Low non-carbonic buffer power amplifies acute respiratory acid-base disorders in septic patients: an in-vitro study

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Running head: Non-carbonic buffer power in sepsis

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Abstract

Rationale: Septic patients have typically reduced concentrations of hemoglobin and albumin, the major components of non-carbonic buffer power (β). This could expose patients to high pH variations during acid-base disorders.

Objectives: To compare, in-vitro, non-carbonic β of septic patients with that of healthy volunteers, and evaluate its distinct components.

Methods: Whole blood and isolated plasma of 18 septic patients and 18 controls were equilibrated with different CO₂ mixtures. Blood gases, pH and electrolytes were measured. Non-carbonic β and non-carbonic β due to variations in Strong Ion Difference (βSID) were calculated for whole blood. Non-carbonic β and non-carbonic β normalized for albumin concentrations (βNORM) were calculated for isolated plasma. Representative values at pH=7.40 were compared. Albumin proteoforms were evaluated via two-dimensional electrophoresis.

Measurements and Main Results: Hemoglobin and albumin concentrations were significantly lower in septic patients. Septic patients had lower non-carbonic β both of whole blood (22.0±1.9 vs. 31.6±2.1 mmol/L, p<0.01) and plasma (0.5±1.0 vs. 3.7±0.8 mmol/L, p<0.01). Non-carbonic βSID was lower in patients (16.8±1.9 vs. 24.4±1.9 mmol/L, p<0.01) and strongly correlated with hemoglobin concentration (r=0.94, p<0.01). Non-carbonic βNORM was lower in patients (0.01 [-0.01 – 0.04] vs. 0.08 [0.06 – 0.09] mmol/g, p <0.01). Septic patients and controls showed different amounts of albumin proteoforms.

Conclusions: Septic patients are exposed to higher pH variations for any given change in CO₂ due to lower concentrations of non-carbonic buffers and, possibly, an altered buffering function of albumin. In both septic patients and healthy controls, electrolyte shifts are the major buffering mechanism during respiratory acid-base disorders.
Septic patients are poorly protected against acute respiratory acid-base derangements due to a lower non-carbonic buffer power, which is caused both by a reduction in the major non-carbonic buffers, *i.e.* hemoglobin and albumin, and by a reduced buffering capacity of albumin. Electrolyte shifts from and to the red blood cells determining acute variations in Strong Ion Difference are the major buffering mechanism during acute respiratory acid-base disorders.
Introduction

Sepsis is a life-threatening disease leading worldwide to 5 million yearly deaths (1, 2). Metabolic acidosis is common in sepsis and is a marker of severity (3, 4). Furthermore, about 40% of septic patients develop secondary respiratory failure and are therefore at risk of respiratory acidosis (5, 6).

In human physiology, two systems limit pH changes in acute acid-base perturbations: the carbonic (carbonic acid/bicarbonate) and non-carbonic buffers (mainly albumin and phosphates in plasma, with the addition of hemoglobin in whole blood) (7).

Both systems contribute to the acute compensation of metabolic derangements, e.g. in case of lactic acidosis both bicarbonate ions (HCO$_3^-$) and the dissociated part of non-carbonic buffers (A$^-$) will be reduced. On the contrary, during acute respiratory acid-base derangements only non-carbonic buffers minimize pH variations, as the change in carbonic acid/bicarbonate is the primary disorder (8). In this context, the ability of non-carbonic buffers to limit pH excursions has been termed as non-carbonic buffer power ($\beta$) and defined as the negative value of the variation in HCO$_3^-$ divided by the corresponding variation in pH, induced by acute changes in partial pressure of carbon dioxide (PCO$_2$) (7, 9-11). Simplifying, this means that, for any given variation in PCO$_2$, a patient with a lower non-carbonic $\beta$ will have greater pH variations as compared to a patient with higher values of non-carbonic $\beta$.

In order to effectively buffer respiratory acid-base derangements, and limit pH variations, non-carbonic buffers have to favor changes in HCO$_3^-$ consensual to the variations in PCO$_2$. According to the electrical neutrality principle, the total concentration of cations has to be equal to the total concentration of anions (12, 13). As a direct consequence, any increase in HCO$_3^-$ observed during acute hypercapnia must be accompanied by a reduction in negative charges and/or an increase in positive charges. This can be achieved in two ways. First, through a reduction in the dissociated part of total non-carbonic weak acids (A$^-$), such as proteins and phosphates, therefore
operating in the buffer base domain (14-16). Second, through a reduction in strong anions, mainly chloride (Cl\(^-\)) or an increase in strong cations, mainly sodium (Na\(^+\)), therefore operating in the Strong Ion Difference (SID) domain (12, 17, 18). The first mechanism is considered the main in isolated plasma. On the other hand, when dealing with a complex solution, such as whole blood, red blood cells gain a key role as they allow electrolytes shifts across their cellular membrane, leading to SID changes consensual to PCO\(_2\) variations (8, 19, 20).

