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Depression of cellular immunity correlated to stress: questions on welfare and protection in italian trotters after running performance

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Abstract

In all Equestrian sports, the welfare of the horse must be paramount and must never be subordinated to competitive or commercial influences. It is necessary to prohibit any training methods which are abusive or cause fear or for which the horse has not been properly trained. The purpose of this study was to examine the effects of prolonged and intensive physical training on the immune response in trotter horses in order to recommend at the legislator the adoption of guidelines for the welfare of these animals. In fact, during their athletic life and some of them undergo lethal lung infections, therefore it is likely that modifications of physiologic cellular parameters could account for the increased susceptibility to microbial disease. Noteworthy, chronic stress has been shown to be immunosuppressive, whereas acute stress seems to lead to immunoenhancing effects. In particular, we have studied some immune parameters as blood cells distribution, hemoglobin, hematocrit, erythrocyte sedimentation rate, phagocytosis activity, macrophage Migration Inhibiting Factor (MIF) and finally the levels of (1-3)-ß-D-Glucan, as indicator of clearance. Taken together, these findings indicate a condition of reduced immune response in seven trotters after race, to identify possible biomarkers of stress dependent on physical exercise.

Key words: stress, immunity**,** trotter, welfare, legislation.

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Introduction

Trotters are exposed to a prolonged and continuous physical stress; some of those horses are subjected to move from different race tracks and it often happens that after long transportation in trailers they became more sensitive to respiratory infections [1]. Several authors [2-8] have showed that strenuous exercise, training and competition may be a factor which predisposes horses to the development of pleuropneumonia.
The lung's immune system is protected by the alveolar macrophages activities and that high concentrations of cortisol in blood suppress the function of these leukocytes [9].

In animals as in humans it's important to evaluate the immune system, considering the scheme of distribution and some blood cells and serum parameters.

In fact**, s**tudies on animal welfare evidenced similarities among species concerning the adaptive response of immune system to different stressing conditions [10-12]. It is known today that different types of stressors, including physical activity, can cause neuro-endocrine and hormonal changes that may mediate alterations in immune cell function and thus influence susceptibility to disease [13-14]. In fact, stress, such as excessive muscular exercise could lead to a number of responses influencing hypothalamic-pituitary-adrenocortical axis and spymphatetic system functions. The activity of this systems is demonstrated by rapid increases in circulating levels of ACTH, adrenaline, noradrenaline and cortisol allowing these hormones to be useful in the evaluation of exercise-induced stress. The exercise induced responses of catecholamines in the horse are much greater than in human [15].

The nervous system reacts to stressors modulating the secretion of neuropeptides [16]. The innervation of specific regions of primary and secondary lymphoid organs estab-

lishes the links necessary for neural modulation of immunity. The expression of beta-adrenoceptors on a variety of immune cells, including lymphocytes and macrophages, provides the molecular bases for these cells to be targets for catecholamine signalling. It is apparent that a multifactorial interaction between the cells of the immune system and the neuroendocrine hormones exists [17-18]. Conversely, there is now evidence of production of neuroendocrine hormones by the cells of immune systems, suggesting that the signal from the immune system can activate the central nervous system and control the function of the neuroendocrine system [19]. The first evidence that the cells of the immune system could produce a peptide was the finding that human lymphocytes could produce ACTH in connection to virus infections [20]. Since then, numerous peptides have been shown to be produced by different immune cells [21]. Moderate stress leads to highly stereotyped changes within leukocyte subsets in peripheral blood in which both neutrophil and lymphocyte concentrations increased markedly [22]. Conversely, strenuous exercise induces lymphocyte reduction. Athletes in endurance are reported to experience increased susceptibility to respiratory tract infections after periods of heavy training. Therefore, strenuous exercise results associated to depression of immunity but the intrinsic molecular mechanisms are not well known. Recently, Hsu et al. [23] observed that stress can induce mitochondrial membrane alterations in leukocytes. Reactive oxygen species produced during vigorous exercise may permeate into cell nuclei and induce oxidative DNA damage. These phenomena may be one of the mechanisms behind the immune dysfunctions after exhaustive exercise [24]. Moderate exercise has potent stimulatory effects on phagocytosis, antitumour activity, reactive oxygen and nitrogen metabolism and chemiotaxis.

