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The effects of dietary N-3 and antioxidant supplementation on erythrocyte membrane fatty acid composition and fluidity in exercising horses

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Summary

***Reasons for performing study*: Fatty acid supplementation could modulate erythrocyte membrane fluidity in horses at rest and during exercise, but information is lacking on the effect of exercise.**

***Objectives*: To assess the effect of exercise with, and without, an oral antioxidant supplementation enriched with n-3 fatty acids on erythrocyte membrane fluidity (EMF) and fatty acid composition in eventing horses.**

***Methods*: Twelve healthy and regularly trained horses were divided randomly into 2 groups: *group S* received an oral antioxidant cocktail enriched in n-3 fatty acid (alphatocopherol, eicosapentaenoic acid [EPA] and docosahexaenoic acid [DHA]) whereas *group P* was placebo-treated. At the end of 4 weeks, all horses performed a standardised exercise test (ET) under field conditions. Venous blood was sampled before starting treatment (T0), immediately before (T1) as well as 15 mins (T2) and 24 h (T3) after ET. Spin labelled (16-DOXYL-stearic acid) red blood cell membranes were characterised using the relaxation correlation time (Tc in inverse proportion to EMF). Fatty acid composition (%) of the membrane was determined by gas-liquid chromatography.**

***Results*: Supplementation (*group S*) did not induce changes in EMF (T1 vs. T0) but significant changes in membrane composition were observed and there were increases in n-3 polyunsaturated fatty acid PUFA, n-3/n-6 ratio, and total n-3 fatty acids. Exercise (T2 vs. T1) induced a significant decrease of EMF in *group P* (Tc: +19%, P<0.05) and nonsignificant decrease in *group S* (Tc: +5%), whereas membrane fatty acid composition did not change in either group. During the recovery period (T3 vs. T2), EMF decreased significantly in *group S* (Tc: +29%, P<0.05) and nonsignificantly in *group P* (Tc: +18%) without any significant changes in fatty acid composition.**

***Conclusion and potential relevance*: An enriched oral antioxidant supplementation induced changes in membrane composition, which modulated the decrease in EMF induced by exercise. Long chain n-3 fatty acid supplementation might therefore be beneficial.**

Introduction

Plasma membranes are fluid structures and the maintenance of their fluidity (indirectly evaluated by the relaxation correlation time [Tc], proportional to rotational and lateral diffusion rates of membrane components) is a prerequisite for function. Indeed, numerous studies have shown that membrane composition influences erythrocytes physical properties and functions (Udilova *et al*. 2003; Zamaria 2004). Essential fatty acids (EFA) and their longer derivates are important constituents of phospholipid membranes and determine cellular membrane fluidity. N-3 fatty acid supplementation has been associated with an increase in membrane fluidity in other species (Simopoulos 1991). Essential fatty acids should be taken with food because they are not synthesised in the body. Erythrocyte fatty acid status, therefore, is a reflection of dietary fat intake. Previous studies have demonstrated that fish oil supplementation (rich in n-3 fatty acids) increases red blood cell deformability, decreases heart rate in horses during exercise (O'Connor and Lawrence 2004) and maintains pH homeostasis during intense exercise (Taylor *et al*. 1995; Dunnett *et al*. 2002). These physiological changes may improve exercise performance (Brilla and Lauderholm 1990).

Exercise alone (increasing metabolism and oxygen uptake), or in combination with an increase in fatty acid insaturation, induces oxidative stress and enhances lipid peroxidation (Oostenbrug *et al*. 1997). In comparison with other cells, erythrocytes of horses have a strong system which protects them against oxidative damage. They act as scavengers of free radicals and their flexibility is high in comparison with erythrocytes in man (Amin and Sirs 1985). Nevertheless, they are more sensitive to oxidative stress than those in man and this reduces their deformability (Baskurt and Meiselman 1999). Accordingly, this may have important consequences for tissue perfusion and cardiovascular adequacy in horses (Baskurt and Meiselman 1999).

The aim of this study was therefore to assess the effects of exercise and dietary n-3 and antioxidant supplementation on erythrocyte membrane fluidity and fatty acid composition.