During sepsis, patients are typically anemic (21) and hypoalbuminemic (22, 23), and have therefore reduced concentrations of non-carbonic buffers. Moreover, albumin is a macromolecule whose structure might vary significantly (24). Indeed, pathological conditions such as inflammation might favor the transition from reduced albumin to “oxidized” albumin (25). If and how these changes of protein structure might affect its buffering function is currently unknown.

As data regarding the non-carbonic \(\beta\) of septic patients are currently lacking, we decided to conduct this prospective, experimental in-vitro acid-base study. We hypothesized that septic patients’ non-carbonic \(\beta\) of whole blood and plasma would be significantly lower as compared to healthy volunteers, thus exposing patients to greater pH variations.
Materials and Methods

Study population

The study was approved by the ethical committee and registered at ClinicalTrials.gov (NCT03503214). Patients admitted to the intensive care unit for sepsis/septic shock (3) were enrolled. Age < 18 years and pregnancy were exclusion criteria. Healthy, age-matched volunteers were recruited for comparison. Informed or deferred informed consent was obtained.

Blood and plasma sample

In both groups, 25 milliliters of venous blood were collected (26, 27). Complete blood count, concentrations of magnesium, phosphate and albumin (Cobas c-702, Roche, Switzerland) were measured. Six milliliters were placed in anti-foam syringes (T310 Syringes, RNA Medical, USA), i.e. plastic syringes pre-treated with an anti-foam material in order to prevent excessive foaming during tonometry. The remaining blood was centrifuged for 10 minutes at 4°C at 3000 rpm to isolate plasma. A sample was frozen at -85°C for subsequent biomolecular analyses.

Tonometry of blood and plasma

The whole blood sample was divided in four aliquots and immediately equilibrated at different CO₂ concentrations through tonometry (Equilibrator, RNA Medical, USA) (27-29). Four gas mixtures containing 2, 5, 12 or 20% of CO₂ with 21% of oxygen and nitrogen for the remaining percentage were used (15 minutes at 37°C). Thereafter, samples were analyzed for blood gases, pH and concentrations of sodium, potassium, calcium, chloride and lactate (ABL 800 FLEX Radiometer, Denmark). Samples in which lactate increased more than 1 mmol/L as compared to baseline values, and samples in which hemoglobin saturation for oxygen was below 94% were discarded. The same procedure was performed on isolated plasma.
**Definitions and calculations**

For each whole blood sample the actual SID obtained at every CO₂ concentration was calculated as:

\[
[SID] = [Na^+] + [K^+] + 2 \times [Ca^{2+}] - [Cl^-] - [lactate^-] \quad \text{[1]}
\]

where \(Na^+, K^+, Ca^{2+}, Cl^-\) and \(\text{Lactate}^-\) refer to plasma sodium, potassium, ionized calcium, chloride and lactate concentrations (expressed in mmol/L) measured with the point-of-care blood gas analyzer. Magnesium was not used for the calculation, as it was measured only in the central laboratory on baseline venous blood. The actual \(HCO_3^-\) concentration of each sample was calculated by applying known values for the solubility of CO₂ in plasma \((S = 0.0307 \text{ mmol/(L·mm Hg)}) (30)\) and the negative logarithm of the first apparent equilibrium dissociation constant of carbonic acid \((pK'_1 = 6.095 \text{ for whole blood and } pK'_1 = 6.105 \text{ for isolated plasma}) (31)\):

\[
[HCO_3^-] = S \cdot PCO_2 \cdot 10^{(pH - pK'_1)} \quad \text{[2]}
\]

where \(PCO_2 = \text{partial pressure of carbon dioxide (expressed in mm Hg)}\) and measured with the point-of-care blood gas analyzer and \(pH = \text{pH measured with the point-of-care blood gas analyzer}\).

Variations of \(HCO_3^-\) and SID over pH in whole blood and plasma were modeled according to a polynomial multilevel model (32) in order to obtain overall \(HCO_3^-/pH\) and \(SID/pH\) curves for the two study groups and individual best fits for all subjects/patients.

Non-carbonic \(\beta\) was defined as \(-\Delta HCO_3^-/\Delta pH\) resulting from a variation in \(PCO_2\) (7). Non-carbonic \(\beta\) is therefore the opposite of the first derivative of the \(HCO_3^-/pH\) curve:

\[
\text{Non-carbonic } \beta = -\frac{d[HCO_3^-]}{dpH} \quad \text{[3]}
\]

Individual non-carbonic \(\beta\) curves were used to obtain representative non-carbonic \(\beta\) values at a pH of 7.40 \((\beta_{7.40})\).

