Effect of time delay and storage temperature on blood gas and acid–base values of bovine venous blood

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Abstract

The aim of this study was to investigate possible changes in the gas composition and acid–base values of bovine venous blood samples stored at different temperatures (+4, 22 and 37 °C) for up to 48 h. Five healthy cattle were used in the study. A total of 15 blood samples collected from the animals were allocated into three groups, which were, respectively, then stored in a refrigerator adjusted to +4 °C (Group I, n = 5), at a room temperature of about 22 °C (Group II, n = 5) and in an incubator adjusted to 37 °C (Group III; n = 5) for up to 48 h. Blood gas and acid–base values were analysed at 0 (baseline), 1, 2, 3, 4, 5, 6, 12, 24, 36 and 48 h of storage. A significant decrease \( p < 0.001 \) was found, in the pH of the refrigerated blood after 5 h and its maximum decrease was recorded at 48 h as 0.04 unit. There were also significant alterations \( p < 0.001 \) in the blood pH of the samples stored at room temperature and in the incubator after 2 and 3 h, respectively. The maximum mean alteration in pCO2 value for Group I was \( \pm 0.72 \) kPa during the assessment, while for groups II and III, maximum alterations in pCO2 were detected as +2.68 and +4.16 kPa, respectively. Mean pO2 values increased significantly \( p < 0.001 \) for Group I after 24 h and for Group II after 6 h, while a significant decrease was recorded for Group III after 24 h \( p < 0.001 \). Base excess (BE) and bicarbonate (HCO3) fractions decreased significantly for all the groups during the study, compared to their baseline values. In conclusion, acid–base values of the samples stored at 22 and +4 °C were found to be within normal range and could be used for clinical purposes for up to 12 and 48 h, respectively, although there were small statistically significant alterations.

Keywords: pO2; pCO2; Acid base parameters; Storage temperature time; Bovine

1. Introduction

Various metabolic and respiratory diseases effect venous blood gas composition and acid–base values of cattle (Radostits et al., 1994; Carlson, 1996). Therefore, accurate measurement of these blood values is of great importance for veterinary clinicians and research workers. Human (Paerregaard et al., 1987; Muller-Plathe and Heyduck, 1992; Beaulieu et al., 1999) and cattle (Poulsen and Surynek, 1977; Szenci and Besser, 1990; Szenci et al., 1991) blood gas composition and acid–base values may change depending on the type of syringe used for sampling, delays in measurement time and variations in storage temperature. Continuous anaerobic and aerobic metabolism between the drawing of the blood sample and its analysis intervals may effect blood acid–base values in humans (Boink et al., 1991; Liss and Payne, 1993), cattle (Jagos et al., 1977; Krokavec et al., 1987; Szenci and Besser, 1990) and dogs (Haskins, 1977). In vitro blood metabolism includes both aerobic metabolism with production of carbon dioxide and anaerobic glycolysis with production of non-gaseous acids such as lactic acid. Oxygen consumption depends on the enzymatic activity of the citric acid and cytochrome systems in leucocytes and reticulocytes and will vary with the concentration of these cell types. Glycolysis is the predominant metabolic process in mature red blood cells. These processes are largely responsible for the rising of pCO2 in the stored blood of dogs (Haskins, 1977), and cattle (Poulsen and Surynek, 1977). These metabolic changes are temperature
dependent (Liss and Payne, 1993). Additionally, severe leucocytosis (Schmidt and Platje, 1992; Liss and Payne, 1993) and anaemia (Haskins, 1977) can lead to alterations in acid–base values.

Since it is difficult to measure the acid–base and gas values of blood under field conditions in a short period of time its necessary to store blood (Szenci and Besser, 1990). Several authors have investigated the alterations in acid–base variables during the storage of blood samples from humans (Paerregaard et al., 1987; Schmidt and Platje, 1992; Beaulieu et al., 1999), cattle (Jagos et al., 1977; Poulsen and Surynek, 1977; Krokavec et al., 1987; Szenci and Besser, 1990; Szenci et al., 1991, 1994) and dogs (Haskins, 1977). It would be useful to determine the extent to which changes in blood acid–base values and gas composition depend on the duration of time its necessary to store blood (Szenci and Besser, 1993) and anaemia (Haskins, 1977) can lead to alterations in acid–base variables.

