Effects of competition on acute phase proteins and lymphocyte subpopulations – oxidative stress markers in eventing horses

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Effects of competition on acute phase proteins, lymphocyte subpopulations, oxidative stress markers in eventing horses.

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Running head: Acute phase response in exercising horses

Summary

The aim of the study was to evaluate markers of the acute phase response (APR) in eventing horses by measuring acute phase proteins (APP) (haptoglobin, Hp, and serum amyloid A, SAA), lysozyme, protein adducts like pentosidine-like adducts (PENT), malondyaldeyde adducts (MDA), hydroxynonenal adducts (HNE) and total advanced glycation/glycoxidation end products (AGEs), complete blood count and lymphocyte subpopulations (CD4+, CD8+ and CD21+) both at rest and at the end of an eventing competition. Blood samples were collected from 8 Warmblood horses (medium age 10 ± 3) during an official national two-day event competition at rest (R) and 10 minutes after arrival of the country-cross test on the second day. Exercise caused a significant increase in red blood cell number, haemoglobin, packed cell volume, neutrophils, white blood cell and lymphocyte number, however these values remained within the normal range. The CD4+ and CD8+ cells significantly increased, whereas the CD21+ lymphocytes decreased; a significant increase in serum SAA, lysozyme and protein carbonyl derivates was also observed. Two day event causes significant changes in APR markers such as lysozyme, protein carbonyl derivates (HNE, AGEs, PENT) and lymphocyte subpopulations. Data support the hypothesis that two-day event may alter significantly APR markers. Limitations of the study were the relatively small sample size and sampling time conditioned by the official regulations of the event. Therefore further studies are needed to investigate the time required for recovery to basal values in order to define the possible effects on the immune function of the athlete horse.

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Keywords: horse, acute phase response, lymphocyte subpopulations, protein carbonyl adducts

Introduction

50 The study of the extent and nature of the acute phase response (APR) to exercise of different intensity
51 and duration is crucial to optimize horse performance and to preserve animal welfare. The APR is
52 an immune-based reaction to several non-specific stimuli including exercise, characterized by
53 oxidative stress and the release of acute phase proteins (APPs) (Scopetta et al., 2012; Fallon, 2001).
54 Among different exercise where horses are involved, eventing, that represents a demanding discipline
55 for horses, has received little attention within the scientific available literature. Nevertheless, the
56 response elicited by this kind of physical stress could have important modulatory effects on
57 immunocyte dynamics and possibly on immune function. These effects are mediated by different
58 factors including exercise-induced release of pro-inflammatory cytokines, classical stress hormones
59 and hemodynamic effects leading to cell redistribution (Pedersen and Hoffman-Goets, 2000).
60 Particularly, during exercise adrenaline and glucocorticoids modulate the immune system. Immune
61 cells support production of potent circulating inflammatory mediators, i.e. interleukin (IL)-6, IL-1
62 and tumour necrosis factor (TNF)-α (Donovan et al., 2007; Holbrook et al., 2010).
63 The response pattern of the APR could be species specific especially for APPs with the exception of
64 serum albumin which decreases by 10–30% to several non specific stimuli in all mammalian species
65 (Suffredini et al., 1999). In horses, serum amyloid A (SAA) is defined as the major APP and
66 haptoglobin (Hp) as a moderate APP (Cywinska et al., 2012). Blood SAA concentrations markedly
67 increase in 24 hours in response to infections and surgical trauma as well as to a long distance rides
68 of 120-160 km (Cywinska et al., 2012; Hulten et al., 1999). Therefore exercise can act as stressor
69 promoting an APR, however no data are available concerning the APP levels following two day event
70 competition.
71 Several reports describe exercise-induced changes in subsets of blood mononuclear cells (McCarthy
72 and Dale, 1988; Pedersen and Hoffman-Goets, 2000; Pedersen and Nieman, 1998). Specific
73 populations within the circulating leucocyte pool vary significantly with exercise and there is some
74 evidence that in humans the CD4+/CD8+ lymphocyte ratio may become significantly reduced (Keast

et al., 1988). According to Malinowski and coworkers (2006) a simulated race test causes changes in the CD4+:CD8+ ratio in Standardbred horses. Nevertheless, few data are available concerning the effects of exercise on lymphocyte subpopulations in horses.

