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In vitro effects of oxygen on physico-chemical properties of horse erythrocyte membrane

Karine Portier[a,*], Michel Guichardant[b], Jean-Claude Debouzy[c], David Crouzier[c], Ingrid Geraud[a], Nathalie Kirschvink[d], Pierre Lekeux[e], Nicole Fellmann[f], Jean Coudert[f]

[a] *Equine Department, National Veterinary School of Lyon, Lyon, France*
[b] *INSERM, Unit 585, INSA-Lyon, IMBL, Villeurbanne, France*
[c] *Centre de Recherche de Santé des Armées, Grenoble, France*
[d] *Animal Physiology, Department for Veterinary Medicine, University of Namur, Namur, Belgium*
[e] *Department for Functional Sciences, Faculty of Veterinary Medicine, University of Liege, Belgium*
[f] *Laboratory of Sport Physiology and Biology, Faculty of Medicine, University of Auvergne, Clermont Ferrand, France*

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Abstract

Whether direct exposure to different concentrations (0%, 13%, 100%) of oxygen may affect horse erythrocyte membrane fluidity (EMF) and fatty acid (FA) composition was studied during 1 (*T*60) and 2 h (*T*120) exposure. EMF was investigated at the head group level and hydrophobic core thanks to phosphorus nucleus 31 (^{31}P) nuclear magnetic resonance (^{31}P NMR) and electronic paramagnetic resonance (EPR) using two spin probes: 5-nitroxydestearic acid and 16-doxylstearic acid. Lipid structure of the membranes was studied by gas liquid chromatography. 4-Hydroxy-2*E*-nonenal was also analyzed as a marker of lipid peroxidation. It increased at *T*120 13% and 100% oxygen whereas there were no significant changes in membrane dynamic or structure. Correlation was demonstrated between EMF and partial pressure of oxygen in the blood (P_{O_2}). In vitro high rate of oxygenation was efficient to induce lipid peroxidation but did not change membrane dynamics. This may be due to a low free radical production in vitro or to the high red blood cells antioxidant properties.
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1. Introduction

Oxygen, which is often used as the sole carrier gas during general anaesthesia, is a chemically very reactive species and can induce oxidation (Sjodin et al., 1990) and free radical generation (Sies, 1985). Lipid peroxidation is a consequence of this oxidative stress (Gibson et al., 2000). Alterations of biophysical properties of membranes caused by free radicals play an important role in cell injury and function. Membranes are particularly sensitive to such damage because of their high polyunsaturated fatty acid content.

Erythrocytes are especially vulnerable since they have no membrane repair and regenerative capacity (Webster and Toothill, 1987) and red cell damages by free radicals would probably be associated with haemolysis (Szweda-Lewandowska et al., 2003). This could also affect their deformability (Shiga and Maeda, 1980) and, as a consequence, impair their transport through the microcirculation (Amin and Sirs, 1985; Bellary et al., 1995; Chung and Ho, 1999). Horses erythrocytes appear to be more sensitive to oxidative stress and have a lower deformability than human erythrocytes (Baskurt et al., 1997).

These changes in membrane properties are characterized by alterations in membrane fluidity (Watanabe et al., 1990) (i.e. rotational and lateral diffusion rates of membrane component, Hollan, 1996) due to lipid peroxidation (Hollan, 1996; Wrobel and Gomulkiewicz, 1999). Indeed, oxidative stress induces membrane lipid peroxidation as a consequence of free radical

* Corresponding author at: Ecole Nationale Veterinaire de Lyon, Departement Hippique, 1 Av Bourgelat, 69280 Marcy l'Etoile, France.
Tel.: +33 4 78 87 26 77; fax: +33 4 78 87 26 75.
E-mail address: k.portier@vet-lyon.fr (K. Portier).

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attacks on polyunsaturated fatty acids and may lead to modifications in membrane fatty acid composition (Oostenbrug et al., 1997). Moreover, significant relationships were demonstrated between membrane fluidity and membrane lipids and fatty acid composition (Shinitzky, 1984).