### 2. Materials and methods

#### 2.1. Animals and sampling

Five clinically healthy, 1–3 years old, crossbreed cattle were used in the study. Following rectal temperature measurement, three jugular venous blood samples were drawn from each animal into 10 ml plastic syringes (Liquemine®-Roche, Istanbul, Turkey). The needle tips were closed with a rubber stopper after the removal of air bubbles from the samples. The samples were placed in a bed of crushed ice, taken immediately to the laboratory and analysed within 15 min. Following the first (0) hour’s laboratory analysis, a total of 15 blood samples were collected from the animals, allocated into three groups and then stored, respectively, in a refrigerator set about 22°C for long periods. Therefore, this study was carried out to detect the changes in gas composition and acid–base values in bovine venous blood samples stored at different temperatures (+4, 22 and 37°C) for up to 48 h.

#### 2.2. Acid–base and blood gas analyses

The measurement of pH, pO₂, pCO₂ and the calculation of standard Base excess (BEstd), actual base excess (BEact), standard bicarbonate (stHCO₃), actual bicarbonate (actHCO₃) concentrations, oxygen saturation (O₂SAT) and oxygen content (O₂CT) were automatically performed on a blood gas analyser (Chiron diagnostics, Rapidlab 248, UK) prior to the study. Blood samples from the five cattle were analysed for blood gas and acid base values four times each within 15 min to determine the imprecision of parameters, which emerged as follows: pH 0.01%; pCO₂ 1.9%; pO₂ 3.52%; ActHCO₃ 1.74%; StdHCO₃ 1.34%, BEact 1.98%, BEstd 1.02%, O₂SAT 0.39% and O₂CT 1.8% on the basis of variation coefficient. Control of accuracy was carried out using a commercial accuracy control solution (Complete, Bayer, East Walpole, USA). Control values were within the normal range as described by the manufacturer. Calculated variables were automatically performed by blood gas analyser according to equations ¹ programed to device by manufacturer (Chiron diagnostics, Rapidlab 248, UK). The values of pH, pCO₂ and pO₂ were automatically adjusted to the animal’s rectal temperatures.

#### 2.3. Haematological analyses

For the initial haematological examinations, peripheral blood samples were collected from jugular vein into ethylenediaminetetraacetic acid (EDTA) treated tubes. These blood samples were used to manually establish total white blood cells (WBCs), total red blood cells (RBCs) and haemoglobin concentration (Hb), as described by Coles (1980).

#### 2.4. Statistical analyses

The effect of temperature on blood gas and acid base values was tested by analysis of variance using SPSS for Windows 6.0. The effects of time on the repeated measurements within each group were also examined using SPSS for Windows 6.0. The values of pH, pCO₂ and pO₂ were automatically adjusted to the animal’s rectal temperatures.

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¹ ActHCO₃ = pH + log(pCO₂ × 0.0307) − 6.105; StdHCO₃ = 24.5 + 0.94 × (A − 2.9)²(2.65 + 0.3ctHb)/1000; A = BEstd − 0.2×ctHb [100 − O₂SAT]/100; BEact = chCO₂ − 24.8 + 1.62(pH − 7.40); BEstd = (1 − 0.014 × ctHb) × (chHCO₃ − 24.8) + (1.43 × ctHb + 7.7) (pH − 7.40).

O₂SAT = N⁻⁴ − 15N⁻³ + 2045N⁻² + 2000N⁻¹

N = pO₂ + 10⁻⁰⁴³(pH−7.4)−0.0001BEstd and BEstd is calculated assuming 100% O₂SAT; O₂CT = O₂SAT × 1.39 × ctHb + 0.00514 × pO₂.
Table 1
Effect of storage time on pCO₂ and pO₂ of bovine venous blood samples stored at +4 °C (Group I), 22 °C (Group II) and 37 °C (Group III) (n = 5) (mean ± SE)