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Materials and methods

Horses

Twelve clinically healthy and regularly trained event horses (6 mares and 6 geldings; mean ± s.d. 10.3 ± 3.5 years) were investigated during a period of one month in spring. All horses were competing at international level (jumping, dressage, cross country). They were divided into 2 groups: one group received during 4 weeks an oral antioxidant cocktail enriched in n-3 fatty acid (*group S*, n = 6) and the second received a placebo (*group P*, n = 6). The horses of each group were matched for sex and age.

Study design

All animals were sampled at the beginning of the study (T0), after 3 weeks of supplementation or placebo administration (T1), 15 mins (T2) and 24 h (T3) after an exercise test (ET) held on a race track. T1 corresponded to the resting value before the ET. Erythrocyte membrane fluidity (EMF) and erythrocyte fatty acids determination were performed at each stage of the study. The following markers were investigated: (i) for EMF determination: relaxation contraction time (Tc) which is negatively proportional to EMF, (ii) for determination of fatty acids in erythrocyte membrane: the relative fraction of saturated fatty acid (SFA), mono-unsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Assessment of a plasmatic oxidant marker, oxidised proteins (prot-ox), was also performed.

Supplement and placebo: composition and administration

All animals were stabled in straw boxes and fed twice daily with hay (4 kg/day) and a commercial cereal blend (2–3 kg/day). In addition, each horse received one dose of TWYDIL[1] competition over a 6 week period before and during the protocol, providing an additional daily intake of Vitamin A: 30000 iu, Vitamin E: 1000 iu, Vitamin C: 750 mg, Cu: 60 mg, Zn: 150 mg and Se: 2.25 mg.

The supplement was administered orally in a 100 ml syringe, once per day, in the morning and prior to feeding. It provided the following quantities of antioxidants and fatty acids: 50 ml of fish oil extract (guaranteed composition: 40% of C20:5n-3 (EPA) and 20% of C22:6n-3 (DHA), α-tocopherol acetate: 5000 mg and copper: 5000 mg). A placebo was used in the control group, containing the same matrix as the supplement without the other ingredients and administered using the same protocol.

Exercise tests (ET)

These were performed on a race track after 3 weeks of dietary supplementation. They consisted of a warm-up at walk (± 5 mins) and trot (3000 m) followed by 3 stages of 1000 m at increasing speed (6.9, 8.3 and 9.7 m/sec) (Serrano *et al*. 2002) and a last stage of 1000 m at individual maximal speed. A cool-down of 5 mins walking finished the ET. The following parameters were assessed during the tests.

Heart rate monitoring: Heart rate (HR) was determined at each stage using a telemetric electro-cardiogram recorder[2].

Venous blood lactate determination: In order to determine exercise intensity, horses were stopped 1 min between each stage and venous blood collected by jugular puncture after each stage for analysis of plasma lactate (LA) and maximal lactate concentration after the last stage (LAmax)[3].

Determination of speed: A GPS system[4] was used in order to determine the speed and confirm that all horses performed the same exercise. This system allowed continuous calculation of mean speed during each stage, calculation of anaerobic threshold, speed at a lactataemia of 4 mmol/l (V_{La4}), speed at a heart rate of 180 and 200 beats/min (V_{180} and V_{200}) and mean speed at the last stage (V_{max}).

Blood sample collection

Venous blood samples (50 ml) were collected at T0, T1, T2 and T3 periods described above, into tubes (5 ml) containing heparin for EMF analysis and disodium EDTA for fatty acids analysis. Tubes were stored at 4°C until analysis of Tc and fatty acids, which were performed within 36 h of collection.