Therefore in Equine Sport Medicine there is an increasing interest in the study of metabolic and neuroendocrine responses to exercise in order to assess physical adaptation to stress elicited by exhaustive training and/or frequent competions and to improve animal welfare. Animal welfare is a relevant theme also within the European Community that has recently presented the "Animal Welfare Action Plan 2006-2010" with the aim to emphasize that well-being of animals represents a scientific discipline that needs to be adequately supported by research.

At present, there isn't corresponding legislation in Italy that provides a mechanism for establishing detailed standards for the welfare of horses and, in particular, of the trotters. In fact, in Italy we have only the following specific rules, produced by associations and federations:

– Vet Regulation F.I.S.E. (Italian Equestrian Federation) (updated to March, 27th, 2006);
– U.N.I.R.E. Regulation (National Union of Improved Horse Species) of harness racing updated to January, 30th, 2004;
– UNIRE Regulation of gallop races updated to February, 1st, 2004;
– Bets Rules, of D.P.R. (Decree) April, 8th 1998, n. 169;
– Code of equestrian behaviour F.E.I. (International Equestrian Federation) destined to all the people who work in the equestrian field. It is a code of good conduct and it acts as guidelines.

Current Italian legislation relating to horse includes:

- Law on Transport of horse (Regulation EC no. 1/2005);
- Law on Veterinary Medicinal Products (Legislative Decree no. 193/2006 following EC Directive 2004/28);
- Law on Identification of equidae (Ministerial Decree 5 May 2006, Commission Decision 2000/68/EC, Regulation EC no. 504/2008);
- Law on Maltreatment of animals (Law 189/2004).

Economic interests that turns around the trotters and their sport are massive and this leads to neglect the health and welfare of these animals.

Considering that the welfare of the horse remains paramount, it is necessary to prohibit any training methods which are abusive or cause fear or for which the horse has not been properly trained.

Although the control of welfare at the physical level was improved by the FEI [32], the problem is that if the interpretation of some types of abuse (such as beating, exhaustion, prolonged and intensive physical training, incompetent poling) is straightforward, other practices need more objective evidence in order to be considered as unethical.

For example, inappropriate physical training is probably a frequent and underestimated source of decreased welfare, but it will be difficult to convince riders because personal pride is involved. There is a need for scientific information in this context.

On the basis of these consideration, the current investigations were undertaken to study the effects of prolonged and intensive physical training on the immune system, considering also some cellular, cytochemical and serum parameters as white and red blood count (WBC and RBC), hemoglobin (HB), hematocrit (HTC), erythrocyte sedimentation rate (ESR), leucocyte enzyme pattern, phagocytosis activity and levels (1-3)-ß-D-Glucan, the last as indicator of clearance.In fact ß-D-Glucans posses their own receptor on macrophages as demonstrated by [25]Brown et al. (2002). Noteworthy the immune response was evaluated by MIF activity, as a reduced cell-mediated immune response, after

race in trotter horses in order to recommend at the legislator the adoption of guidelines for the welfare of these animals.

Materials and Methods

Trotters
This study was carried out on no. 7 trotters, [five females (age range 2-4 years) and two males (age range 5-8 years)] weighing approximately 300-350 kilos, kept in stall housing in the province of Bari (Italy). All animals, clinically healthy, were investigated for the physiological stress before and after race.

Blood samples were taken with and without anticoagulant from the jugular vein - before and after race - for haematological and biochemical analysis - using 18 gauge needles (Venoject Terumo, Haasrode, Belgium); sera stored at –30°C until use. Heparinized blood was used for cellular studies.

Cellular parameters and cytochemical methods
The following cellular parameters, WBC, RBC,HB,HTC and ESR, were performed by means of a hemocytometer (K 1000 SYSMEX-TOA, DASIT S.p.A., Milan, Italy) using Sysmex-Dasit reagents.

Lymphocyte, neutrophils and monocyte counts were microscopically read on peripheral blood films stained with May-Grünwald-Giemsa (MGG).