The same analysis was performed substituting SID to \(HCO_3^-\) in order to quantify the contribution of the electrolyte shifts to total non-carbonic \(\beta\) in whole blood:
\[ \beta_{\text{SID}} = -\frac{d\text{SID}}{dpH} \]  

Individual values of non-carbonic $\beta$ of isolated plasma obtained at pH=7.40 were divided by the albumin concentration in order to normalize for different albumin concentrations and investigate the molecular buffering function:

\[ \beta_{\text{NORM}} = \frac{\beta_{7.40}}{\text{Albumin} \ [g/L]} \]  

**Polynomial multilevel model**

Variations of dependent variables over pH were modeled according to a polynomial multilevel model (general linear mixed models) with random intercept at subject level and random slope at pH level. Statistical analyses were performed as previously described (29) and carried out by SAS 9.4 statistical package. The exponential power of pH was decided according to model-based likelihood ratio tests (cut-off $P$-value $\leq 0.100$). Interaction between the independent variables, pH and subjects’ group (controls and patients), was also included according to model-based likelihood ratio tests.

**Two-dimensional electrophoresis (2-DE)**

Total proteins in plasma samples were quantified and separated by two-dimensional electrophoresis (2-DE). The total protein content in plasma samples was assessed by the bicinchoninic acid (BCA) protein assay (EuroClone). After normalization based on albumin concentration (assessed by monodimensional gel electrophoresis), the same amount (10 µg) of proteins per sample was separated by 2-DE (33). Briefly, plasma samples were diluted in 2.5% dithiothreitol (DTT) /5% sodium dodecyl sulfate (SDS) and denatured at 95°C for 5 minutes. Then, they were diluted (250 µl) in UTC buffer (7M urea, 2M thiourea, 4% CHAPS) added with 0.04% ASB-14, 5% glycerol, 0.2% Tween-20, 100 mM DTT and 2% IPG buffer pH 4-7 (GE Healthcare). Samples were loaded on 13 cm Immobiline™ DryStrip pH 4-7 (GE Healthcare) and isoelectric focusing was performed...
with an Ettan™ IPGphor II system (Amersham Biosciences). Strips were equilibrated in 50 mM Tris-HCl pH 8.8, 36% urea w/v, 2% SDS w/v, 67% glycerol v/v, bromophenol blue added with 1% DTT (30 minutes incubation) and then with 2.5% IAA (30 minutes). Second dimension (based on MW) was then performed on 12.5% polyacrylamide gels by SDS-PAGE. Gels were stained with ProteinStain Fluo-R (SERVA) and acquired with a GelDoc-It™310 Imaging System (UVP), at four different apertures (4.0, 5.6, 8.0 and 11).

Albumin proteoforms were identified, aligned and quantified using the ImageJ software. Briefly, a linear background correction was applied (10 pixels rolling ball, sliding paraboloids) and all images were aligned based on the main albumin protein spot (Registration plugin, alignment by line ROI). Then, signals intensity was calculated for all albumin spots. The integrated density values of each albumin proteoforms were normalized based on the sum of the intensities of all albumin spots per sample. Results were expressed as mean±SEM in the two groups (Controls vs. Patients).

Statistical analysis
Data are expressed as mean±SD unless otherwise specified. Baseline data from patients and controls were compared via t test or Mann-Whitney rank sum test, as appropriate. Different albumin proteoforms were compared via two-tailed t-test, applying Benjamini-Hochberg correction for multiple testing (FDR<0.05 as threshold). Pearson’s correlation coefficient was employed to assess the degree of linear relationship between two variables. Analysis was performed with SAS 9.4 (SAS Institute Inc., USA). A P value <0.05 was considered statistically significant.
Results

Eighteen healthy controls and 18 septic patients were enrolled. Age (52 [47 – 60] vs. 58 [47 – 74] years p=0.32), body mass index (23.4 [20.9 – 25.1] vs. 23.5 [20.8 – 26.0] kg/m², p=0.54) and gender (8 (44%) vs. 5 (28%) n (%) of females, p=0.49) of healthy volunteers did not differ from those of patients with sepsis. Septic patients had a SOFA score of 9 ± 2 points; eleven (61%) had septic shock and were on vasopressors at the time of study. Infection sites were the lung in 9 (50%), abdomen in 5 (28%), genitourinary tract in 3 (17%) and “other” in 1 (5%) case. Twelve patients (67%) where mechanically ventilated, 3 (17%) where supported also by extracorporeal membrane oxygenation and 2 (11%) where undergoing continuous renal replacement therapy. Overall mortality of the studied population was 6 (33%). Patients received a median amount of 100 [0 – 300] ml of commercial 20% albumin (Albital, Kedrion, Italy) before the study time.