Blood marker analysis

Erythrocyte membrane fluidity: After centrifugation (5 mins, 1000 ***g***) of the heparin tube, a buffer solution (PBS, 100 μl) was added to the erythrocytes (100 μl). Erythrocytes were washed and suspended to 30% haematocrit and 100 μl of the suspension labeled with 1 μl of a radical nitroxyde marked probe, (10^{-2} mol) (16 DOXYL-stearic-acid (16-NS), 253596[5]) in dimethylsulphoxyde (DMSO, 2.10^{-4}mol) and transferred to Pyrex capillary tubes. The tubes were placed in a quartz holder, and inserted into the cavity of a Bruker ECS 106 spectrometer[6] operating at 9.79 GHz. The spectra were recorded at 37°C with the following conditions: microwave power 20 mW, modulation frequency 100 kHz, modulation amplitude 2.868 G, time constant 327 ms. Tc or the rotational correlation time was calculated from Keith's equation (Keith *et al*. 1973) and expressed in nanoseconds. Tc is in inverse proportion to EMF.

Oxidised proteins

Plasma concentration of oxidised proteins was analysed spectrophotometrically allowing determination of carbonyl structures according to the method described by Reznick and Packer (1994).

Fatty acids

Fatty acid composition was determined by gas-liquid chromatography after lipid extraction and preparation of methyl esters.

Lipid extraction: Whole blood was collected by jugular puncture into tubes containing disodium EDTA as anticoagulant. Blood samples (1 ml in glass vial) were centrifuged for 5 mins at 3500 ***g*** and 4°C. Plasma was removed and kept (500 μl) and erythrocytes washed 2 times with 0.85 mol/l NaCl. HCl 3 mol/l (1 ml), C.15.0 (2.2 mg/ml) (20 μl) and Hexane/Isopropanol (3/2) (4 ml) were added to the pellet of erythrocytes, which remained after the final wash and to the plasma and the samples were vortexed for 1 min. These 2 solutions were centrifuged separately for 10 mins at 4000 ***g*** and 4°C.

After the upper layer was drawn-off and kept in closed glass vials, the lower layer was added to Hexane/Isopropanol (3/2) (2 ml), vortexed for 1 min and centrifuged for 10 mins at 4000 ***g*** and 4°C. The upper layer was added to NaCl 0.9%, vortexed for 5 mins at 2000 ***g*** and 4°C and kept in a closed glass vial.

Preparation of methyl esters: These samples, which contained the lipid extracts, were evaporated to dryness (50°C) under a stream of nitrogen. After the samples were dried, 1 ml NaOH 0.5 mol/l in

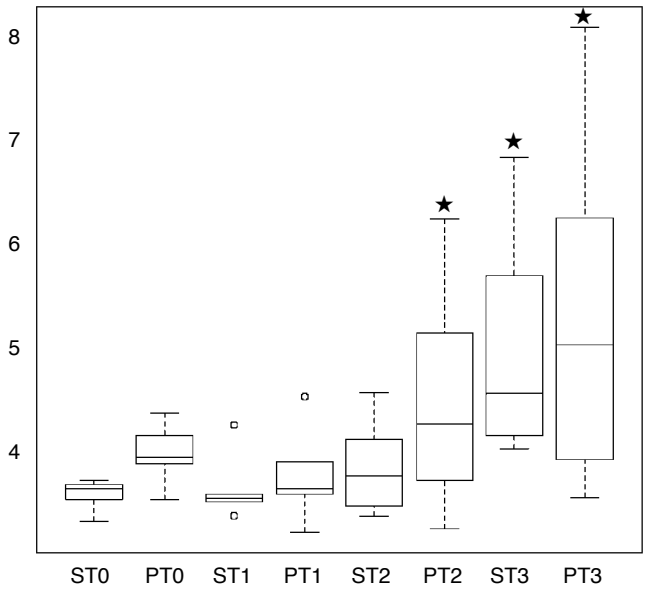

*Fig 1: Tc (correlation-relaxation time, nsecs) determined in jugular venous blood of healthy trained event horses receiving during 3 weeks either an oral placebo (P, n = 6) or an antioxidant supplement enriched in (n-3) fatty acids (S, n = 6). T0: before placebo or supplement treatment, T1: after 3 weeks of placebo or supplement treatment, T2: 15 mins and T3: 24 h after an exercise test. Data are shown as median and range [minimum, maximum]. * significantly different from respective T1 value, P<0.05.*

methanol (2 ***g***/100 ml) and incubated at 70°C for 30 mins and then 1 ml of BF3 14% methanol added. Samples were incubated again at 70°C for 15 mins, then added to pentane (4 ml) and NaCl 0.9% (4 ml) and vortexed. The upper layer was drawn-off and the lower layer added to pentane 2 ml and vortexed.