No previous works has demonstrated any relationship between direct exposure to oxygen and horse erythrocyte membrane fluidity (EMF) or fatty acid (FA) composition. Therefore, the aim of this study was to assess the in vitro effects of oxygen at different concentrations (100%, 13% and 0% to mimic hyperoxemia, normoxemia and hypoxemia, respectively) and durations (60 and 120 min empirically estimated as anaesthesia mean time) on horse erythrocyte membrane lipid composition and fluidity. Correlations between membrane structure and dynamics were also evaluated.

2. Material and methods

Six healthy horses, receiving the same feeding and exercise regime, were matched for age and gender.

Jugular venous blood samples (140 ml) were taken simultaneously in all horses. The samples were put into sterile vacuum tubes (5 ml) containing heparin for EMF, NMR and blood gas analysis and EDTA for fatty acids and phospholipids analysis, respectively.

Samples were bubbled at 37 °C with three different gas mixtures: hyperoxia: 100% O_2 (O_{100}), normoxia: 13% O_2 (O_{13}) and hypoxia: 0% O_2 (O_0) (air liquid Santé, Venissieux, France). For each gas mixture, analyses were performed at a control time ($T0$), after 60 min ($T60$) and 120 min ($T120$) of oxygen exposure. Samples were stored at 4 °C until phospholipids, EPR and RMN analysis, which were performed within 5 h of collection. Blood gas analysis was performed immediately.

2.1. Blood gas analysis

Samples were analyzed for partial pressure of oxygen (P_{O_2}), immediately post-sampling, by a blood gas analyser (Opti CCA, AVL, Schaffhausen, Switzerland). Quality control samples were analyzed before the experiment to ensure that the equipment was working within acceptable parameters. P_{O_2} is measured by optode based on fluorescence extinction (Kautsky, 1939) and does not consume O_2 during the measurement. The values were corrected for samples temperature (37 °C) and mean barometric pressure was 736 mmHg.

2.2. EPR

The membrane fluidity at the acyl chain level was investigated in whole intact erythrocyte by electron paramagnetic resonance (EPR).

The erythrocytes were isolated from fresh blood by centrifugation at 4 °C (5 min, 1000 × g), then rinsed using a buffer solution (PBS), recentrifugated, this procedure being repeated until a clear centrifugation supernatant was obtained, then brought to 30% packed cell volume with the same buffer before labelling. Each 100 μL sample of this suspension was then labelled with 2 μL of a radical nitroxide marked probe solution, (10^{-2} M in dimethylsulfoxyde); the probes were either 16 DOXYL-stearic-acid (16-NS) or 5 DOXYL-stearic-acid (5NS), SIGMA–Aldrich). After labelling, the samples were transferred by capillarity into 20 μL Pyrex capillary tubes. These tubes were placed in a 3 mm diameter quartz holder, and inserted into the cavity of a Bruker ESP 380 spectrometer (Karlsruhe, Germany) operating at 9.79 GHz. The complete membrane incorporation of the spin labels was ascertained by the absence of the extremely resolved EPR lines corresponding to free rotating markers. The spectra were recorded at 37 °C under the following conditions: microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 2.868 G, time constant 327 ms. For 16NS experiments, the rotational correlation time, T_c, was calculated from Keith's equation (Keith et al., 1975) and expressed in nanoseconds. T_c is in inverse proportion to EMF. The order parameter, S, was calculated for 5NS spctra from Gaffney's formula (Gaffney and Mich, 1976). Changes of the order parameter values (in gauss) correspond with changes of membrane local viscosity, its increase means a decrease of viscosity (Gornicki and Gutsze, 2000).

2.3. NMR

Erythrocyte ghost preparation: whole blood was centrifuged (5 min, 699 × g, Sigma 1K15), 1 mL of erythrocyte was kept and added to PO_4^{3-} (50 nM, pH 8.0) and then centrifuged (5 min 18,894 × g). The supernatant fluid was removed, and this operation was repeated three times after addition of NaCl 0.9% in excess and the washing–centrifugation cycle repeated until red colour disappear in the supernatant while a white pellet was collected for NMR experiment.