Baseline laboratory data and results of blood gas analysis performed on whole blood at 5% of CO₂ are reported in Table 1. Of note, among non-carbonic buffers, hemoglobin and albumin concentrations were significantly lower in septic patients (p<0.01), while phosphate concentrations were similar in the two groups. Values of SID, Base Excess (BE) and HCO₃⁻ were significantly lower in septic patients, while lactate concentration had a significantly higher value in this population.

Non-carbonic β of whole blood

Tonometry of whole blood with 2, 5, 12 or 20% of CO₂ was performed to study in-vitro the capacity of whole blood to resist to respiratory acid-base disorders. The experiments allowed to explore Pco₂ values between 13 and 138 mm Hg, with a resulting pH range of 6.83 to 7.81. As expected, the increase in Pco₂ caused a decrease in pH and an increase in Hco₃⁻ (Figure E1, Panel A and B, https://doi.org/10.6084/m9.figshare.14398211.v1). Consequently, there was a negative relationship between Hco₃⁻ and pH. Individual pH and Hco₃⁻ values resulting from the equilibration process in
whole blood of healthy controls and septic patients are reported in Figure 1A and 1B. Overall HCO₃⁻/pH curves for whole blood of the two study populations obtained by the polynomial multilevel model are reported in Figure E1, Panel C. The resulting non-carbonic β/pH curves of the populations, for the explored pH range, are two straight lines with equal slope and different intercept (p<0.01, see Table E1, https://doi.org/10.6084/m9.figshare.14398250.v1), resulting in lower absolute β values in septic patients (Figure E1, Panel D).

Non-carbonic β of isolated plasma

Subsequently, to exclude red blood cells from the system, tonometry with 2, 5, 12 or 20% of CO₂ was performed on isolated plasma. This allowed to explore PCO₂ values between 16 and 130 mm Hg, with a resulting pH range of 6.73 to 7.90. Individual experimental points of PCO₂ and the resulting pH and HCO₃⁻ values for isolated plasma are reported in Figure E2, Panels A and B (https://doi.org/10.6084/m9.figshare.14398226.v1). Absolute pH and HCO₃⁻ values of the study populations resulting from the equilibration process in isolated plasma are reported in Figure 2A and 2B. Overall HCO₃⁻/pH curves for isolated plasma of the two study populations obtained by the polynomial multilevel model, are reported in Figure E2, Panel C. As for whole blood, the resulting non-carbonic β/pH curves of the study populations, for the explored pH range, are two straight lines with equal slope and different intercept (p<0.01, see Table E2, https://doi.org/10.6084/m9.figshare.14398247.v1), resulting in lower absolute non-carbonic β values in septic patients (Figure E2, Panel D).

Non-carbonic β of whole blood and isolated plasma at pH of 7.40

To describe the capacity to resist to acid-base variations in normal conditions, absolute values of non-carbonic β of both whole blood and isolated plasma were calculated at a pH value of 7.40. Values of healthy volunteers were significantly higher than those of septic patients both for whole
blood (31.6±2.1 vs. 22.0±1.9 mmol/L, p<0.01) and isolated plasma (3.7±0.8 vs. 0.5±1.0 mmol/L, p<0.01), as shown in Figure 3. Of note, for both patients and controls, isolated plasma had significantly lower values of non-carbonic β as compared to whole blood (p<0.01, for both).

Buffering mechanisms

a. Whole blood

The mechanisms underlying the buffering capacity of whole blood where then investigated and changes in sodium, chloride and SID consequent to the applied PCO₂ were assessed. When passing from the lowest (around 20 mm Hg) to the highest PCO₂ (around 120 mm Hg), a significant increase in sodium concentration (5±1 mmol/L vs. 4±1 mmol/L, p<0.01, in healthy volunteers and septic patients, respectively) and reduction in chloride concentration (7±1 mmol/L and 5±1 mmol/L, p<0.01, in healthy volunteers and septic patients, respectively) was observed (Figure E3, Panel A and B, https://doi.org/10.6084/m9.figshare.14398244.v1). On the contrary, no significant variations in potassium were observed. Changes in sodium and chloride in the two populations, according to the pH changes resulting from CO₂-tonometry are provided in Figure E4 (https://doi.org/10.6084/m9.figshare.14398238.v2 ). As a result, a significant increase in SID (13.5±1.2 mEq/L and 9.8±1.4 mEq/L, p<0.01, in healthy volunteers and septic patients, respectively) was observed with the maximum PCO₂ variation (Figure 4A and 4B).