Methylesters, contained in the upper layer, were concentrated by evaporation under flowing nitrogen just before being injected into a gas chromatograph (CHROMPACK CP 9001 link to integrator SP 4290, capillary column BPX70 0.25 μm with flame ionisation detectors: helium flow as the carrier gas; azote, air and hydrogen were used as the gases for the flame ionisation detectors).

Statistical analysis

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

For evaluation of the effect of complementation, the means of the differences between values calculated at the end of supplementation period (T1) and values calculated before (T0) were calculated for each parameter in each group (T1p–T0p and T1s–T0s). Differences between means of the differences of each group were tested for being significantly different from zero using a Mann-Whitney test.

Evaluation of the impact of exercise and supplementation was performed between T2 and T1 and T3 and T1 as described before. Nonparametric analytical techniques (Wilcoxon signed rank test for matched pairs) were used to compare data within each group at different times (T2s vs. T1s for example). Differences were considered significant if P was less than 0.05. Results are expressed as median and range.

Data concerning speed and physiological parameters during exercise test had normal distributions and are expressed as parametric data, mean ± s.e.

Results

Supplementation period (T0–T1)

No significant difference was observed during this period regardless all parameters in *group P* (Table 1, Fig 1).

Impact on membrane fluidity: The correlation-relaxation time (Tc in nanoseconds) at T0, T1, T2, T3 in *P* and *S* groups is shown in

TABLE 1:

Data	Group	T0	T1	T2	T3
Protox	S	4.81 (4.67–5.62)^{ac}	3.98 (2.24–4.21)^{ac}	3.89 (2.25–4.68)^{ac}	5.52 (4.20–7.61)^{ad}
10^{-2} nmol/mg prot	P	4.96(2.76–6.42)^{ac}	3.89 (3.11–5.20)^{ac}	4.43 (2.57–5.20)^{ac}	4.97 (4.47–5.69)^{ad}
% C20:5n-3	S	0.59 (0.40–0.80)^{ac}	1.14 (1.03–1.83)^{ad}	1.27 (1.16–2.27)^{ad}	1.41 (1.07–2.05)^{ad}
	P	0.52 (0.13–0.96)^{ac}	0.48 (0.23–1.08)^{bc}	0.65 (0.28–1.14)^{ad}	0.59 (0.01–1.20)^{ad}
% C22:6n-3	S	0.17 (0.11–0.32)^{ac}	0.76 (0.51–0.95)^{ad}	0.66 (0.53–1.05)^{ad}	0.71 (0.50–0.84)^{ad}
	P	0.19 (0.11–0.75)^{ac}	0.12 (0.01–0.22)^{bc}	0.19 (0.01–0.76)^{ac}	0.19 (0.10–0.31)^{ac}
% n-3	S	2.62 (2.34–2.74)^{ac}	4.23 (3.72–4.82)^{ad}	4.76 (3.59–5.83)^{ad}	4.63 (3.55–5.10)^{ac}
	P	3.05 (2.07–3.60)^{ac}	3.22 (3.00–3.90)^{bc}	3.58 (1.67–4.09)^{ac}	3.11 (1.21–4.27)^{ac}
n-3/n-6	S	0.07 (0.05–0.08)^{ac}	0.13 (0.11–0.15)^{ad}	0.12 (0.10–0.16)^{ad}	0.13 (0.10–0.16)^{ad}
	P	0.08 (0.05–0.10)^{ac}	0.09 (0.08–0.11)^{bc}	0.10 (0.08–0.11)^{ac}	0.09 (0.06–0.13)^{ac}
% SFA	S	34.37 (31.75–36.43)^{ac}	38.77 (36.94–44.78)^{ac}	32.00 (23.77–41.74)^{ac}	36.56 (30.96–46.53)^{ad}
	P	34.20 (31.39–36.29)^{ac}	34.29 (31.70–42.33)^{ac}	32.33 (28.82–51.75)^{ac}	36.5 (31.75–52.02)^{ac}
% MUFA	S	24.65 (22.83–25.17)^{ac}	22.2 (21.17–24.09)^{ac}	23.56 (19.80–24.95)^{ac}	23.20 (20.3–27.32)^{ad}
	P	24.03 (22.86–27.05)^{ac}	22.95 (21.23–25.14)^{ac}	24.30 (23.81–35.6)^{ac}	25.04 (19.53–32.16)^{ac}
% PUFA	S	41.2 (38.64–45.41)^{ac}	39.19 (34.07–40.33)^{ac}	43.32 (36.65–45.55)^{ac}	39.48 (33.18–45.87)^{ac}
	P	41.19 (39.65–43.75)^{ac}	41.53 (35.35–44.62)^{ac}	42.64 (16.61–47.37)^{ac}	40.05 (15.81–43.44)^{ac}
% UFA	S	65.64 (63.57–68.24)^{ac}	61.23 (55.24–63.03)^{ac}	67.98 (58.27–68.30)^{ac}	63.44 (53.48–69.03)^{ad}
	P	65.79 (63.69–68.6)^{ac}	65.71 (57.69–68.29)^{ac}	67.68 (48.24–78.52)^{ac}	65.09 (47.97–68.25)^{ac}
% PUFA/SFA	S	1.21 (1.06–1.43)^{ac}	1.02 (0.76–1.09)^{ac}	1.35 (0.90–1.83)^{ac}	1.09 (0.71–1.48)^{ac}
	P	1.23 (1.11–1.32)^{ac}	1.23 (0.83–1.36)^{ac}	1.32 (0.32–1.64)^{ac}	1.10 (0.30–1.37)^{ac}