A heavy acute exercise is also known to induce oxidative stress (OS) (Vider et al., 2001). In this condition a high concentration of free radicals can damage molecules such as proteins. Specifically, protein oxidative modification can be due to direct damage by reactive oxygen species or indirectly by reaction of secondary by-products of OS (Dalle et al., 2003a). In this case, for example, lipid peroxidation breakdown products such as hydroxynonenal (HNE) and malondialdehyde (MDA) or advanced glycation/glycoxidation end products (AGEs) such as pentosidine (PENT) (Valle et al., 2012) are able to link to protein aminoacid residues leading to the formation of protein carbonyl derivates (Dalle Donne et al., 2003; Traverso et al., 1998). These compounds may be used as measure of protein damage (Odetti et al., 1999) and markers of oxidative stress. In fact, they are stable adducts produced early and circulate in the blood for long periods (Dalle Donne et al., 2003 b). In human plasma and serum samples many of these adducts have been effectively quantified thanks to their fluorescent properties attributable to double bonds and ring structures in a number of moieties using fluorometry (Forbes et al., 2005). In humans there are some evidence demonstrating changes in AGEs following physical activity (Kotani et al., 2011); however, no data are available on sport horses.

On the basis of these premises, the aim of the study was to evaluate possible markers of the APR in eventing horses by measuring APPs (SAA, Hp), lysozime, protein adducts (HNE, MDA, AGEs, PENT), complete blood count (CBC) and lymphocyte subpopulations (CD4+, CD8+, CD21+) both at rest and at the end of a 2-day event competition.

Materials and methods

Animals

99 Eight experienced Warmblood horses (5 gelding, one stallion and 2 females), medium age 10
100 ± 3 (mean ± standard deviation (s.d.)), mean body weight 580±41 kg were included in the study. All
101 horses were clinically healthy and had not received any medication during the last 15 days before the
102 beginning of the study. The animals were kept in the same stable and fed with first cut meadow hay
103 and commercial cereal-mix feed for sport horses (crude protein 12.5%, crude fat 5.2%, crude ash
104 8.5%, crude fiber 8.5%, Vitamin E 200 UI/kg and Selenium 0.35 mg/kg) in a ratio forage:concentrate
105 corresponding to 60:40. They were trained six times a week with a typical conditioning program for
106 eventing horses: flat work (50 min/day, 3 days/wk.), flat work and jump (50 min/day, 2 days/wk.)
107 and conditioning (warm up and gallop at 450-480 m/min for 5-8 minutes once a week).
108
109 Competition feature
110 Data were collected during an official national two-day event competition organized by the
111 Italian Equestrian Federation at Cameri sport club (Italy). The competition was held in May, the
112 weather conditions remained relatively constant throughout the two days of competition
113 (environmental temperature 19°C, relative humidity 66 %). The ground conditions made up of grass
114 were similar for all horses, irrespective of starting order. On day 1, horses performed the dressage
115 (marks given for medium walk, working-medium-extended trot, working canter, working-medium-
116 collected canter, shoulder in at trot and rein back) and the show jumping phase (maximum fence 1.20
117 m; speed 350 m/min). On day 2, animals performed the cross country test on a distance of 3100 m
118 (maximum distance) (minimum speed 530 m/min and fence 1.10 m maximum height). All horses
119 competed between 8:30 am and 11:30 am.
120
121 Blood samples collection
122 Blood samples were collected by jugular venipuncture at rest (R), between 8:00 and 9:00 a.m.,
123 in the original barn, before being moved to the competition site. Horses were moved the day before

the competition with a travel of about 50 minutes. A second blood sample was performed within 10 minutes after the end (PC) of the cross country phase on day 2. This time frame was influenced by the official regulations of the competition. Samples for the determination of APPs and lysozyme were placed into 10 mL vacutainer tube contain clot activator, allowed to clot at room temperature, centrifuged at 2500 g for 10 minutes and aliquots of the obtained sera stored at -80°C until analysis. Blood for the determination of complete blood cell count (CBC), lymphocyte subpopulations was placed into a 10 mL EDTA vacutainer tube, refrigerated and immediately transferred to the laboratory for analysis. For protein adducts plasma aliquots were obtained by centrifugation (2500 *g* for 10 minutes at 4°C) immediately after collection and then stored at -80° C until analysis.