The contribution of electrolyte shifts to total non-carbonic β of blood (non-carbonic βSID) was then computed. Overall SID/pH curves for the two study groups are reported in Figure E3, Panel C. The resulting function describing non-carbonic βSID in the explored pH range was a horizontal line (Figure E3, Panel D). According to this model (Table E3, https://doi.org/10.6084/m9.figshare.14398223.v1 ), βSID of whole blood is therefore independent from pH values. Furthermore, significantly lower values of βSID were observed in septic patients as compared to controls (16.8±1.9 vs. 24.4±1.9 mmol/L, p<0.01, Figure 5). A strong correlation
(r=0.94, p<0.01) between hemoglobin concentration and βSID was found when pooling data from both groups (Figure 6).

b. Isolated plasma

A possible correlation between albumin concentration and non-carbonic β of isolated plasma was investigated to understand the mechanisms underlying the buffering capacity of isolated plasma. A strong correlation (r=0.93, p<0.01) was observed when pooling data from both groups (Figure E5, https://doi.org/10.6084/m9.figshare.14720898.v1). In addition, the representative values obtained at pH = 7.40 of non-carbonic β of isolated plasma normalized for albumin concentration (βNORM) were compared in order to assess possible differences of albumin buffering capacity. Septic patients had significantly lower values of βNORM (0.01 [-0.01 – 0.04] vs. 0.08 [0.06 – 0.09] mmol/g, p <0.01) as compared to controls (Figure 7). In addition, a different amount of specific albumin proteoforms was observed by two-dimensional electrophoretic separation of plasma samples (Figure 8A). In particular, amongst the nine detected albumin proteoforms, in septic patients a significant increase (FDR<0.05) in the amount of the acidic ones was observed (spots 7 and 9), accompanied by a downward trend of the alkaline ones (spots 1, 2 and 3) (Figure 8B).
In the present study, respiratory acid-base derangements were induced \textit{in-vitro}. We simulated hypoventilation/hypercapnia by equilibrating our samples with gases containing high CO$_2$ concentrations. Moreover, hyperventilation/hypocapnia was simulated using gases containing a low CO$_2$ concentration. This allowed to measure the \textit{in-vitro} non-carbonic $\beta$, \textit{i.e.} the capacity of blood/plasma to limit pH changes secondary to acute PCO$_2$ variations, in septic patients and healthy volunteers. Our main finding is that septic patients’ capacity to buffer respiratory acid-base disorders is markedly reduced (approximately by 30%) as compared to healthy volunteers. This, of course, exposes this category of patients to more pronounced pH variations for any given respiratory and/or metabolic acid-base derangement. The reduced non-carbonic $\beta$ of septic patients is due to two major factors. First, and more importantly, this category of patients has lower concentrations of non-carbonic buffers. Indeed, both hemoglobin and albumin concentrations were significantly lower (Table 1). Second, it appears that septic patients’ albumin has a reduced buffering capacity. Indeed, when normalizing the non-carbonic $\beta$ of isolated plasma by the measured albumin concentration, we found significantly lower buffering capacity for every gram of plasma albumin (Figure 7). This finding suggests that the molar buffering capacity of the protein macromolecule might be altered by critical illness and/or by the administration of exogenous albumin. The difference observed in albumin proteoforms (Figure 8) supports this finding. In other words, these results suggest that the protein buffering system of septic patients might have different acid dissociation constants. In addition, it has been demonstrated that commercial albumin, frequently administered to our patients, has an altered red-ox state (41). However, how this red-ox state alteration affects albumin buffering capacity and its acid dissociation constant needs to be determined in further studies, properly designed to address this question.
In addition, the present study shed light on the physiologic and pathophysiologic mechanisms of buffering during acute respiratory acid-base derangements. In line with previous studies performed in the setting of metabolic acid-base disorders (34, 35), our data show, overall, that whole blood has a significantly higher buffering capacity as compared to isolated plasma (Figure 3). To understand the underlying mechanisms, we partitioned and quantified the two components of non-carbonic buffer power, i.e. i) changes in electrolytes induced by PCO$_2$ variations, resulting in variations in SID and ii) variations in the dissociation/association of plasma proteins.

We observed that electrolytes vary remarkably, when PCO$_2$ is changed acutely in whole blood. Indeed, when the PCO$_2$ of whole blood is increased through tonometry, chloride decreases significantly (Figure E3, Panel B). Moreover, a concomitant significant increase in sodium concentration was observed. Interestingly, similar results were reported by Giebisch in in-vivo experiments performed in dogs (8). When analyzing intracellular fluid, the authors did not find a reduced sodium concentration, suggesting that the increase in sodium observed during respiratory acidosis was not caused by a shift from the red blood cell. This difference between our results and the finding of Giebisch and colleagues could be explained by known differences between canine and human red blood cell physiology (36). Another factor potentially at play is the salt-type binding/unbinding of electrolytes to large proteins, which appears to be influenced by PCO$_2$ and pH (27, 37-39).

