivation of either value from the other depends upon a normal oxyhemoglobin dissociation curve in the patient at the time of the measurement. To the extent that the feline oxyhemoglobin dissociation curve is different from that of humans, these calculations will be erroneous. For instance, if the curve is shifted to the right, the SO_2 for any given PO_2 would be lower than that which would be predicted using the human oxyhemoglobin dissociation curve, or the PO_2, for any given SO_2 would be higher. The opposite would be true with a leftward shift of the oxyhemoglobin curve.

The oxyhemoglobin dissociation curve is shifted by conditions that alter the affinity of Hb for oxygen. Hypothermia, hypocapnia, alkalosis, a decreased intraerythrocytic 2,3-DPG or adenosine triphosphate (ATP), carboxyhemoglobinemia, and methemoglobinemia increase Hb affinity for oxygen and shift the curve to the left. This left shift facilitates oxygen loading in the lungs but impedes oxygen unloading in the tissues. Capillary PO_2 and oxygen driving pressure would, therefore,

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be reduced. Hyperthermia, hypercapnia, acidosis, increased intra-erythrocytic 2,3-DPG or ATP decrease hemoglobin affinity and shifts the curve to the right. This right shift impedes hemoglobin oxygenation in the lungs but aids release of oxygen to the tissues and will increase the capillary PO_2, if the animal is not hypoxemic. Hypoxemia, of course, also diminishes driving pressure at the tissue level and an arterial PO_2 below about 38 mmHg would offset any improvement in oxygen off-loading by a right-shifted curve.[1]

The P_{50} for humans has been reported as 26.8,[2] 29.2,[3] 26.0,[4] and 27.1 mmHg[5] and for the dog as 29.1 [3,4] and 31.5 mmHg.[3,4,6] The oxyhemoglobin saturation curve for the cat has been reported to be shifted to the right, with a P_{50} of 36.3[3] and 36.4 mmHg.[4] This would have important implications with regard to extrapolation of SO_2 or Cont O_2 from PO_2, or vice versa; for interpretation of pulse oximeter measurements, for calculation of oxygen content, and for the use of the calculated SO_2 parameters on printouts from blood gas analyzers in clinical feline patients.

The goal of this study was to measure the P_{50} of hemoglobin in normal cats and to compare these findings with those previously reported.

Materials and Methods

The project was approved by the campus Animal Care and Use Committee. Thirty milliliters of blood were collected from each of 10 clinically healthy, domestic cats. Eight cats came from a cat colony and 2 cats from private homes. Heparin was used as the anticoagulant. The individual blood samples were placed in a tonometer[a] immediately after collection. The water bath in the tonometer was maintained at 37 °C throughout the experiment. The pH of the blood was maintained between 7.36 and 7.44 by the addition of sodium bicarbonate.[b] After each addition of sodium bicarbonate, the blood sample was equilibrated in the tonometer for 60 minutes before any measurements were made. The blood sample was placed in a tonometer with a calibrated gas mixture of 95% oxygen and 5% carbon dioxide for 60 minutes to achieve a PO_2 of at least 500 mmHg. After equilibration, a 2 mL blood sample was removed and the following measurements were made: (1) oxygen content;[c] (2) carboxyhemoglobin percentage (%COHb);[d] (3) methemoglobin percentage (% MetHb);[d] (4) pH and blood gases;[e] (5) hemoglobin concentration;[f] and (6) packed cell volume (PCV).[g] All measurements were then repeated. The blood samples were then successively mixed in the tonometer with a calibrated gas containing 21% oxygen to achieve a PO_2 of about 150 mmHg, 8% oxygen to achieve a PO_2 of about 60 mmHg, 5% oxygen to achieve a PO_2 of about 40 mmHg, 4% oxygen to achieve a PO_2 of about 30 mmHg, and 2.5% oxygen to achieve a PO_2 of about 20 mmHg. The gas mixtures all contained 5% carbon dioxide with the balance being nitrogen. The measurements were performed in duplicate.