Besides, same smears were stained with cytoenzymatic methods: α-naphyl acetate esterase (ANAE) (Sigma Diagnostics, St. Louis, MO, USA) and Peroxidase (Perox) (Sigma Diagnostics).

Determination of (1-3)-β-D-Glucan in serum
The G-test was a generous gift from Associates of Cape Cod/Seikagaku (Cape Cod MA). Equine sera were assayed for (1-3)–β-D-Glucan according to the manufacturer's instructions.

Peripheral blood mononuclear cell (PBMC) isolation
PBMC were isolated from heparinized blood diluted 1:4 with Hanks' Balanced Salt Solution (HBSS) by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) density gradient centrifugation [26]. Cell suspensions recovered at the interface were resuspended in RPMI 1640 (Flow Laboratories, Milan, Italy) supplemented with penicillin 100 IU/ml, streptomycin 100 µg/ml, glutamine 2mM and 5% heat-inactivated foetal calf serum (FCS) (complete medium).

Monocyte (MØ) isolation
Aliquots of PBMC, resuspended in complete medium at a concentration of $10x10^6$ cells/ml, were introduced into FCS-pretreated plastic Petri dishes and incubated for 2 hrs at 37°C to allow Mo adhesion [27]. After gentle agitation, the nonadherent cells (purified lymphocytes) were discarded; the purity of the resulting enriched plastic adherent Mo population was about 99%, as evaluated by non-specific esterase staining and immunofluorescence, using fluorescein isothiocyanate (FITC)-conjugated anti-CD14 monoclonal antibody (Becton Dickinson, Milan, Italy).

MØ phagocytosis
At 1 ml of medium containing MØ (15×10^6 cells) 400 µl of horse serum and a known volume of Candida albicans (Ca) (cell/micete ratio 1:2) were added.

The suspensions were incubated for 30 minutes at 14 °C, cytocentrifuged on slides at 1200g, and, finally, cells were fixed with methyl alcohol and stained with MGG. Percent of phagocytosis was microscopically evaluated and at least 300 elements for slides, that have engulfed 1 or 2 or 3 Ca, were counted.

PBMC stimulation with Phytohemagglutinin (PHA)
In parallel experiments, aliquots of PBMC ($2x10^6$/ml) were stimulated with PHA (1µg/ml), and incubated for 1 h in 5% CO_2 -5% humidified air atmosphere at 37°C. At the end of this incubation period, the mononuclear cells were centrifuged at 400X g for 10 min and the PHA-containing supernatant was discarded. Mononuclear cells were resuspended at the concentration of 5×10^6cell/ml in complete medium and transferred to plastic tissue culture flasks for a 48 hrs incubation. Control supernatants were prepared by incubating mononuclear cells without the mitogen. At the end of incubation period, samples were centrifuged at 400X g for 10 min and, then, supernatants were collected.

Preparation of agarose
The solution of agarose contained 1% agarose, 1X medium199, 10% human AB+ serum, 200U of penicillin/ml, 200µg of streptomycin/ml, and the HEPES and bicarbonate buffer (pH 7.20 to 7.40). The final pH of the agarose need to be maintained between 7.20 and 7.40, as measured MIF activity is consistently reduced at a low plate pH (< 7.10) and the ability of monocytes (MØ) to migrate is inhibited above pH 7.40. Fractions (5.5 ml) of the complete agarose were placed in Petri dishes and allowed to solidify for 5 to 10 min. Six-nine wells were cut into the agarose with a 2.5mm gel punch [27].

Incubation of MØ with supernatants
An aliquot of cells (15×10^6) were incubated with activated and control supernatants. To cells were added 15µl of AB + serum and 85 µl of the test supernatant. The sample were then placed in the incubator for 30 min at 37° C with gentle shaking every 5 min. After the incubation, 10 µl samples (containing 1.5×10^6 cells) were pipetted into the

wells cut in agarose plates. All samples were tested in triplicate. The Petri dishes were incubated for 18 h at 37°C in 5% CO_2-95% humidified air atmosphere.

Measurement of MIF activity

The areas of migration were measured microscopically at low magnification by using a microscope with a grid attachment.

The percentage of migration inhibition was determined by the formula [28].