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<th>Parameter</th>
<th>Group</th>
<th>Time (h)</th>
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<tbody>
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<td>pCO₂ (kPa)</td>
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<td></td>
<td></td>
<td>a,b</td>
<td>6.59±0.11</td>
<td>5.95±0.09</td>
<td>5.94±0.10</td>
<td>5.87±0.12</td>
<td>6.04±0.10</td>
<td>6.01±0.11</td>
<td>6.17±0.10</td>
<td>5.86±0.11</td>
<td>6.34±0.12</td>
<td>6.40±0.12</td>
<td>6.90±0.10</td>
<td>p &lt; 0.001</td>
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<td></td>
<td></td>
<td>c,d</td>
<td>6.59±0.11</td>
<td>6.34±0.14</td>
<td>6.50±0.16</td>
<td>6.59±0.12</td>
<td>6.81±0.14</td>
<td>6.88±0.10</td>
<td>7.34±0.12</td>
<td>7.07±0.13</td>
<td>8.06±0.16</td>
<td>8.82±0.19</td>
<td>9.27±0.47</td>
<td>p &lt; 0.001</td>
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<td></td>
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<td>e</td>
<td>6.59±0.11</td>
<td>6.20±0.15</td>
<td>6.41±0.15</td>
<td>6.61±0.12</td>
<td>7.01±0.16</td>
<td>7.37±0.18</td>
<td>9.01±0.26</td>
<td>8.88±0.29</td>
<td>10.75±0.69</td>
<td>9.29±0.19</td>
<td>9.05±0.34</td>
<td>p &lt; 0.001</td>
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<td></td>
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<td>f</td>
<td>6.59±0.11</td>
<td>6.20±0.15</td>
<td>6.41±0.15</td>
<td>6.61±0.12</td>
<td>7.01±0.16</td>
<td>7.37±0.18</td>
<td>9.01±0.26</td>
<td>8.88±0.29</td>
<td>10.75±0.69</td>
<td>9.29±0.19</td>
<td>9.05±0.34</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

P1, significant differences within group compared to baseline value (a,b,c,d,e,f), (p < 0.001); P2, in each column different letters (A, B, C) indicated significant between groups (p < 0.01), (p < 0.001); pCO₂, partial pressure of carbondioxide (kPa); pO₂, partial pressure of oxygen (kPa).

Table 2
Effect of storage time on O₂SAT and O₂ct of bovine venous blood samples stored at +4 °C (Group I), 22 °C (Group II) and 37 °C (Group III) (n = 5) (mean ± SE)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Time (h)</th>
<th>0</th>
<th>1</th>
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<th>48</th>
<th>P1</th>
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<tbody>
<tr>
<td>O₂SAT</td>
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<td></td>
<td></td>
<td>a,b</td>
<td>62.32±1.20</td>
<td>62.80±2.46</td>
<td>63.28±2.16</td>
<td>64.36±2.04</td>
<td>63.78±2.24</td>
<td>64.56±2.19</td>
<td>66.62±2.09</td>
<td>66.56±2.27</td>
<td>71.52±2.72</td>
<td>69.82±2.89</td>
<td>72.78±2.00</td>
<td>p &lt; 0.005</td>
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<tr>
<td></td>
<td></td>
<td>c,d</td>
<td>62.32±1.20</td>
<td>61.82±1.02</td>
<td>62.56±1.02</td>
<td>62.44±1.02</td>
<td>62.44±1.02</td>
<td>62.06±1.21</td>
<td>60.67±1.32</td>
<td>65.54±2.71</td>
<td>53.22±0.41</td>
<td>50.17±3.38</td>
<td>1.85±0.05</td>
<td>p &lt; 0.001</td>
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<td></td>
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<td>e</td>
<td>62.32±1.20</td>
<td>61.10±1.42</td>
<td>60.16±1.55</td>
<td>59.30±1.70</td>
<td>57.48±1.82</td>
<td>55.58±2.00</td>
<td>51.82±2.30</td>
<td>45.54±2.71</td>
<td>32.22±0.41</td>
<td>26.18±3.38</td>
<td>1.85±0.05</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>f</td>
<td>62.32±1.20</td>
<td>61.10±1.42</td>
<td>60.16±1.55</td>
<td>59.30±1.70</td>
<td>57.48±1.82</td>
<td>55.58±2.00</td>
<td>51.82±2.30</td>
<td>45.54±2.71</td>
<td>32.22±0.41</td>
<td>26.18±3.38</td>
<td>1.85±0.05</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>