Data Analysis

The mean and standard deviation were calculated for all subjects for each parameter at each measurement. The acid base values were analyzed by the repeated measures analysis of variance. If a significant difference was detected, the Bonferroni (all-pairs) multiple-comparison test was used to locate the significant differences.[h]

Oxygen content per gram of hemoglobin (Cont O_2/Hb) was calculated by subtracting the dissolved oxygen, calculated as $0.003 \times PO_2$, from the measured whole blood oxygen content, and then dividing that number by the measured grams of hemoglobin. PO_2 *versus* hemoglobin saturation and PO_2 *versus* oxygen content per gram of hemoglobin plots were constructed.[h] The log $(SO_2/1 - SO_2)$ *versus* log PO_2 and the oxygen content per gram of hemoglobin *versus* log PO_2 were plotted for all cats for the determination of the P_{50}.[I] A linear regression was plotted for each data set and the P_{50} was calculated. In order to statistically compare the P_{50} determined from saturation and content, the P_{50} was then calculated for each individual cat and a two-tailed, paired *t*-test was used to compare the P_{50} values.

Results

Acid–base values were maintained within the prescribed ranges (Table 1). There were no statistically significant changes over the course of the experiment, except for bicarbonate anion (HCO_3).

The results for PO_2 and Cont O_2 indexed to the respective hemoglobin saturation and SO_2 are provided in Table 2.

The functional hemoglobin saturation, which is the ratio of hemoglobin saturated with oxygen (oxyhemoglobin) to the total hemoglobin available for binding with oxygen (which excludes methemoglobin and carboxyhemoglobin) was used. The fractional hemoglobin saturation is the ratio of hemoglobin bound with oxygen compared with the total hemoglobin (including methemoglobin and carboxyhemoglobin). Hemoglobin saturations calculated by the blood gas analyzer, using the human oxygen–hemoglobin saturation curve, are provided in Table 2.

Two hemoglobin saturation curves were generated. The plot of PO_2 *versus* hemoglobin saturation curve is shown in Figure 1. The plot of PO_2 *versus* oxygen content indexed per gram of hemoglobin is shown in

Table 1: Acid–base variables from 10 feline blood samples equilibrated with 6 different oxygen concentrations

Oxygen concentrations analyzed with tonometry (%)						
Parameter	**95**	**21**	**8**	**5**	**4**	**2.5**
pH	7.41 ± 0.03	7.41 ± 0.03	7.39 ± 0.02	7.38 ± 0.03	7.39 ± 0.03	7.38 ± 0.03
PCO_2	39.5 ± 1.9	42.0 ± 2.3	40.8 ± 1.4	42.0 ± 4.4	41.3 ± 1.7	41.8 ± 1.5
BD	0.1 ± 2	1.6 ± 2	− 0.6 ± 1	− 0.6 ± 2	0.1 ± 2	− 0.4 ± 3
HCO_3	24.7 ± 1.8[b,c]	26.0 ± 1.8[b]	23.7 ± 0.9[a,c]	23.6 ± 1.6[a,c]	23.5 ± 2.1[a,c]	22.4 ± 2.3[a]

Values are reported as mean ± 1 standard deviation, pH, PCO_2 (partial pressure of carbon dioxide; mmHg), BD (Base deficit; mEq/L), HCO_3 (bicarbonate; mEq/L). Values with no superscripts were not statistically different. Values with superscripts in common are not significantly different. Values with superscripts not in common are significantly different.

Table 2: PO_2, oxygen content, oxyhemoglobin, methemoglobin and carboxyhemoglobin concentrations from 10 feline blood samples equilibrated with calibrated gases of 6 different oxygen concentrations