Complete blood count

Complete blood count was performed by using an automated laser cell counter hematology System (Advia 120, Siemens Diagnostics Inc., Tarrytown, USA) to assess the following parameters: white blood cell count (WBC, $10^3/\mu L$), neutrophils (NEUT, $10^3/\mu L$), lymphocytes (LYM, $10^3/\mu L$), red blood cells (RBC, $10^6/\mu L$), hemoglobin (Hb, g/dL), packed cell volumes (PCV, %), platelets (PLT $10^3/\mu L$).

Lymphocyte subpopulations

Lymphocyte subpopulations were characterized by flow cytometry using monoclonal antibodies against the following antigens: CD21+ (CA2.1D6, Serotec, B cells, Serotec, Kidlington, UK), CD4+ (CVS4, Serotec, T-helper, Serotec, Kidlington, UK), CD8+ (CVS8, Serotec, T-cytotoxic/suppressor, Serotec, Kidlington, UK). For surface staining, 100 µL of whole blood were incubated with monoclonal antibodies at the appropriate dilution for 30 min at 4 °C. After two washes with phosphate-buffered saline (PBS), cells were incubated with 10 µL of 1:5 diluted fluorescein isothiocyanate (FITC)-labeled goat anti mouse immunoglobulin F (ab') 2 fragment (SouthernBiotech,

149 Birmingham, USA) for 30 min at 4 °C. Negative controls consisted of cells incubated with
150 fluoresceinated goat anti mouse antibody alone. Two milliliters of lysing solution (ammonium
151 chloride) were added and left for 20 min at room temperature. Cells were then washed twice and
152 resuspended in 0.5 mL of PBS.
153 A minimum of 10000 events from a gated lymphocyte population was acquired and analyzed on a
154 Epics XL-MCL flow cytometer (Coulter, cCoulter® Epics® XL-MCLTM Miami, USA), thus
155 obtaining percentages of the different subpopulations. Absolute lymphocyte subset counts were
156 calculated as the product of the lymphocyte absolute count and the lymphocyte subset percentage.
157
158 Protein carbonyl adducts
159 Delipidated plasma samples were analyzed for fluorescence modification with
160 spectrophotofluorometer at wave-length of 370-nm excitation (EX)/440-nm emission (EM) for total
161 AGE adducts (AGEs), 335-nm EX/385-nm EM for pentosidine-like adducts (PENT), 390-nm
162 EX/460-nm EM for malondyaldeyde adducts (MDA), 356-nm EX/460-nm EM for hydroxynonenal
163 adducts (HNE) and 280EX/390-nm EM for triptophane (Trip).
164 Control of the absorbance of the protein solution was performed in order to rule out any possible
165 interference with the evaluation of fluorescence: the absorbance of the protein solution at the
166 wavelengths used for fluorescence excitation never exceeded 0.1; therefore absorbance should not
167 have been significant as possible interference on the evaluation of fluorescence. Fluorescence
168 intensity was expressed in arbitrary units (AU) of fluorescence per g of total proteins.
169
170 Acute phase proteins and lysozyme
171 Serum SAA and Hp concentrations were measured using a commercial assay kits (Phase, Tridelta
172 Ltd. Ireland). Serum lysozyme was assessed by the lyso-plate assay method according to Osserman
173 and Lawlor (1966); briefly, serum samples were reacted with a suspension of *Micrococcus*

174 *lysodeikticus* in agar gel in 10-cm Petri dishes. Under these conditions, lysozyme concentration
175 (μg/mL) is proportional to the diameter of lysis areas and is determined from a standard curve created
176 with a standard lysozyme preparation (Sigma-Aldrich SRL, Milano).
177
178 Protein content determination
179 Plasma protein content was determined using the BCA protein assay kit according to the
180 manufacturer's instructions (Pierce, BCA Protein Assay).
181
182 Statistical analysis
183 Statistical analyses were performed with SPSS software package (SPSS, Chicago, Illinois, USA).
184 After checking the normality, a paired sample t-test was used to compare the values for lymphocyte
185 subpopulation data, protein carbonyl adducts data, acute phase proteins and lysozyme and complete
186 blood count data at rest and after exercise. Statistical significance was set at $p \leq 0.05$. All measurements
187 were performed in triplicate and data are expressed as mean ± SD
188
189 **Results**
190 Complete blood cell count
191 Exercise caused a significant increase in red blood cell number, haemoglobin concentration, packed
192 cell volume percentage, neutrophils, white blood cells numbers, and lymphocytes and a decrease in
193 platelet number (Table 1). However all the values were in the reference range values according to
194 the Laboratory of the Department of Veterinary Sciences, University of Turin
195
196 Lymphocyte subpopulation
197 The absolute number of CD4+ cells was significantly higher after exercise ($1650 \pm 110\ 10^3$ cell/μL,
198 $p = 0.01$) compared to basal values ($1260 \pm 80\ 10^3$ cell/μL), while the relative percentage with respect