^{31}P NMR spectra were recorded using a 5 mm diameter NMR tubes on a Bruker AMX 400 spectrometer operated in unlocked mode. ^{31}P NMR experiments were performed at 162 MHz. Phosphorus spectra were recorded using a dipolar echo sequence $(\pi/2\text{-}t\text{-}\pi\text{-}t)^i$ with a t-value of 12 μs and a broadband two levels proton decoupling. Phosphoric acid (85%) was used as external reference. The chemical shift difference between the low field and the high field edges of the ^{31}P NMR spectrum is called Chemical Shift Anisotropy (CSA, ppm) and is directly related to the fluidity-reorientation at the polar head level where the phosphorus nuclei are located. Hence a mobile phosphorus group give a single narrow resonance (several Hz) as detected in true solution or for small structures (micelles), while solid state phosphorus gives extremely broad contributions (more than 120 ppm). The partly motional averaging present in membrane bilayer structures results in the asymmetrical lineshape with a highfield component corresponding to the contributions perpendicular to the membrane surface and a low field broad shoulder related with parallel orientations. Thus, faster motional reorientation of the phosphorus head group results in reduction of the position difference (i.e. the CSA, in ppm) of these contributions.

2.4. Lipid extraction

Membrane suspensions were spiked with diheptadecanoylphosphatidyletholamine choline and [2-13C]-cholesterol, as internal standards. They were then extracted twice by 3 volumes ethanol and 6 volumes chloroform. After decantation, organic phases were pooled and the solvent was removed under vacuum.

2.5. Lipid purification

Lipid residues were dissolved in a solvent mixture of chloroform/ethanol (2:1, v/v) and purified by silicagel TLC plates. The eluant was a mixture of hexane/diethylether/methanol (80:20:10, v/v/v). Separated lipid classes were detected by fluorescein spraying and identified using their corresponding standard eluted on the same plate. Both the sterol and phospholipid fractions were extracted with a mixture of chloroform/ethanol (2:1, v/v).

2.6. Separation of phospholipid classes

The different phopholipid classes (sphingomyeline (sph), phosphatidylcholine (PC) and phosphatidylethanolamine (PE)) were separated on TLC using a solvent mixture chloroform/methanol/water (63/27/4, v/v/v). They were then extracted with a mixture of chloroform/ethanol (2:1, v/v).

2.7. Fatty acid content in phospholipids classes

Sph, PC and PE fractions were transesterified by treatment with BF3/methanol (14%) for 90 min at 100 °C. Reaction was halted by adding a solution of K_2CO_3 (5%) in ice and the fatty acid methyl esters (FAMEs) were then extracted with isooctane.

2.8. Gas chromatography of FAMEs

FAMEs were analyzed on a Agilent Technologies chromatograph model 6890 equipped with a BPX 70 fused silica capillary column (60 m × 0.25 mm

i.d., 0.25 μm film thickness). Oven temperature was set at 80 °C for 1.5 min and increase to 150 °C at 20 °C/min min and then to 250 °C at 2 °C/min. This temperature was maintained for 10 min before returning to the initial conditions. Helium was used as the carrier gas at 1 ml/min. Temperature of the split/splitless injector and the flame ionization detector were set at 230 °C and 280 °C, respectively.

2.9. Cholesterol derivatization

Cholesterol was treated by *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) for 30 min at 60 °C.

2.10. GC–MS of derivatized cholesterol

GC–MS was carried out on a Hewlett-Packard quadrupole mass spectrometer interfaced with a Hewlett-Packard gas chromatograph (Les Ullis-France). The gas chromatograph was equipped with a DB-17MS fused-silica capillary column (60 m × 0.25 mm i.d. 0.25 μm film thickness) (Agilent Technologies) which was maintained at 57 °C. The following oven temperature programme was used: 5 min at 57 °C, then increased to 200 °C at a rate of 40°/min followed by an increase to 310 °C at a rate of 10 °C/min and hold for 20 min at 310 °C. The interface, injector and ion source were kept at 280, 280 and 150 °C, respectively. Electron energy was set at 70 eV and helium was used as carrier gas. The electron multiplier voltage was set at 1400 V. Mass spectra were acquired from 100 to 600 Da using electronic impact ionization mode (EI).

Sterols were identified by comparison of their retention times and their mass spectra to commercial standard sterols derivatized as previously described and quantified using their total ion current signals.