Per cent inhibition= 1- area of migration in xperimental wells x 100 area of migration in control wells

Statistical studies

Statistical differences were determined by the Student's t test.

Results

Cellular and serum parameters

Increased values of RBC, HCT and HB may depend on the high hemoconcentration resulting from the fluid loss through sweating (Table 1).

In trotters an increased counts of lymphocytes and monocytes were showed, while neutrophils were decreased (Fig. 1).

In Tablw 2, MØ were evaluated morphometrically; they appear smaller than what is reported in literature [31].

While from the point of cytochemical view, the pattern enzyme did not show any particular interest because a classic citoplasmatic positivity to Perox and ANAE enzymes were showed in monocytes and neutrophils to reveal its lisosomial activity (Figs. 2-3).

In Fig. 4 it showed the activity of phagocytosis by MØ, against a common micete as Ca, after the race was strongly declined (Fig. 5) to confirm this result is the test of MIF (Tab. 3 and Fig. 6).

Table 1. *A Series of Blood Parameters in Trotters Before (1) and After (2) a Race*

Horse	ESR		HB		RBCx1000	
	1	2	1	2	1	2
1	60	16	15,7	20,3	8980	11840
2	20	8	18,0	19,6	10650	11960
3	32	5	18,2	23,1	10060	12940
4	66	20	13,6	18,6	7860	10790
5	44	6	15,1	22,8	7550	11500
6	56	22	15,8	18,0	9070	10540
7	36	18	15,8	16,8	9630	10250
Mean	44,86	13,57	16,03	19,89	9114,29	11402,9
± SD	16,65	7,07	1,61	2,37	1122,42	941,89
T-test	p<0,001		p<0,05		p<0,05	

Horse	WBC		HCT		ßGlucan	
	1	2	1	2	1	2
1	9100	11400	40	55	57	106
2	8300	8600	45	54	0	896
3	7600	9400	46	62	93	281
4	11400	13700	34	50	504	2664
5	7400	8700	38	59	75	21
6	8500	9300	39	46	836	516
7	8400	8450	40	43	n.d.	n.d.
Mean	8671,43	9935,71	40,29	52,71		
± SD	1331,31	1937,51	4,11	6,82		
T-test	p<0,05		p<0,05			

n.d. : not determined

Table 2. *Morphometric measures of monocytes*

	Area μ^2	**Length** µ	**Width** µ	**Perimeter** µ	**Rounded**	**Eq. Diam.** µ
Mean	119.3	13.4	11.6	40.7	1.04	12.4
SD	10.4	0.7	0.8	2.2	0.03	0.5
Min	99.5	12.3	10.1	37.1	1.01	11.3
Max	130.8	14.3	12.3	43.7	1.09	12.9

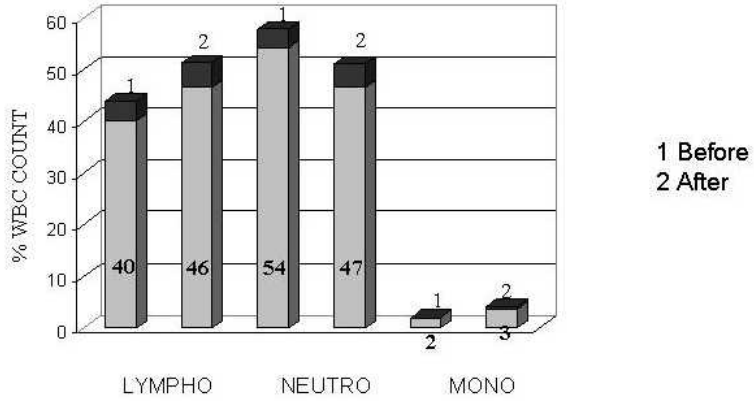


Figure 1. *Lymphocyte, neutrophil and monocyte counts in 7 trotters before (1) and after (2) race. At least 300 cells were counted for each horse on peripheral blood films stained with May-Grünwald-*Giemsa.