	Oxygen concentration analyzed with tonometry (%)					
Parameter	**95**	**21**	**8**	**5**	**4**	**2.5**
PO_2	621 ± 34	151 ± 10	60 ± 2	41 ± 3	31 ± 1	22 ± 1
Cont O_2m	21.1 ± 2.5	21.0 ± 3.0	15.6 ± 1.7	10.2 ± 1.4	7.6 ± 1.9	3.7 ± 0.7
Hb	14.9 ± 1.6	14.6 ± 2.0	14.1 ± 1.3	12.2 ± 1.4	14.8 ± 2.8	14.3 ± 2.4
Cont O_2m/Hb	1.39 ± 0.1	1.36 ± 0.1	1.1 ± 0.04	8.5 ± 0.1	0.51 ± 0.1	0.26 ± 0.04
$\%SO_2$m	102 ± 1.3	100 ± 1.4	83 ± 1.5	58 ± 5.0	37 ± 2.4	22 ± 3.0
$\%SO_2$ calc	100 ±	99 ± 0.2	90 ± 3.0	72 ± 3.4	55 ± 3.3	34 ± 2.7
%MetHb	2.0 ± 0.9	2.0 ± 0.8	2.3 ± 0.7	2.6 ± 0.7	2.4 ± 0.6	2.4 ± 0.7
%COHb	9.0 ± 0.8	8.9 ± 2.3	6.8 ± 1.1	4.9 ± 1.3	3.6 ± 1.5	1.7 ± 1.1
PCV	42 ± 3.4	42 ± 4.7	41 ± 3.8	34 ± 3.4	40 ± 5.8	41 ± 6.9
Hb/PCV ratio	2.6 ± 0.6	2.9 ± 0.2	2.9 ± 0.1	2.8 ± 0.2	2.7 ± 0.2	2.8 ± 0.1

Values are reported as mean ± 1 standard deviation, PO_2 (partial pressure of oxygen; mmHg), Cont O_2m (measured oxygen content; mL/dL), Hb (hemoglobin concentration; g/dL), Cont O_2m/Hb (oxygen content per gram of hemoglobin), $\%SO_2$m (measured oxygen saturation; %), $\%SO_2$ calc (calculated oxyhemoglobin saturation; %), %MetHb (methemoglobin percentage; %), %COHb (percent carboxyhemoglobin; %), PCV (packed cell volume; %), Hb/PCV ratio (hemoglobin packed cell volume ratio).
The SO_2 calc was oxyhemoglobin saturation calculated from a normal human oxyhemoglobin dissociation curve.

Figure 2. The P_{50} value derived from 50% hemoglobin saturation was 35.6 mmHg. The P_{50} value using half of the maximum oxygen content per gram of hemoglobin was 36.2 mmHg. These values were not statistically significantly different.

Methemoglobin measurements varied between 2.0 ± 0.9 at the beginning of the trial and 2.4 ± 0.7 at the end of the trial (Table 2). Carboxyhemoglobin was measured to be 9.0 ± 0.8 at the beginning of the trial and decreased to 1.7 ± 1.1 at the end of the trial (Table 2).

Hemoglobin and PCV values are reported in Table 2. The Hb-to-PCV ratio varied between 3.6 and 2.7 for each measurement and averaged 2.78 over the entire experiment.

Discussion

The pH and PCO_2 were maintained within prescribed limits and were not statistically different over the course of the experiment. The bicarbonate values did exhibit statistically significant variations, however, this was not considered to be important since the actual numerical variance was small, and the pH and PCO_2 did not change.

The P_{50} of feline hemoglobin obtained from the hemoglobin saturation curve and from the oxygen content per gram of hemoglobin curve was estimated to be 35.6 and 36.2 mmHg, respectively. Those values were very similar to previous investigations reporting P_{50} values of 36.3[3] and 36.4 mmHg.[4]

Hemolysates of hemoglobin, when dialyzed to remove all effector molecules, which have an influence on the affinity of hemoglobin for oxygen, have a high oxygen affinity. Most mammalian red blood cells (RBCs) contain large amounts of 2,3-DPG (4–2 mmol of 2,3-DPG/mL of RBCs[7]) as the primary effector molecule of hemoglobin. Hemolysates of human hemoglobin have a P_{50} of about 5 mmHg.[7] Normal amounts of 2,3-DPG, CO_2, and chloride (Cl^-) decrease oxy-

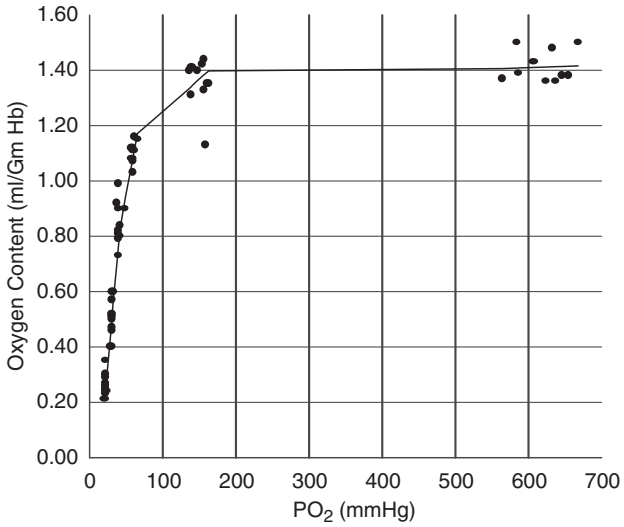

Figure 1: Partial pressure of oxygen (PO_2) *versus* measured hemoglobin saturation from 10 feline blood samples analyzed with tonometry with 6 different oxygen concentrations.