The result of the observed electrolyte shifts secondary to a primary increase in PCO$_2$ was an increase in SID up to 15 mmol/L (Figure 4). As an increase in SID favors an increase in HCO$_3^-$, and shifts the system towards alkalosis, it is clear that this mechanism of interdependence between PCO$_2$ and SID during respiratory acid-base disorders limits the resulting pH changes of whole blood (19). Of note, in the present study hemoglobin was always completely saturated with oxygen. The contribution of the Haldane effect, with the related chloride shift due to the transition of hemoglobin
from the deoxygenated tense (T-state) to the oxygenated relaxed (R-state), could thus be excluded as contributing factor to the observed electrolyte shifts (40, 41).

We therefore quantified the contribution of SID variations to the non-carbonic $\beta$ and called this term $\beta_{\text{SID}}$. The aim of this analysis was to quantify the changes in $\text{HCO}_3^-$ secondary to $\text{CO}_2$ variations determined by electrolyte shifts. It is worth underlining that the SID changes observed in whole blood samples were secondary to electrolyte shifts from and to the red blood cells, as no electrolytes were added or removed from the sample. Moreover, it is important to state that the $\beta_{\text{SID}}$ concept used in the present study differs significantly from other studies, in which $\text{PCO}_2$ was kept constant, SID was changed through the addition of strong acids or bases, and the resulting pH variation was assessed (42).

In both healthy volunteers and septic patients, we found that the electrolyte shift and therefore $\beta_{\text{SID}}$ is undoubtedly the most important buffering mechanism of blood, accounting for approximately 80% of the total non-carbonic buffering capacity (Figure 5).

Interestingly, the value of $\beta_{\text{SID}}$ was found to be, in the explored pH range, independent from pH (Figure E3, Panel D), i.e. in our model the “hemoglobin-red blood cell” buffering system does not seem to behave as a weak acid, but seems to have a fixed, pH-independent buffering effect.

Finally, we were able to demonstrate that the interindividual differences in $\text{PCO}_2$-induced SID variations (Figure 4) and therefore the differences in $\beta_{\text{SID}}$ were strongly correlated with hemoglobin concentration, a reasonable proxy of red blood cell intracellular volume (Figure 6).

When studying isolated plasma, we were able to exclude the buffering function of the “hemoglobin-red blood cell” system, and therefore focused our attention on a second physiologic mechanism, i.e. the variations in dissociation/association of plasma proteins (15, 16). Also in the experiments performed on isolated plasma, we found significant differences in the buffering capacities of healthy volunteers and septic patients (Figure 3). This finding is certainly explained,
at least in part, by a lower albumin concentration in septic patients (Table 1). Nevertheless, also when normalizing the non-carbonic $\beta$ for measured albumin (the major non-carbonic buffer of plasma) we found lower values in patients. This finding suggests that the molar buffering capacity of the protein macromolecule might be altered by critical illness and/or by the administration of exogenous albumin. The difference observed in albumin proteoforms (Figure 8) supports this finding. In other words, these results suggest that the protein buffering system of septic patients might have different acid dissociation constants. In addition, it has been demonstrated that commercial albumin, frequently administered to our patients, has an altered red-ox state (43). However, how this red-ox state alteration affects albumin buffering capacity and its acid dissociation constant needs to be determined in further studies, properly designed to address this question.

Clinical implications

Our study suggests that septic patients, due to lower buffer concentration and, possibly, an altered buffering function of albumin, are more exposed to acute acid-base derangements. In line with previous studies (44, 45), we clearly observed that the “hemoglobin-red blood cell” system is by far the most important buffering mechanism. It is thus clear that the red blood cell transfusion strategy (46, 47) will have a significant impact on the ability of blood to resist to acid-base perturbations, with a more liberal strategy being associated with higher non-carbonic buffer power and a more restrictive strategy with a lower capacity to resist to respiratory acid-base derangements. In addition, the in-vitro carbon dioxide titration curve that we performed clearly underlines the importance of tailoring nomograms to the specific clinical condition. Indeed, rules frequently applied in critical care to predict changes in $\text{HCO}_3^-$ following an acute hypercapnia (48) or hypocapnia (49) are derived from healthy volunteers or from healthy surgical patients, i.e. not from critically ill patients. In addition, the studies that derived these rules hardly discussed the buffering
role of the “hemoglobin-red blood cell” system, which, as previously observed (44, 45) and confirmed by our data, is by far the major non-carbonic buffer. Finally, it is important to mention that non-carbonic buffer power is employed in all equations used to calculate base excess (50-52). Many equations estimate the non-carbonic buffer power according to the hemoglobin concentration, other equations, such as the “Van Slyke equation” introduced by Siggaard-Andersen and suggested by the Clinical and Laboratory Standards Institute (CLSI) (31) assume a constant value of 16.2 mmol/L for the extracellular fluid (50). This value was derived from in-vivo experiments performed in 8 healthy subjects undergoing either voluntary hyperventilation or inhalation of CO₂ (53). The author thus derived the following equation: \( \beta = 2.3 \cdot \text{ctHb (Ecf)} - 7.7 \) mmol/L, which yields a value of -16.2 mmol/L for a blood hemoglobin concentration of 16 g/dL and a total protein concentration of 7 g/dL (53, 54). While changes in the non-carbonic buffer power have a small effect in the computation of Base Excess (50), our study describes the variability of whole blood non-carbonic buffer power in septic patients (range between 20 and 28 mmol/L), caused both by the variability of hemoglobin and albumin concentration, and by different acid-base characteristics (\( \beta_{\text{NORM}} \)) of albumin. These findings hence underline the importance of using personalized values in order to compute accurate base excess values.