Oxidised protein (prot-ox) in nmol/mg of proteins and composition in fatty acids in percent for n-3 fatty acids (n-3) (%),n-3 fatty acids/n-6 fatty acids ratio (n-3/n-6), saturated fatty acids (SFA) (%), monounsaturated fatty acids (MUFA) (%), polyunsaturated fatty acids (PUFA) (%), unsaturated fatty acids (UFA) (%), polysaturated/saturated fatty acids ratio (PUFA/SFA) (%), determined in jugular venous blood of 12 healthy trained event horses before (T1), 15 mins (T2) and 24 h (T3) after a exercise test. The horses were treated during 3 weeks (T0–T1) with an antioxidant supplement enriched in (n-3) fatty acids (S, n = 6) or with an oral placebo (P, n = 6). Data are shown as median and range. Differences between the placebo and supplement at each sampling point are significant (P<0.05) if the first superscript is different (^{a} or ^{b} for with-in column comparisons), whereas differences between sampling times are significant (P<0.05) if the second superscript is different (^{c} or ^{d}) for within-line comparisons.

Fig 1. No time (T0 vs. T1)- or treatment (*S* vs. *P*)-related changes were observed for *S* and *P*.

Impact on fatty acids membrane composition: Significant differences (T0 vs. T1) were observed (Table 1) between the groups for the following parameters (expressed in percentage of total fatty acids): for SFA, C14 decreased in *P* from 0.84 [0.62–1.25]% to 0.42 [0.18–0.97]% (P<0.05) and C20 increased in S from 1.23 [1.18–1.49]% to 2.16 [1.71–2.62]% (P<0.05); for MUFA, C16:1n-9 increased in *S* from 0.49 [0.33–0.53]% to 1.09 [0.18–1.86]% (P<0.05); n-3 PUFA increased in *S*: C18:3n-3 from 1.64 [1.41–1.77]% to 1.78 [1.61–2.07]%, C20:5n-3 (+124%), C22:6n-3 (+311%) and total n-3 fatty acids (+64%), (P<0.05); n-6 PUFA decreased in *S*: C18:2n-6 from 35.36 [32.55–39.80]% to 31.27 [26.70–32.78]% and C18:3n-6 from 0.32 [0.23–0,56]% to 0.11 [0.01–0.27]%, (P<0.05); n-3/n-6 ratio increased in *S* (+86%; P<0.05).