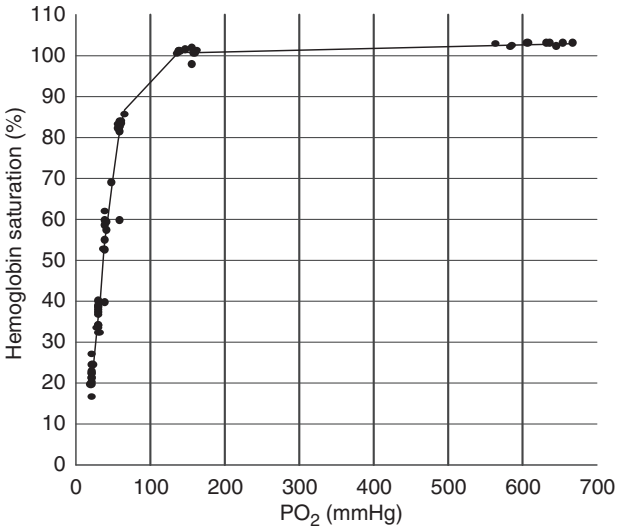

Figure 2: Partial pressure of oxygen (PO_2) *versus* oxygen content/g of hemoglobin from 10 feline blood samples analyzed with tonometry with 6 different oxygen concentrations.

hemoglobin affinity, thus shifting the oxyhemoglobin dissociation curve to the right. The 2,3-DPG binds to the beta (β) chain of hemoglobin, resulting in a conformational change, which reduces Hb affinity for oxygen. In chronic anemia, for example, increases in 2,3-DPG further decrease oxyhemoglobin affinity and shift the curve further to the right. Decreases in 2,3-DPG occur with storage of blood, increasing oxyhemoglobin affinity and shifting the curve to the left.

A unique feature of normal feline RBC is that they contain only small amounts of 2,3-DPG, averaging about 0.5–1.0 μmol/mL of RBCs;[8] 2,3-DPG increases to 2.78 ± 0.39 μmol/mL of RBCs in the anemic cat,[7] but remains far below normal concentrations for other mammalian species.

Hemoglobin hemolysates from species with low intra-erythrocytic 2,3-DPG have an oxygen affinity, which is considerably lower compared with species with a normally high intra-erythrocytic 2,3-DPG. Hemolysates of feline hemoglobin have a P_{50} of 15 mmHg, which is threefold higher than human hemoglobin, but still not high enough to facilitate oxygen unloading to the tissues. Furthermore, hemoglobin affinity in these species is only minimally affected by the addition of 2,3-DPG.[9] These species depend upon chloride as the major effector molecule of the affinity of hemoglobin for oxygen. Chloride, which is a weak effector molecule in the human RBC, is very important in feline oxyhemoglobin affinity.

The feline family has 2 major hemoglobin components, HbA and HbB, which are present in different proportions in different individuals[8,2] (1/1, 2.3/1, and 9/1[8]). These 2 components differ only with respect to their β chains.[10] HbA is 2,3-DPG responsive, although this interaction is relatively weak *in vitro*. HbB is completely unaffected by 2,3-DPG.[2,8] There is no difference in 2,3-DPG or ATP concentrations in the RBCs of cats with different proportions of HbA/HbB.[8,2] The different phenotypes of feline Hb also exhibit the same oxyhemoglobin dissociation curve.[8,2]

Even with the 2,3-DPG concentrations increasing within RBCs of anemic cats, the P_{50} increased only marginally, suggesting a minimal shift of the oxyhemoglobin disscociation curve. However, Taketa[8] also reported a shift to the right in the upper part of the curve and a shift to the left in the lower part of the curve. This represents an interesting perspective because it implies a change in the shape of the oxyhemoglobin dissociation curve such that perhaps greater amounts of oxygen can still be unloaded to the tissues without a change in the P_{50}, the traditional way to characterize shifts in the oxyhemoglobin dissociation curve. The rightward shift of the upper part of the curve was attributed to the effect of the small increase in 2,3-DPG on HbA. The leftward shift of the lower part of the curve was attributed to a decrease in HbB and an increase in HbB_I and HbB_{II}, which have a relatively higher oxygen affinity and are not responsive to 2,3-DPG.