to the total lymphocyte population was decreased (54.51 ± 6.05 % vs 60.73 ± 5.50 %, p = 0.002) (Fig 1a-1d). Similar results were obtained also for the CD8+ cell subpopulation (300 ± 30 10^3 cell/µL, R, vs 620 ± 90 10^3 cell/µL, PC, p = 0.007) (Fig 1b). However, the relative percentage was increased after competition (20.66 ± 7.90 % vs 13.71 ± 2.28 %, p=0.02) (Fig 1d). The CD4+:CD8+ ratio was significantly (p = 0.047) decreased at the end of the event compared to its value at rest (3.03 ± 0.6 vs 4.62 ± 0.4, p = 0.047). From rest to after the event, the CD21+ subpopulation significantly decreased (270 ± 30 10^3 cell/µL, R, vs.160 ± 20 10^3 cell/µL, PC, p = 0.01, Figure 1c) and represented a smaller portion of lymphocytes in percentage (12.80 ± 4.27 % vs. 5.25 ± 1.49 %, p = 0.002; Figure 1d).

Acute phase proteins and lysozyme

After exercise, a significant increase in serum SAA (1.96 ± 0.55 µg/mL, R, vs 3.91 ± 0.73 µg/mL, PC; p = 0.001) and lysozyme (3.35 ± 0.85 µg/mL vs 4.52 ± 1.57 µg/mL; p = 0.05) was observed. No significant changes of HP concentration was observed (0.91±0.42 mg/mL vs. 1.29±0.55 mg/mL; p = 0.169)

Protein carbonyl adducts

Exercise caused a significant ($p < 0.05$) increase of blood concentration in total AGE adducts, pentosidine-like adducts, hydroxynonenal adducts and tryptophan. For MDA adducts, besides a trend suggesting an increase at the end of competition, no significant differences were observed (p = 0.058) (Fig 2).

4. Discussion

To our knowledge, this is the first study investigating the effects of exercise on acute phase response in eventing horses. The modifications observed in complete blood cell count may be considered as typical expected exercise-induced responses in performance horse (Jumping Endurance and

Standardbred horses) (Cuniberti et al., 2012). White blood cells and neutrophils increase during and after exercise, whereas lymphocyte concentrations increase during acute exercise and fall below basal values after long-duration physical exercise (Pedersen and Hoffman-Goets, 2000). In our study, both neutrophil and lymphocyte number increase after exercise. However, according to Malinowski et al., (2006) in the horse the magnitude of lymphocyte number modifications due to exercise depends on age and training. The increase in the total number of red blood cells, packed cell volume and haemoglobin concentration can be ascribed both to spleen contraction regulated by catecholamine and to sweating that causes fluids loss (Cuniberti et al., 2012).

In our in-field study, for practical reasons, we did not measure horse body weight at the end of competition, therefore we did not estimate the possible dehydration percentage. However, protein concentration before and at the end of competition, that could reflect dehydration and fluid loss (Rose and Hodgson, 2005) is not significant and minimal (5.75±0.38 vs 6.14 ±0.45). For this reason we can assume that the effect of dehydration is negligible and the variation of the observed parameters is not masked by this effect. This is also confirmed by the fact that the lymphocyte undergo to a real redistribution. In fact interestingly, we found a significant increase in the number of CD4+ and CD8+ cells after exercise. It has been postulated that the activation of the sympathetic system and the increased levels of circulating catecholamines during exercise leads to increased lymphocyte concentration due to the recruitment of lymphocyte subpopulations to the vascular compartment (Gannon et al., 2001). Although the number of lymphocyte subpopulation increases, the percentage of CD4+ decreases probably due to the fact that NK cells increase more than other lymphocyte subpopulations such as the helper CD4+ (Fry et al., 1992). As a consequence, in our study the CD4+:CD8+ ratio significantly decreased in response to exercise. This finding agrees with the results obtained by Malinowski and coll., (2006) where the acute response to exercise influence the percent CD4+ and CD8+ both in unfit and trained Standardbred mares.