No significant differences during this period were recorded between and within the groups for the other fatty acids neither for total n-6 fatty acids, SFA, MUFA, PUFA, UFA (unsaturated fatty acids), PUFA/SFA ratio.

Exercise (T2–T1) period

ET: There were no significant differences (Table 2) between the groups for all parameters measured during the exercise tests (inframaximal criteria). Mean speeds at the first step was 7.7 ± 1.1 m/sec, at the second step 8.5 ± 0.3 m/sec, at the third step 9.6 ± 0.3 m/sec and at the last step 11.1 ± 0.8 m/sec.

Impact on membrane fluidity: Tc increased (Fig 1) significantly after ET period in P: (T1 vs. T2) (3.71 [3.30–4.61] ns vs. 4.35 [3.33–6.32] ns, P<0.05), whereas no significant difference was observed in S: (T1 vs. T2) (3.62 [3.46–4.33] ns vs. 3.84 [3.46–4.65] ns). Tc did not differ significantly between groups during this period at T1, T2 and for the difference T2-T1 in P: 0.38 [0.03–2.34] ns and *S*: 0.22[-0.04–0.53] ns).

Impact on membrane composition in fatty acids (Table 1)

Total percentage of PUFA, MUFA, SFA and UFA did not change significantly after exercise but the distribution of fatty acid within each category varied between T1 and T2: for SFA, C12 increased from 0.07 [0.04–0.18]% to 0.17 [0.13–0.31]% (P<0.05), C22 decreased from 2.92 [0.48–7.62]% to 2.32 [0.01–5.55]% (P<0.05); for MUFA, C20:1n-9 increased from 0.69 [0.39–1.05]% to 0.97 [0.64–2.88]% (P<0.05) and for PUFA, C20:5n-3 was increased (+24%; P<0.05; Table 1). Total percentage of PUFA, MUFA, SFA and UFA did not change significantly after exercise but the distribution of fatty acid within each category varied between T1 and T2: for SFA, C18 decreased from 18.87 [17.38–19.55]% to 16.91 [16.23–18.36]% (P<0.05), for MUFA: C16:1n-9 decreased from 1.09 [0.18–1.86]% to 0.25 [0.14–0.43]% (P<0.05) and C16:1n-7 increased from 0.79 [0.48–1.16]% to 0.94 [0.49–1.38]% (P<0.05); for PUFA, C18:3n-3 increased from 1.78 [1.61–2.07]% to 1.99 [1.65–2.27]% (P<0.05).

Significant differences were observed between *P* and *S* in response to exercise (T2–T1) for C16.1n-9 (-0.04 [-0.21–0.76]% vs. -0.88 [-1.58– -0.04]%, P<0.05) and C20.1n-9 (0.12 [0.07–2.49]% vs. 0 [-0.51–0.13]% , P<0.05).

Recovery (T3–T2) period

Impact on membrane fluidity: Tc increased significantly (Fig 1) during this period in both groups, and no significant differences between the groups were observed. In *group P*, no further increase of Tc was noted between T2 and T3, whereas in *group S*, where no changes of Tc had been recorded between T1 and T2, a significant increase of Tc occurred at T3. The magnitude of the Tc changes did not significantly differ between *P* and *S* for the period T3–T2 (0.42 [-0.42–2.53] ns vs. 0.88 [0.28–3.15] ns) and T3-T1 (0.97 [0.33–3.86] ns vs. 1.01 [0.62–3.29] ns).

Impact on oxidised proteins: Concentration in prot-ox increased significantly in both groups after exercise. This increase was nonsignificantly higher in *P*. Results are shown in Table 1.

Impact on fatty acid membrane composition: Significant differences (T3 vs. T2) were observed in *P* during recovery period for SFA: C20 increased from 1.28 [0.01–2.32]% to 1.79 [0.83–2.86]% (P<0.05). Significant differences (T3 vs. T2) were observed in *S* during exercise period for MUFA: C16:1n-7 decrease from 0.94 [0.49–1.38]% to 0.83 [0.45–1.23]% (P<0.05). The changes between the recovery period (T3–T2) were significantly different between *P* and *S* for C16:1n-9 (-0.05 [-1.15–0.01]% vs. 0.19 [-0.05–0.86]%, P<0.05).