**Limitations**

We need to mention some limitation of our study. The in-vitro nature of the study, while allowing to exclude confounding factors, such as intravenous fluid therapy (55-58), did exclude the interaction with the interstitium which is certainly clinically relevant. Brackett et al (48) suggest that the in-vivo non-carbonic \( \beta \) is lower than the equivalent in-vitro determination due to a higher volume of distribution (59). This aspect, which needs to be clarified in future clinical studies, might be exacerbated in critically ill patients, frequently characterized by an expanded extracellular volume. Another limitation of our study relies in the fact that we analyzed whole blood, i.e. the
“plasma-red blood cell system”. Our data therefore do not allow to draw conclusions on the "buffering" role of free hemoglobin. Moreover, we have no information on the intracellular effects of CO₂- tonometry. Finally, our data suggest that plasma proteins of septic patients might have a lower acid dissociation constant. However, in our experimental design we explored only 4 different partial pressures of CO₂ which did not allow us to determine experimentally the dissociation constant (27). Future studies are warranted to determine the acid dissociation constant of septic patients’ plasma proteins.

Conclusions

Septic patients, as compared to healthy volunteers, have a reduced non-carbonic β, which exposes this category of patients to more pronounced pH shifts for any given respiratory acid-base derangement. Moreover, the reduced non-carbonic β likely exposes these patients also to greater pH shifts during metabolic acid-base derangements. The “hemoglobin-red blood cell” system is by far the most important buffering mechanism and seems to be pH-independent, i.e. it does not behave as a classic buffer in the explored pH range. Indeed, this system limits pH changes through electrolyte shifts determining variations in SID, proportional to the variation in PCO₂. This finding, besides underlining the interdependence of PCO₂ and SID, suggests that the pathophysiology of acid-base equilibrium is far from being fully understood.
Acknowledgments

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Availability of data and materials

The complete dataset is available at the following link:

https://doi.org/10.6084/m9.figshare.14709609.v2
References


**FIGURE AND TABLE LEGEND**

**Figure 1. pH to bicarbonate relationship in whole blood.**
Experimental points of pH and HCO$_3^-$ concentration obtained through equilibration of whole blood at different PCO$_2$ values in healthy controls (Panel A) and septic patients (Panel B). Each subject/patient is identified by a different symbol.

**Figure 2. pH to bicarbonate relationship in isolated plasma**
Experimental points of pH and HCO$_3^-$ concentration obtained through equilibration of isolated plasma at different PCO$_2$ values in healthy controls (Panel A) and septic patients (Panel B). Each subject/patient is identified by a different symbol.

**Figure 3. Non-carbonic β at pH 7.40.**
Representative values at pH = 7.40 for non-carbonic buffer power (β) in healthy controls and septic patients for whole blood (left side of the graph) and isolated plasma (right side of the graph). Dots represent 5$^{th}$ and 95$^{th}$ percentiles.

**Figure 4. PCO$_2$ to SID relationship in whole blood**
Experimental points of PCO$_2$ and Strong Ion Difference (SID) obtained through equilibration of whole blood at different PCO$_2$ values in healthy controls (Panel A) and septic patients (Panel B). Each subject/patient is identified by a different symbol.

**Figure 5. Non-carbonic β due to SID variations in whole blood**
Values of the buffer component due to SID variation ($\beta_{SID}$) in whole blood of healthy controls and septic patients. This values are independent of the applied pH, as $\beta_{SID}$ did not change with pH in the explored PCO$_2$ range. Dots represent 5$^{th}$ and 95$^{th}$ percentiles.
**Figure 6. Hemoglobin to $\beta_{SID}$ variation relationship**

Relationship between hemoglobin and $\beta_{SID}$ in the whole study population. Black circles represent data from control subjects, while white circles represent data from septic patients. A strong correlation was found for the overall population. A similar finding was observed, when analyzing Patients ($r = 0.74$, $p < 0.01$) and Controls ($r = 0.61$, $p < 0.01$) separately.