P1, significant differences within group compared to baseline value (a,b,c,d,e,f), (p < 0.05, p < 0.001); P2, in each column different letters (A, B, C) indicated significant between groups (p < 0.05, p < 0.01, p < 0.001); O₂SAT, oxyhaemoglobin saturation (%); O₂CT, oxygen content (mmol/l).
Table 3
Effect of storage time on ActHCO₃ and StdHCO₃ of bovine venous blood samples stored at +4 °C (Group I), 22 °C (Group II) and 37 °C (Group III) (n = 5) (mean ± SE)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Time (h)</th>
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<th>P1</th>
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</thead>
<tbody>
<tr>
<td>ActHCO₃ (mmol/l)</td>
<td>I</td>
<td>a</td>
<td>31.38 ± 0.35</td>
<td>29.04 ± 0.27</td>
<td>28.00 ± 0.30</td>
<td>27.62 ± 0.40</td>
<td>28.12 ± 0.34</td>
<td>27.88 ± 0.33</td>
<td>28.42 ± 0.43</td>
<td>26.90 ± 0.31</td>
<td>28.74 ± 0.31</td>
<td>28.56 ± 0.54</td>
<td>30.20 ± 0.29</td>
<td>p &gt; 0.001</td>
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<td>B,c</td>
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<td>B,cd</td>
<td>C,de</td>
<td>B,cd</td>
<td>e</td>
<td>B,cd</td>
<td>31.20 ± 0.52</td>
<td>30.24 ± 0.29</td>
<td>30.55 ± 0.35</td>
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<tr>
<td>StdHCO₃ (mmol/l)</td>
<td>I</td>
<td>a</td>
<td>29.24 ± 0.29</td>
<td>26.42 ± 0.97</td>
<td>26.40 ± 0.26</td>
<td>26.32 ± 0.27</td>
<td>26.88 ± 0.20</td>
<td>26.40 ± 0.22</td>
<td>26.80 ± 0.36</td>
<td>25.58 ± 0.20</td>
<td>27.00 ± 0.24</td>
<td>27.30 ± 0.20</td>
<td>27.78 ± 0.29</td>
<td>p &lt; 0.001</td>
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P1, significant differences within group compared to baseline value (a,b,c,d,e,f), (p < 0.05, p < 0.01, p < 0.001); ActHCO₃, actual bicarbonate concentrations (mmol/l); StdHCO₃, standard bicarbonate concentrations (mmol/l).

Table 4
Effect of storage time on BEact and BEstd of bovine venous blood samples stored at +4 °C (Group I), 22 °C (Group II) and 37 °C (Group III) (n = 5) (mean ± SE)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Time (h)</th>
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<th>P1</th>
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</thead>
<tbody>
<tr>
<td>BEact (mmol/l)</td>
<td>I</td>
<td>a</td>
<td>7.04 ± 0.39</td>
<td>4.74 ± 0.32</td>
<td>3.58 ± 0.35</td>
<td>3.2 ± 0.04</td>
<td>2.36 ± 0.36</td>
<td>3.32 ± 0.35</td>
<td>3.86 ± 0.49</td>
<td>2.24 ± 0.31</td>
<td>4.06 ± 0.31</td>
<td>3.76 ± 0.57</td>
<td>5.24 ± 0.34</td>
<td>p &lt; 0.001</td>
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<td></td>
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<td>B,b,c</td>
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<td>BEstd (mmol/l)</td>
<td>I</td>
<td>a</td>
<td>6.12 ± 0.34</td>
<td>4.16 ± 0.26</td>
<td>3.24 ± 0.25</td>
<td>2.84 ± 0.34</td>
<td>3.16 ± 0.31</td>
<td>2.90 ± 0.32</td>
<td>3.22 ± 0.43</td>
<td>1.98 ± 0.29</td>
<td>3.46 ± 0.27</td>
<td>3.14 ± 0.50</td>
<td>4.28 ± 0.32</td>
<td>p &lt; 0.001</td>
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P1, significant differences within group compared to baseline value (a,b,c,d,e,f), (p < 0.05, p < 0.01, p < 0.001); BEact, extracellular base excess (mmol/l); BEstd, blood base excess (mmol/l).
Blood acid–base and blood gas values are summarised in Tables 1–4 and Fig. 1. The pH values of the samples kept at different temperatures tended to decrease significantly with time in all groups \( (p < 0.001) \) (Fig. 1). Compared to initial values, pH values were found to decrease significantly after 5, 3 and 2 h \( (p < 0.001) \) for the samples stored at +4, 22 and 37 °C, respectively.