2.11. 4-Hydroxy-alkenal measurement in plasma

Crude plasma was treated with 200 μL of *O*-2,3,4,5,6-pentafluorobenzyl hydroxylamine hydrochloride (PFBHA) (50 mM in 0.1 M PIPES buffer pH 6.5) for 30 min at room temperature, as described by (Van Kuijk et al., 1990). After acidification with 100 μL of 98 % H_2SO_4, pentafluorobenzyloxime derivatives were extracted with 500 μL methanol and 2 mL hexane. The solvent was removed under nitrogen and the hydroxyl group was converted into trimethylsilylether after an overnight treatment with *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) at room temperature. The pentafluorobenzyl oxime, trimethylsilylether-4-HNE derivative (*O*-PFB-TMS-4-HNE) was then analyzed by gas chromatography–mass spectrometry (GC–MS).

2.12. GC–MS of 4-hydroxy-alkenals

GC–MS was carried out as previously described (Bacot et al., 2003) on a Hewlett-Packard quadrupole mass spectrometer interfaced with a Hewlett Packard gas chromatograph (Les Ullis-France). The gas chromatograph was equipped with a DB-17MS fused-silica capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) (Hewlett-Packard) which was held at 57 °C. The following oven temperature program was used: 2 min at 57 °C, then increased to 180 °C at 20 °C/min, followed by an increase to 280 °C at 4 °C/min. Samples were injected with a splitless injector with a head pressure of 7.9 psi. The interface, injector and ion source were kept at 280, 260 and 130 °C, respectively. Electron energy was set at 70 eV. Helium and methane were used as carrier and reagent gases, respectively. Quantification was performed in SIM using the negative ion chemical ionization (NICI) modes. The electron multiplier voltage was usually set at 1400 V.

2.13. Statistical analysis

Statistical analysis was performed using IR©(http://cran.cict.fr/) statistical software (Ihaka and Gentleman, 1996). Data did not fulfil requirements for parametric analysis (all parameters were tested for normal distribution using a Shapiro-Wilks test).

Nonparametric analytical techniques (Wilcoxon signed rank test for matched pairs) were used to compare data between each group (0%, 13%, 100%) at different times (*T*60 and *T*120). Differences were considered significant if *p* was less than 0.05. Results are expressed as median and range [minimum, maximum]. Correlation analyses were performed using a Spearman non-parametric test.

3. Results

3.1. P_{O_2}

Data are shown in Fig. 1. Duration and percentage of oxygen exposure had a significant effect on (P_{O_2}) ($p < 0.05$). After 1 h (*T*60) of contact between blood samples and gas mixture containing 100% O_2 (O_{100}), P_{O_2} (481 [430, 603] mmHg) was significantly different from P_{O_2} (85 [84, 91] mmHg) measured in blood in contact with a gas mixture containing 13% O_2 (O_{13}) and from P_{O_2} (42 [37–49] mmHg) measured in blood in contact with a gas mixture containing 0% O2 (O_0). Similar results were obtained after 2 h of contact, P_{O_2} (*T*120 O_{100}) was (478 [403, 595] mmHg), P_{O_2} (*T*120 O_{13}) was (85 [81, 88] mmHg) and P_{O_2} (*T*120 O_0) was (39 [30, 43] mmHg). Differences were also significant between P_{O_2} (*T*60 O_0) and P_{O_2} (60 O_{13}) and between P_{O_2} (*T*120 O_0) and P_{O_2} (*T*120 O_{13}).

3.2. 4HNE

Data are shown in Fig. 2. Concentrations of the lipid peroxidation product, 4HNE (arbitrary units), were significantly higher at *T*120 (O_{13}) (114.4 [106.1, 141.8]) and *T*120 (O_{100}) (125.5[110.1, 149.5]) than at *T*120 (O_0) (99.2 [95.9, 104.3]).

3.3. Effects of P_{O_2} on membrane fluidity (T_c) and membrane rigidity (S)

The membrane fluidity assessed close to the hydrophobic part of the membrane bilayer (monitored by 16-NS) showed quite similar correlation times (around 33.3 ns, $\log(H°/H^{-1}) = 0.61$ at 37 °C). The membrane rigidity assessed close to the polar head (monitored by 5-NS provided order parameters of $S = 0.62 \pm 0.006$ without any statistically difference (with spectra value of 2A// of 52.2G ± 0.1G). Thus, no significant effect of the variation in oxygen concentration on membrane fluidity was demonstrated. T_c values are shown in Fig. 3.