Discussion

Effect of oral antioxidant and n-3 fatty acid supplementation

As expected, our results give evidence that enriched oral supplementation with n-3 fatty acids induced changes on erythrocyte membrane composition. Indeed, supplementation increased significantly the percentage of n-3 fatty acid content in erythrocyte membrane and increased the n-3/n-6 ratio. This was a result of the dramatic supply in C20:5n-3 (EPA) and C22:6n-3 (DHA) from fish oil and a selective incorporation of these PUFA in the erythrocyte membrane. In this study, the 3 weeks duration of the supplementation period was long enough to induce changes in fatty acids membrane content. Most dietary fat supplementation studies in horses have been for more than one month (Henry *et al.* 1991; Hansen *et al.* 2002), but some lasting only 3 weeks, have been previously reported in man (Oostenbrug *et al.* 1997). The quality and quantity of the supplementation were also sufficient to induce changes in fatty acid membrane concentration. Supplementation with n-3 might have beneficial effects in health and sport performance. Indeed, in man, several studies have

TABLE 2:

Group	V_{La4} (m/sec)	V_{180} (m/sec)	V_{200} (m/sec)	V_{max} (m/sec)	LA_{max} (mmol/l)	HR_{max} beats/min
S	8.75 ± 0.28	8.86 ± 0.36	10.5 ± 0.39	11 ± 0.29	13.1 ± 1.81	204 ± 23
P	8.53 ± 0.32	8.9 ± 0.44	10.4 ± 0.29	11.3 ± 0.42	13.9 ± 2.63	202 ± 16

V_{La4} (speed at a lactataemia of 4 mmol/l); V_{180} and V_{200} (speed at a heart rate of 180 beats/min and 200 beats/min); Vmax (mean speed at the last step, LAmax (maximal lactate concentration after the last maximal speed), HRmax (maximal heart rate) determined during exercise test which consisted in 3 stages of 1000m at increasing speed (7.7 ± 1.1 m/sec, at the second step 8.5 ± 0.3 m/sec, at the third step 9.6 ± 0.3 m/sec and at the last step 11.1 ± 0.8 m/sec) undertaken by healthy trained event horses receiving during 3 weeks, just before the test, either an oral placebo (P, n = 6) or an antioxidant supplement enriched in (n-3) fatty acids (S, n = 6).

indicated that dietary fish oil increased the deformability of erythrocyte as a result of the incorporation of n-3 fatty acids in the membrane and facilitated the transport of erythrocyte in the microcirculation (Shiga and Maeda 1980). This could lead to improved exercise performance (Oostenbrug *et al*. 1997) and it is now well known that n-3 PUFA have anti-inflammatory, antithrombotic and antiarrhythmic effects, and immuno-modulating properties (Zamaria 2004).

N-3 might also have beneficial effects improving EMF. Indeed, numerous studies in animal species demonstrated that n-3 fatty acid had beneficial effects by increasing membrane fluidity and suggested that it could provide benefits during exercise by lowering heart rate and decreasing serum cholesterol in horses (O'Connor and Lawrence 2004). In our study, despite the structural differences observed in erythrocyte membrane, the fish oil supplementation had no effect on membrane fluidity observed during this period. These results do not correspond with previous data obtained in various species (Shinitzky 1984; Keddad *et al*. 1996; Cazzola *et al*. 2004). The lack of change in EMF despite membrane structural modifications may be explained by the accumulation of n-3 PUFA, especially DHA (docosahexaenoic acid), which could counteract the effect of enrichment in n-3 fatty acid content. Indeed, their high degree of unsaturation could enhance the degree of membrane unsaturation and consequently make the membrane more sensitive to peroxidation (Udilova *et al*. 2003), providing a variety of lipid peroxides and aldehydic breakdown products with pro-oxidant properties (Delton-Vandenbroucke *et al*. 2001). Furthermore, the anti-inflammatory effect of n-3 PUFA is related to oxygen radical production (Costabile *et al*. 2005).