Chloride is the major regulator of oxygen affinity in the cat, cow and other ruminants.[11] Chloride and 2,3-DPG appear to compete for the same binding sites on deoxyhemoglobin. Chloride has a minimal effect on oxyhemoglobin affinity in species with high intra-erythrocytic 2,3-DPG, but acts as the primary regulator in species where the intracellular 2,3-DPG is low.

The oxygen content per gram of Hb at a PO_2 of 621 mmHg was 1.39 ± 0.06 mL/dL. When calculating oxygen content in human blood, it is common clinical practice to calculate Hb capacity as from 1.31–1.39 mL of oxygen/g of Hb.[5,1] Our data would support using the factor 1.39 to calculate oxygen content from PO_2 and Hb saturation in the cat.

Methemoglobin concentrations were measured to be approximately 2% and did not change over the course of the experiment. Methemoglobin is Hb in which the iron has been oxidized to the ferric (+3) form. Methemoglobin impairs the delivery of oxygen to the tissues by its inability to bind and, therefore, transport oxygen and by impairing the unloading of oxygen, shifting the oxyhemoglobin dissociation curve to the left.[12] Approximately 3% of Hb is oxidized to methemoglobin each day from spontaneous autoxidation of oxyhemoglobin and secondarily to oxidants produced in normal metabolic reactions.[13]

Methemoglobin usually accounts for less than 1% of total Hb because the formed methemoglobin is continuously reduced back to Hb by the reduced form of nicotinamide adenine dinucleotide (NADH)-methemoglobin reductase or the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) dehydrogenase.[13] Our values were slightly higher than has previously been reported for the cat.[14] It is possible that exposure to 95% oxygen in the initial trial in the present study accounted for some methemoglobin formation since methemoglobin production can be increased by oxidants. However, methemoglobin concentrations in blood samples from 3 cats from the same colony used in the experiment that had not been analyzed by tonometry were subsequently measured at 2.5%, so oxygen-induced oxidant damage is an unlikely explanation for our results. In the present experiment a co-oximeter[d] that was calibrated for human hemoglobin was used. As it was not possible to find a feline coefficient for this instrument, it is possible that the methemoglobin results could be attributed to analytical error. Subsequently, access was gained to a new analyzer[g] with the feline coefficients. Blood samples from 3 cats from the same colony as those used in the experiment were measured 4 times and the average methemoglobin concentration was $0.35 \pm 0.1\%$. Those results corresponded with previously published values of $0.33 \pm 0.2\%$.[16] It was concluded that the methemoglobin values reported here and obtained from the first co-oximeter, using the human coefficient, were erroneously high.

The molar absorptivity of feline Hb (for all species of hemoglobin: reduced hemoglobin, oxyhemoglobin, methemoglobin, and carboxyhemoglobin) is slightly different from those of humans. The use of species-specific constants is important for the accurate calculation of each type of hemoglobin from the measured light absorption.[15]

Carboxyhemoglobin concentrations were consistently high ($9 \pm 0.75\%$) at the beginning of the experiment. The carboxyhemoglobin concentrations progressively decreased to approximately 1.7% at the end of the experiment. Normal carboxyhemoglobin levels in humans range from 0 to 5%.[15] For smokers consuming 1 pack of cigarettes per day, levels can range from 6–10%.[16] Normal carboxyhemoglobin levels for the cat were previously reported to be $1.0 \pm 0.2\%$.[15] Eight of the cats in the present experiment were normal colony cats and 2 were healthy house pets, and all 10 had elevated carbon monoxide levels. One possible explanation was contamination of the first tank of calibrated gas with carbon monoxide, but blood samples from 3 cats from the same colony that had not been analyzed by tonometry had elevated values of $6.9 \pm 0.27\%$. Again, in the experiment reported here, a co-oximeter[d] calibrated for human hemoglobin was used. The feline coefficient for this instrument could not be found at the time of the experiment. Blood samples from 3 cats from the same colony were measured with the second co-oximeter,[g] which had the feline coefficient, and the average carboxyhemoglobin concentration was $6.97 \pm 0.57\%$. It was concluded that these cats truly had very high carboxyhemoglobin concentrations and that this was not a methodological or analytical error. This conclusion was supported by the finding that the carboxyhemoglobin concentration gradually decreased over the course of the experiment.