**Figure 7. Normalized non-carbonic $\beta$ of plasma at pH = 7.40**

Representative values of non-carbonic $\beta$ of isolated plasma at pH = 7.40 normalized for albumin concentrations ($\beta_{\text{NORM}}$). Dots represent 5th and 95th percentiles.

**Figure 8. Albumin proteoforms.**

A) Representative map of plasma proteins after two-dimensional electrophoresis (2-DE). Proteins were separated horizontally by their isoelectric point (plus end: acidic; minus end: alkaline) and then vertically by their molecular weight (MW). Lane 1: protein marker. Lane 2: loading control (10 µg). Inset image: magnification of albumin proteoforms, identified by nine adjacent spots. B) Quantification of albumin proteoforms after 2-DE. *FDR<0.05.

**Table 1. Baseline laboratory values and blood gases and electrolytes obtained at 5% of CO$_2$.**

Baseline laboratory data for albumin, total proteins, phosphates and hemoglobin concentrations, and results of gas analysis performed on whole blood at 5% of CO$_2$. P values refer to t-test or Mann-Whitney rank sum test, as appropriate.
BLOOD

p < 0.01

35
30
25
20
15
10
5
0
-5

$\beta$ at pH = 7.40 [mmol/L]

Controls
Patients

PLASMA

p < 0.01

20
15
10
5
0
-5

Controls
Patients
$y = 1.90x - 2.86$
$r = 0.942$
$p = <0.0001$
B

Albumin proteoforms

![Image of gel electrophoresis with labeled spots and normalized intensity density graph showing controls and patients.](image-url)
<table>
<thead>
<tr>
<th></th>
<th>Controls (n=18)</th>
<th>Patients (n=18)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin [g/dL]</td>
<td>4.7 [4.6 – 4.9]</td>
<td>3.0 [2.8 – 3.2]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hemoglobin [g/dL]</td>
<td>14.3 ± 1.0</td>
<td>10.4 ± 0.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hematocrit [%]</td>
<td>41 [40 – 44]</td>
<td>30 [29 – 32]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Phosphate [mg/dL]</td>
<td>3.4 [2.9 – 3.5]</td>
<td>4.4 [2.8 – 5.3]</td>
<td>0.12</td>
</tr>
<tr>
<td>PCO2 [mm Hg]</td>
<td>31.2 [30.0 – 33.2]</td>
<td>30.2 [27.3 – 31.5]</td>
<td>0.05</td>
</tr>
<tr>
<td>HCO3⁻ [mmol/L]</td>
<td>20.9 [19.9 – 21.7]</td>
<td>17.9 [14.3 – 21.4]</td>
<td>0.03</td>
</tr>
<tr>
<td>Na⁺ [mmol/L]</td>
<td>139 [137 - 140]</td>
<td>139 [135 - 144]</td>
<td>0.78</td>
</tr>
<tr>
<td>K⁺ [mmol/L]</td>
<td>4.3 ± 0.4</td>
<td>4.3 ± 0.6</td>
<td>0.65</td>
</tr>
<tr>
<td>Ionized Ca²⁺ [mmol/L]</td>
<td>1.16 [1.13 – 1.20]</td>
<td>1.08 [1.06 – 1.14]</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total Calcium [mg/dL]</td>
<td>9.5 ± 0.3</td>
<td>7.8 ± 0.2</td>
<td>&lt;0.01</td>
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<td>Magnesium [mg/dL]</td>
<td>2.1 [2.0 – 2.2]</td>
<td>2.0 [1.9 – 2.3]</td>
<td>0.40</td>
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<tr>
<td>Cl⁻ [mEq/L]</td>
<td>108 [108 – 110]</td>
<td>111 [107 – 113]</td>
<td>0.31</td>
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<tr>
<td>Lactate⁻ [mmol/L]</td>
<td>1.7 [1.1 – 2.1]</td>
<td>2.0 [1.7 – 5.2]</td>
<td>0.01</td>
</tr>
<tr>
<td>SID [mEq/L]</td>
<td>34.0 [33.2 – 36.6]</td>
<td>32.2 [29.6 – 35.0]</td>
<td>0.04</td>
</tr>
<tr>
<td>BE [mmol/L]</td>
<td>-1.2 [-2.0 – -0.1]</td>
<td>-4.8 [-9.2 – -0.5]</td>
<td>0.02</td>
</tr>
</tbody>
</table>