3.4. Effects of P_{O_2} on the membrane structure at the head group level (CSA)

The membrane structure at the head group level was not affected by the variation in oxygen concentration. Hence the classical value of CSA (around 70 ppm, at 310 K) was measured in all samples, with only small nonsignificant variations.

3.5. Effects of P_{O_2} on phospholipids and cholesterol

PC, PE, sph and CHOL were not significantly modified by the different concentration of oxygen. Both the ratio of $n-3/n-6$ fatty acids and saturated/polyunsaturated fatty acids were not significantly modified by P_{O_2}. Also PE and PC plasmalogen

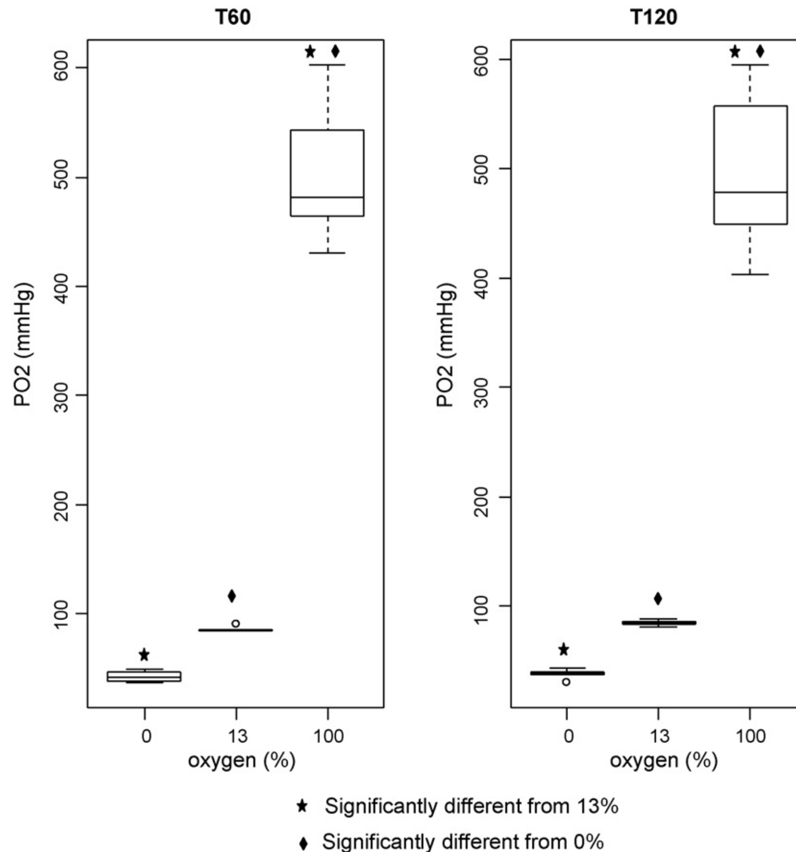

Fig. 1. Partial pressure of oxygen (P_{O_2}) measured in jugular vein blood of six healthy horses and submitted to different oxygen gas mixture flow (0%, 13% and 100%) during 1 (T60) or 2 h (T120). Results were significant if $p < 0.05$.

contents were not significantly correlated with the P_{O_2} variations.

3.6. Correlations

Correlations analyses were performed between markers of membrane dynamic (T_c, S, CSA), P_{O_2}, and membrane lipids (PE, PC, SP, CHOL, plasmalogen in PC and PE) and fatty acids ratios (SFA/PUFA, $n-3/n-6$). A positive correlation was found between P_{O_2} and T_c at T120 O_{100} ($r = 0.83$, $p = 0.05$).

4. Discussion

Oxygenation at 13% and 100% was efficient enough to induce an oxidative stress as demonstrated by an increase in lipid peroxidation products 4-HNE at T120 (Yamada et al., 2004).

The dynamic of the membrane was studied at three different levels. T_c evaluated the membrane fluidity at the hydrophobic core (Cazzola et al., 2003). S, the order parameter, assessed membrane viscosity (Gornicki and Gutsze, 2000) at the hydrophilic portion. CSA was used to observe the membrane structure, especially at the head group level, on ghosts and provided a good overview of membrane dynamics at the polar head level where phosphorus nuclei are located (Debouzy et al., 2002). It is now well known that these parameters are correlated to membrane structure.