As far as CD21+ lymphocytes are concerned, in our study a significant exercise-induced reduction was observed. In humans several reports have documented either increased or unchanged B lymphocyte numbers after exercise (Tvede et al., 1989; Pedersen and Hoffman-Goets, 2000). No data concerning CD21+ lymphocyte modifications related to exercise in horses are available in literature. According to Stull and coll. (2008), in horses long-distance road transport induces a decline in CD21+ lymphocyte subpopulation. Both during transport and exercise the redistribution of lymphocyte subpopulations may represent a cortisol-mediated stress response.

According to our results, eventing causes a significant increase in SAA and lysozime levels. It has been shown that during the acute phase response, SAA increases within 4 hours reaching the maximum serum levels 24-48 hours after the initiating stimulus (Petersen et al., 2004). Although the precise biological function of increased SAA expression has not been fully elucidated, it has been suggested that SAA is involved in chemotaxis of leucocytes and T cells, inhibition of platelet activation, and induction of calcium mobilization by monocytes (Zimlichman et al., 1990; Badolato et al., 1995). A 10-fold increased in circulating SAA concentrations has been observed in horses after long-distance ride (Cywinska et al., 2012). In our study, at the end of competition a 2-fold increase in SAA concentrations was found compared to pre-race values. It should be considered that in humans and horses individual variations in SAA levels have been described (Hulten et al., 1999, 32). Moreover, while the effects of endurance exercise on APPs have been extensively documented and the hypothesis that strenuous exercise is analogous to the APR elicited by tissue injury has been confirmed, no data concerning eventing horses are available. In humans, for example, an acute phase response does not seem to occur in a variety of field and court sports, whereas in ultramarathon runners acute phase reactants are significantly affected by exercise (Fallon, 2001, Fallon et al., 2001). Therefore, our results confirm that SAA is the most sensitive APP in horses although eventing seems to elicit a mild response compared with long-distance endurance races.

Studies of the exercise/stress influence on lysozyme concentrations in horses are scarce. According to Sotirov and coworkers (2004) exercise has no negative effects on equine innate immune factors. In our study a significant increase of circulating lysozyme was found at the end of competition (Sotirov et al., 2004). It has been demonstrated that increased levels of glucocorticoids and catecholamines during exercise stimulate the secretion of different neutrophil granule proteins such as lysozyme in blood (Morozow et al., 2003). In swimming rats and treadmill runner athletes the plasma lysozyme content is increased by 41% following intensive exercise load. In humans, several studies have documented the effects of exercise on salivary lysozyme concentration (Papacosta and Nassis 2011). It has been suggested that the lysozyme response to exercise depends on exercise intensity with a significant increase following acute bouts of intense exercise and no changes at lower workload (West et al., 2010).

The main biological functions of Hp are represented by binding hemoglobin and preventing iron loss, bacteriostatic and immunomodulatory effects and regulation of lipid metabolism (Murata et al., 1993). In horses fluctuations in blood Hp concentrations have been observed in case of natural diseases, experimental-induced inflammation and after surgery (Pollock et al., 2005). In unhealthy horses the circulating Hp levels increase slowly after surgery and they are still elevated by 72 hours after the initial stimulus (Pollock et al., 2005). Controversial data have been published on Hp levels in horse after physical exercise (Pellegrini Masini et al., 2003; Fazio et al., 2010). According to Fazio and coworkers (2010), serum Hp concentrations significantly increase during a typical thoroughbreds training. By contrast, in endurance horses neither limited nor long distance rides seem to significantly affect peripheral Hp concentrations (Cywinska et al., 2012). In another study, Scoppetta et al., (2012) found that Hp is highly reduced immediately after the race. The decreased Hp levels may be related to its binding with hemoglobin released into circulation following exercise-induced haemolysis. Haptoglobin levels in our study are in line with those measured by Cywinska and coworkers (Cywinska et al., 2012) and do not seem to be significantly affected by eventing exercise.