Alterations in pCO\(_2\) and pO\(_2\) values are summarised in Table 1. The mean pCO\(_2\) values of the samples stored at +4 °C decreased significantly between 1 and 24 h, but later increased again to their initial levels. Moreover, the pCO\(_2\) values of the samples kept at 22 and 37 °C increased significantly \( (p < 0.001) \) after the fourth hour and continued to increase until the end of the study (Table 1). The maximum mean alteration in pCO\(_2\) value for Group I was +0.72 kPa during the assessment, while for groups II and III maximum alterations for pCO\(_2\) were detected as +2.68 and +4.16 kPa, respectively. The pO\(_2\) values of the samples kept at +4 and 22 °C increased with time \( (p < 0.001) \). However, a significant decrease was recorded after 24 h for the samples kept at 37 °C \( (p < 0.001) \). Values of O\(_2\)SAT in group I increased \( (p < 0.05) \) after 24 h storage, on the other hand it was significantly decreased in group III after 5 h \( (p < 0.001) \) and there were no changes in group II \( (p > 0.33) \) (Table 2). The O\(_2\)CT values were significantly increased \( (p < 0.05) \) after 24 h of storage at +4 °C, but significantly decreased after 4 h of storage at 37 °C \( (p < 0.001) \) (Table 2). However, no significant alterations were observed in the O\(_2\)CT values of the samples stored at 22 °C. BE and HCO\(_3^-\) concentrations decreased significantly for all three groups during the study compared to their baseline values. The most prominent decrease in BE and HCO\(_3^-\) concentrations was recorded for the samples kept at 37 °C (Tables 3 and 4).

4. Discussion

The effects of storage time and temperature on blood gases and acid–base values in humans (Mahoney et al., 1991; Liss and Payne, 1993; Beaulieu et al., 1999; Lenfant and Aucutt, 1965) and in cattle (Poulsen and Surynek, 1977; Szenci and Besser, 1990; Szenci et al., 1991) have been investigated intensively. Previous studies have revealed that the blood gas and acid–base values of human blood samples usually stabilised between 15 min and 2 h (Paerregaard et al., 1987; Mahoney et al., 1991; Beaulieu et al., 1999). The effect of storage temperature and storage time on blood gas and acid–base values in cattle are disputed (Poulsen and Surynek, 1977; Szenci and Besser, 1990; Szenci et al., 1991). Szenci and Besser (1990) reported that cattle venous blood acid–base values could be used for diagnostic purposes up to 24 h. On the other hand, the pH values of blood samples taken from cattle and stored at +4 °C stabilised after for 5–6 h (Poulsen and Surynek, 1977). In the present study, the pH values of the blood samples stored at +4 °C were found to be suitable for clinical usage up to 48 h (Fig. 1). Furthermore, storage time and temperature were found to significantly alter the blood pH and bicarbonate values of all groups.
Particularly, rapid and significant decreases in blood pH ($p < 0.001$) were observed for those samples stored at $37 \, ^\circ\mathrm{C}$, compared to the other groups (Groups I and II) (Fig. 1). Metabolism of in vitro blood samples continues during storage. Oxygen consumption occurs due to anaerobic metabolism and CO$_2$ generation in the tricarboxylic acid cycles (TCA). Additionally, lactic acid is accumulated via glycolysis depending on anaerobic metabolism. These metabolic activities cause a decrease in blood pH (Andersen, 1961; Szenci and Besser, 1990; Boink et al., 1991; Liss and Payne, 1993). Since sampling error was reduced to a minimal level in the present study the decrease in blood pH and HCO$_3$ was attributed to the formation of lactic acid due to glycolysis and to an increase in the level of pCO$_2$. Moreover, the much greater decrease in the pH of the blood samples stored at $37 \, ^\circ\mathrm{C}$ may be attributed to lactic acid generation due to rapid anaerobic metabolism as a result of the high storage temperature (Liss and Payne, 1993).