CHOL is the main lipid rigidifier in natural membrane and index of cholesterol/phospholipids can be correlated with lipid fluidity (Rock et al., 1995). The CHOL/PL ratio was not affected by oxygenation rate and remained constant perhaps as the result of esterification and de-esterification of serum cholesterol. The constant CHOL/PL could also be responsible for the maintenance of membrane fluidity (Liu and Shan, 2006; Shinitzky, 1984).

Oxidation can induce modifications in membrane structure as a result of free radical attack on fatty acid double bonds. As described above changes in membrane composition induce modifications in membrane dynamic. In this study a correlation was effectively found between T_c and P_{O_2} after 2 h of 100% oxygenation. This means that at this time an increase in P_{O_2} was associated to a decrease in membrane fluidity.

In this study, oxygen failed to induce ‘in vitro’ constant and important modifications on horses’ erythrocytes. In comparison, ‘in vivo’ experiments demonstrated some significant modifications. For example, Vento showed that the use of 100% oxygen during resuscitation of newly born human infants increased arterial P_{O_2} and this hyperoxemia was correlated with an increase of the concentration in oxidized glutathione (GSSG). They also described an increase in antioxidant enzyme activity and conclued that the antioxidant capacity of the newly born infant may have been surpassed (Vento et al., 2002). But the great erythrocyte antioxidant properties, supported by a strong

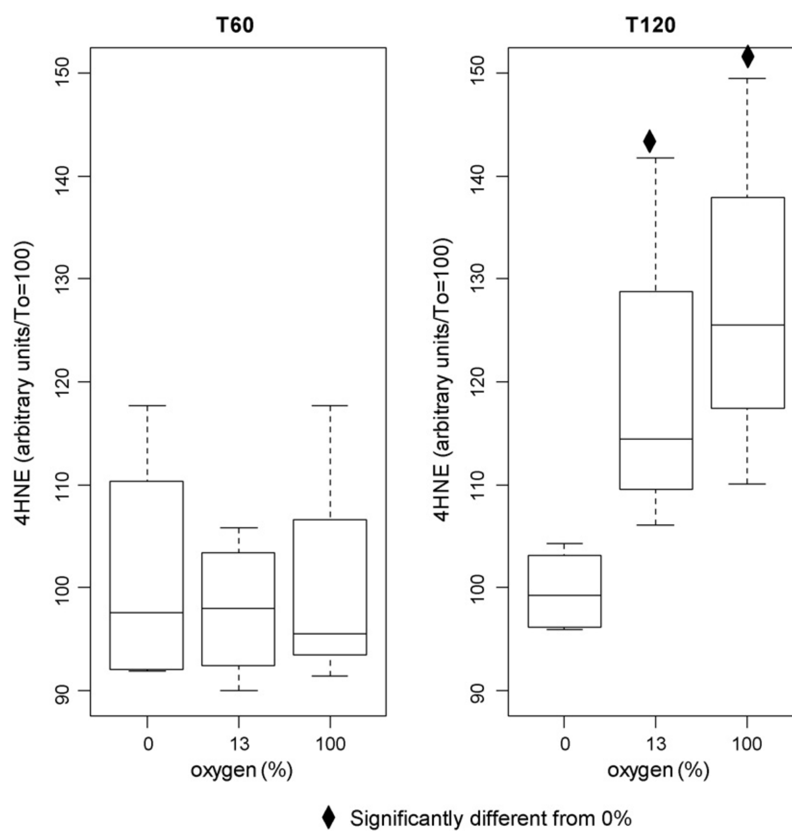

Fig. 2. Concentration of 4-hydroxy-2*E*-nonenal (4-HNE) expressed in arbitrary units (*T*0 controls = 100) measured in jugular vein blood of five healthy horses and submitted to different oxygen gas mixture flow (0%, 13% and 100%) during 1 (*T*60) or 2 h (*T*120). Results were significant if $p < 0.05$.