Therefore, a balanced intake of both n-6 and n-3 PUFA are essential for good health. In this study no significant change was observed in n-6 fatty acids irrespective of the group, whereas n-3 (and n-3/n-6) increased significantly in the treated group. A decrease in n-6 fatty acids was expected because it has previously been described that the n-3 fatty acid series competes with the n-6 fatty acid series for incorporation into the cell membrane (Hansen *et al*. 2002).

On the other hand, many other variables such as type of phospholipid, fatty acid chain length, degree of unsaturation and cholesterol content may induce changes in EMF (as the cholesterol content of the RBC increases, EMF decreases (Dumas *et al*. 1997; Buchwald *et al*. 2000)), and then explain this mismatch (Shinitzky 1984). The PUFA/SFA ratio, another fluidity modulator (Shinitzky 1984), did not change after the supplementation period, which is consistent with the failure of change in EMF.

Impact of exercise

Correlation was demonstrated between the physiological response to exercise and some markers of oxidation in equine plasma and erythrocytes (Ono *et al*. 1990; Kirschvink *et al*. 2002). Oxidative stress may also depend on exercise intensity in the equine athlete (Mills *et al*. 1996).

The exercise protocol designed in this study aimed to simulate equine competition at high intensity. Immediately following cessation of exercise, EMF was only found to have decreased in the placebo group. After exposure to oxidative stress induced by exercise (prot-ox as a plasmatic marker of proteins oxidation (Kinnunen *et al*. 2005) increased after ET in both groups), electron spin resonance (ESR) study on intact erythrocyte revealed an increase in correlation time of 16-DOXYL-stearic acid. The 16NS probe was near the interface between the two membrane hemilayer and was directly related to the whole membrane fluidity (Keddad *et al*. 1996). Indeed, it has been shown that the nitroxylic radical of 16-DS penetrates no less than 2.2 nm from the membrane surface (Schreier-Mucillo 1976). This feature of spin-labelled fatty acid derivatives allowed us to evaluate membrane fluidity at an area close to the inner core of plasma membrane. Consequently, the increase in Tc gives strong evidence of a significant decrease in erythrocyte membrane fluidity (EMF) in the untreated group. Changes in membrane fluidity have yet to be described in man after exercise as a result of oxidative stress (Cazzola *et al*. 2003). However, as a result of training, higher levels of erythrocyte membrane fluidity were found in human athletes (Kamada *et al*. 1993).

The significant decrease in EMF after exercise was associated with only a few membrane structural changes. Therefore, other changes might explain the Tc increase. For example, the ratio of cholesterol to phospholipid should have been a better index to correlate to EMF (Rock *et al*. 1995). Another important factor to be taken into consideration is ageing because it has been previously found to induce a decrease in membrane fluidity (Shiga and Maeda 1980).

Unlike the placebo group, EMF in the treated group remained unchanged 15 mins after exercise. As a result of the peroxide process a decrease in PUFA could have been expected together with a decrease in the PUFA/SFA ratio, as previously reported (Keddad *et al*. 1996). In the placebo group there was a nonsignificant decrease in PUFA and we did not find any change of PUFA and PUFA/SFA ratio in the treated group. It can therefore be hypothesised that a supplement might limit peroxidation in PUFA and consequently reduce changes of EMF.

Recovery period (24 h post exercise)

A delayed significant increase in Tc 24 h after cessation of exercise (T3 vs. T2) was observed in *S* while the value in *P* remained elevated without further significant increase. We hypothesise that the supplementation delayed, but did not prevent, changes in membrane fluidity.

Conclusion

Our study found that erythrocyte membranes in horses are less fluid after exercise and that a fat diet enriched with n-3 fatty acid might delay this alteration. Further studies should be undertaken to assess whether exercise-induced oxidative stress also affects membrane fluidity of erythrocytes.

Manufacturers' addresses

[1]Pavesco AG, Basel, Switzerland.
[2]Life Scope Monitor, Nihon, rosbach, Germany.
[3]Accusport, Boehringer, Mannheim, Germany.
[4]Equipilot, Fidelak, Kamen, Germany.
[5]Sigma-Aldrich.
[6]Bruker, Karlsruhe, Germany.

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