In the present study, increases in the pCO$_2$ values of the blood samples kept at $37 \, ^\circ\mathrm{C}$ and at room temperature and a decrease in those of the samples stored at $+4 \, ^\circ\mathrm{C}$ were recorded as compared to their baseline values. Researchers have reported that in vitro blood pCO$_2$ value rises depending on aerobic and anaerobic metabolism in blood cells (Sandhagen et al., 1988; Szenci and Besser, 1990; Beaulieu et al., 1999). Unlike O$_2$, CO$_2$ does not influx through plastic syringe walls (Mahoney et al., 1991), and therefore the significant rises of pCO$_2$ in groups II and III may be the result of high cell metabolic activities at $+22$ and $37 \, ^\circ\mathrm{C}$ (Foster and Terry, 1967). Moreover the most prominent increase in pCO$_2$ was observed in the sample stored at $37 \, ^\circ\mathrm{C}$. The significant decrease in the pCO$_2$ values of the samples stored at $+4 \, ^\circ\mathrm{C}$ may be due to the blood being transferred to a lower than body temperature medium (Mahoney et al., 1991).

In the case of blood samples stored over time, the type of syringe used for sampling (Mahoney et al., 1991; Beaulieu et al., 1999) and the aerobic metabolism of leucocytes (Poulsen and Surynek, 1977; Haskins, 1977) in the blood have been determined to influence alterations in the levels of pO$_2$. It has been revealed that pO$_2$ in plastic syringes increases by the diffusion of oxygen through the plastic wall of the syringe (Paerregaard et al., 1987; Beaulieu et al., 1999). Leucocytes are responsible for most of the aerobic metabolism in the blood (Liss and Payne, 1993) and cause O$_2$ consumption in blood samples stored under in vitro anaerobic conditions (Sandhagen et al., 1988; Szenci and Besser, 1990). A decrease in pO$_2$ values has been found in blood samples with high leucocyte counts (Schimidt and Plathe, 1992) and in samples with anaemia (Haskins, 1977). Since the initial mean leucocyte and erythrocyte numbers and haemoglobin concentrations of the blood samples were within normal range in the present study, the significant decrease ($p < 0.001$) in pO$_2$ level found in the blood samples stored at $37 \, ^\circ\mathrm{C}$ after 24 h may be attributed to an increase in aerobic metabolism and O$_2$ consumption by leucocytes due to the high storage temperature (Liss and Payne, 1993). A significant increase in the level of pO$_2$ was detected after 6 h in the samples stored at room temperature and in the refrigerated samples. This may have been due to the release of O$_2$ from haemoglobin as a result of the reduction in blood pH (Szenci and Besser, 1990) and to the diffusion of O$_2$ through the plastic syringe wall (Beaulieu et al., 1999; Mahoney et al., 1991) although oxygen consumption occurs during aerobic cell metabolism. Moreover, this finding reflects the fact that aerobic metabolism of leucocytes was lower at room temperature and under $+4 \, ^\circ\mathrm{C}$ in vitro conditions than at $37 \, ^\circ\mathrm{C}$.

The SAT O$_2$ value also decreased together with pH in the blood samples kept at $37 \, ^\circ\mathrm{C}$. This can be explained by the shifting of the haemoglobin–O$_2$ dissociation curve to the right (Bohr effect) due to decrease in blood pH (Szenci and Besser, 1990; Carlson, 1996). The O$_2$CT and O$_2$SAT value displayed the least alteration within the parameters for the blood samples kept at room temperature and at $+4 \, ^\circ\mathrm{C}$, which is consisted the findings of Beaulieu et al. (1999) in a study carried out on human blood. Therefore, we suggest that the O$_2$CT and O$_2$SAT value should be taken into consideration for blood samples stored at room temperature when asssessing blood O$_2$.

In conclusion, the acid–base values of the samples stored at $22$ and $37 \, ^\circ\mathrm{C}$ changed significantly when compared to the samples stored at $+4 \, ^\circ\mathrm{C}$. However, the acid–base values of the samples stored at $22$ and $+4 \, ^\circ\mathrm{C}$ were within normal range and could be used for clinical purposes for up to 12 and 48 h, respectively.

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