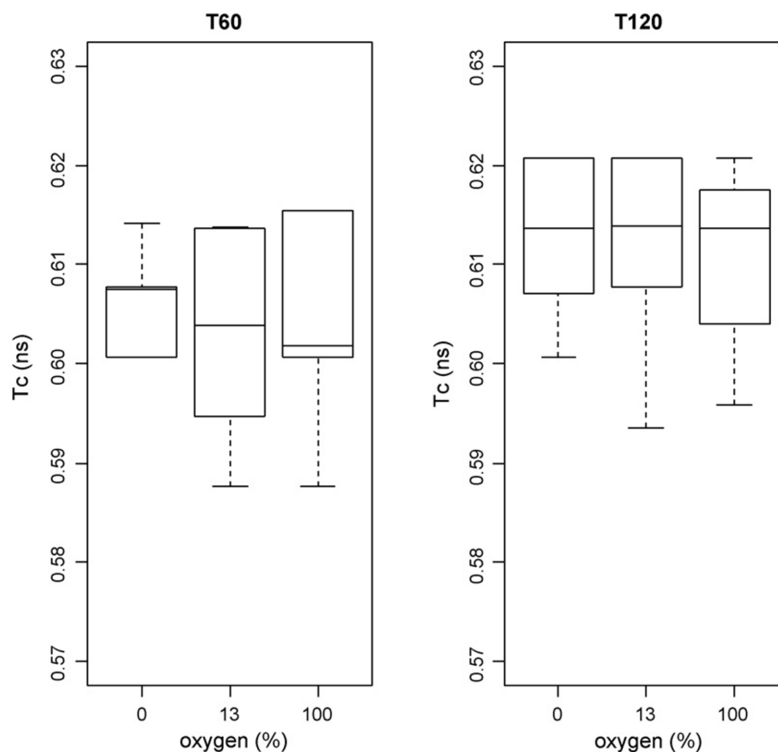

Fig. 3. Correlation relaxation time (T_c), expressed in nanosecond of the 16NS spin probe measured blood withdraw from jugular vein of six healthy horses and submitted to different oxygen gas mixture flow (0%, 13% and 100%) during 1 (*T*60) or 2 h (*T*120). Results were significant if $p < 0.05$.

antioxidant potential in the GSH system (Klemm et al., 2001; Siems et al., 2000) may protect them against oxidative stress induced by O_2, in agreement with their oxygen carrier function.

An increase in oxygen consumption during exercise can also induce oxidative stress and alters the membrane fluidity (Cazzola et al., 2003; De Moffarts et al., 2006; Portier et al., 2006). Indeed free radical formation results in an increased energy demand during intensive muscle activity. This demand leads to an increased electron flow through the electron transport chain in the mitochondria. Mitochondrial electron transport chain is one of the most potent sites for the release of oxygen free radicals (Balogh et al., 2001; Sjodin et al., 1990). The point is that free radical formation, peroxidation and then modification of membrane dynamics are the result of increased energetic mechanisms and of other factors that occur during exercise (increase in temperature for example). Furthermore a study demonstrated that an increase in P_{O_2} in air-exposed storage units of blood, in comparison to anaerobic storage, resulted in a faster decrease in red cell fluidity and in ATP energy change. It was concluded in this study that there was an increased need of high energy phosphate group use for replacement of the phospholipids in membrane bilayer (Hogman et al., 1986). Taken together, these data could explain why an increase in oxygen concentration in the cell environment not associated with an increase in energy consumption, failed to induce strong oxidations and changes in membrane dynamics.

The length of exposure may also have been too short because red cells of rats exposed to 80% oxygen:20% nitrogen for 5 days and compared with red cells of air-breathing animals showed evidence of destruction (Webster and Toothill, 1987). Partial pressure of oxygen may have been to low too because it has been demonstrated that exposure to hyperbaric oxygen leads to oxidative stress too (Benedetti et al., 2004; Dise et al., 1987). Oxygen alone is not as efficient as other oxygen species like ozone that seems to be more efficient in inducing oxidative stress in erythrocyte (Bialas et al., 2001; Gornicki and Gutsze, 2000).

5. Conclusion

In vitro oxidations induced by high oxygen percent flowing in whole blood were efficient enough to induce a peroxidation in erythrocyte membrane lipids but not efficient enough to induce changes in membrane dynamics. This may be due to the absence of increased energy demand in vitro and so to the absence of major free radical formation and/or to the high red blood cells antioxidant properties. The correlation between membrane fluidity and P_{O_2} encourages further in vivo studies on erythrocytes of horses at rest breathing 100% O_2, for example during anaesthesia.

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