The carbon monoxide levels in the housing facility of the cat colony were measured and no carbon monoxide was detected. The only endogenous source of carbon monoxide is from the conversion of α-methene carbon to carbon monoxide generated by the metabolism of heme in the reticuloendothelial cells.[17] A fraction of the carbon monoxide is released via the respiratory tract. The measurement of carbon monoxide in the exhaled breath has, in fact, been proposed as a measure of the quantity of heme that is being catabolized in an individual.[17] No reasonable explanation was found to explain the high measured carboxyhemoglobin levels for the cats in this study.

Modern blood gas analyzers calculate the Hb saturation and Cont O_2 from PO_2 using an algorithm for normal human hemoglobin. These calculated values are not accurate for the cat. In the present study, while SO_2 values calculated by the blood gas analyzer were similar to those measured by the co-oximeter on the upper, flat portion of the oxyhemoglobin dissociation curve (SO_2 values near 100%), they were, as expected, considerably higher on the steep part of the curve (90

versus 83, 72 *versus* 58, 55 *versus* 37, and 34 *versus* 22). Likewise, the calculation of oxygen content in the cat using SO_2 values derived from the human oxyhemoglobin dissociation curve would also be inaccurate.

Guidelines for the clinical interpretation of the oxygen concentration measured by pulse oximeters (SpO_2) have been correlated to corresponding PO_2 values using the human oxyhemoglobin dissociation curve.[18] Since the oxyhemoglobin dissociation curve is shifted to the right in the cat, in comparison with the human and the dog, a given SpO_2 would be associated with a higher PO_2. The PO_2 values between 60 and 160 mmHg were not measured in this experiment and so clinically relevant SO_2/PO_2 or Cont O_2/PO_2 associations for the cat cannot be made within this range.

It is common practice to derive Hb concentration from PCV by dividing the PCV by 3. The cat has a smaller RBC diameter and mean corpuscular volume (MCV),[19] lower mean corpuscular hemoglobin concentration (MCHC),[19] and a similar MCH[19] compared with those in humans[20] or dogs[21] and one might have some concern as to whether this factor of 1/3 is appropriate for cats. Since both PCV and Hb were measured in this study, it was possible to calculate the PCV/Hb ratio. In the present study, the calculated ratio averaged 2.8. These data support the practice of using the factor of 1/3 for approximating Hb from PCV in cats.

In summary, this study confirmed that the oxyhemoglobin saturation curve in the cat is shifted to the right, compared with humans and dogs, with a P_{50} of approximately 36 mmHg. The aforementioned differences in feline Hb may have important implications with regard to interpreting calculated SO_2 and Cont O_2 values, which are based on the human oxyhemoglobin dissociation algorithms.

Footnotes

[a] Modified Instrumentation Laboratory 137, three-flask, rotating tonometer, Lexington, MA.
[b] Sodium Bicarbonate, 8.4% solution, Abbott, North Chicago, IL.
[c] LEX-O_2-CON-K, total blood oxygen content analyzer, Hospex, Chestnut Hill, MA.
[d] CO Oximeter IL 482, Instrumentation Laboratories, Lexington, MA.
[e] Rapid Lab 248 pH/Blood Gas Analyzer, Chiron Diagnostics, Halstead, Essex, UK.
[f] HemoCue, blood Hemoglobin Photometer, Hemocue Inc., Mission Viejo, CA.
[g] OSM™3 Hemooximeter, Radiometer Medical A/S, Copenhagen, Denmark.
[h] NCSS 2000, Statistical System for Windows, NCSS, Kaysville, UT.
[I] Microsoft Excel 2000, Microsoft Corporation.

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