297 Even though horses can be considered as excellent models for studying reactive species production
298 due to their high aerobic capacity, data on exercise-induced oxidative stress in horses are
299 heterogeneous and mainly concern racehorses. In the present research an increase in the fluorescence
300 related to lipoxidation (HNE adducts) and glycoxidation adducts (total AGE and PENT) was
301 observed. Exercise-induced protein carbonylation, has previously been described both in
302 Thoroughbred (Duberstein et al., 2009) and Standardbred horses (Kinnunen et al., 2005). Kinnunen
303 and coworkers 2005 showed that an increased concentration of plasma and muscle protein carbonyls
304 can be detected after a single session of moderate intensity exercise. The increased protein carbonyl
305 derivates levels are detectable immediately after exercise and are still elevated after 4 h of recovery,
306 returning to pre-exercise values within 24 h of recovery. MDA adducts in our study tended to increase
307 after exercise, although the modification was not statistically significant. We therefore hypothesize
308 that lipid peroxidation plays a minor role in protein damage and/or that the generation of protein
309 carbonyl derivates from this pathways occurs later. The increase in tryptophan fluorescence observed
310 at the end of competition could be ascribed to the alteration in protein organization which leads the
311 exposition of tryptophan residues due to the bound of the aldehyde in other positions in the protein
312 (Traverso et al. 2004). Our results suggest that competition causes increased protein carbonyl
313 derivates that may predispose eventing horses to tissue damage and inflammation. However, the life
314 span of the stress response in terms of oxidation end products should be further investigated to
315 estimate the quantity of dietary antioxidants required by horses especially during the competition
316 season.
317
318 **5. Conclusions**
319 Our data support the hypothesis that two-day event may alter significantly APR markers such as acute
320 phase proteins, lysozime, protein carbonyl derivatives, complete blood count and lymphocyte
321 subpopulations. These preliminary findings were obtained during an official competition on a

restricted number of horses and the variation of parameters studied could be influenced by sampling time, that was conditioned by the official regulations of the event. Further studies are needed to investigate the time required for recovery to basal values in order to define the possible effects on the immune function of the athlete horse.

Conflict of interest statement

All authors declare they have no financial or commercial conflicts of interest.

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461 **Table 1**
462 Complete blood cell count in eventing horses at rest and after competition

	Rest	After competition	p*	Reference values*[1]
WBC ($x10^3$ cell/µL)	5.76 ± 0.74	7.74 ± 0 .74	0.002	4.30-14.80
Neutrophils ($x10^3$ cell/µL)	3.21 ± 0.52	4.30 ± 1.02	0.005	2.20-8.10

Lymphocytes (x10^3 cell/µL)	2.12 ± 0.38	3.02 ± 0.52	0.002	1.70-5.80
RBC (x10^6 cell/µL)	8.05 ± 0.76	10.01 ± 0.77	0.003	4.45-12.0
Hemoglobin (g/dL)	13.88 ± 1.08	17.56 ± 1.54	0.002	11.30-18.90
PCV %	34.94 ± 2.77	45.24 ± 4.93	0.003	31.10-50.50
Platelets (x10^3 cell/µL)	131.75 ± 23.63	89.25 ± 36.63	0.003	69.90-250.80

Results are mean values ± sd. WBC, white blood cell count; lymphocytes; RBC, red blood cells; PCV, Packed Cell Volume;

*Statistical analysis performed by paired sample *t* test.

*[1] Reference range values according to the Laboratory of the Department of Veterinary Sciences, University of Turin

Fig. 1

a) Number of CD4+ cells at rest (R, dot filled bar) and after competition (PC, line filled bar) (*$p \leq 0.05$)

b) Number of CD8+ cells at rest (R) and after competition (PC) (*$p \leq 0.05$)

c) Number of CD21+ cells at rest (R) and after competition (PC) (*$p \leq 0.05$)

d) Lymphocyte subpopulation percentage at rest (R) and after competition (PC) (*$p \leq 0.05$)

Fig 2

Mean ± SEM values for protein carbonyl adducts (AGEs, total AGE adducts; PENT, pentosidine-like adducts; MDA, malondyaldeyde adducts; HNE, hydroxynonenal adducts; Trip, triptophane) before (R, dot filled bar) and after competition (PC, line filled bar) (*$p \leq 0.05$)