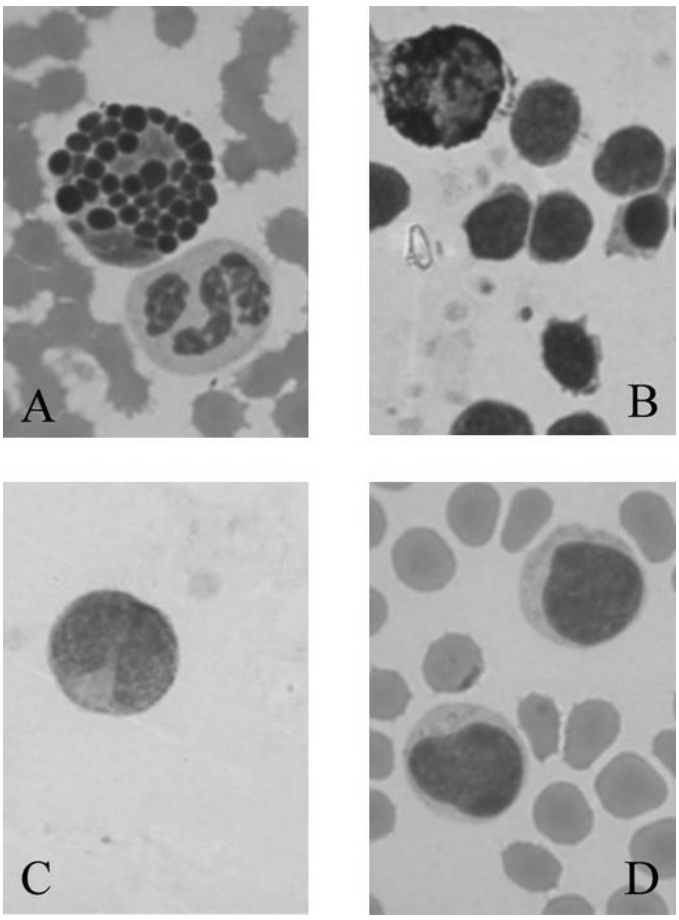


Figure 2. *Horse blood cells (MGG, 1000X).*

A*: an eosinophil and a granulocyte neutrophil in a blood smear.*
B*: a basophil and several small limphocytes in a cyto-centrifuged.*
C*: a classic monocyte in a blood smear.*
D*: two large limphocytes in a blood smear.*

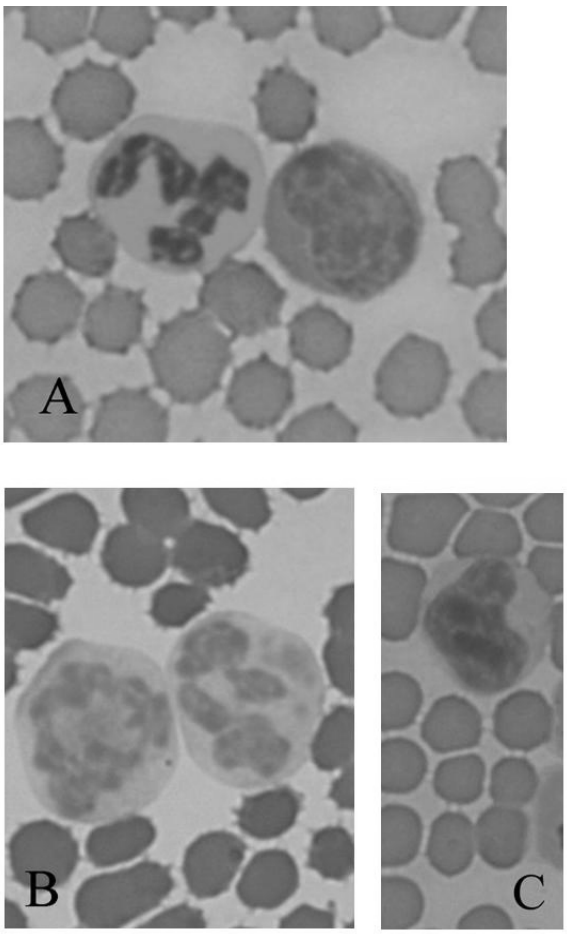


Figure 3. *In horse blood smear (1000X).*

A*: a monocyte with strongly citoplasmatic positivity to ANAE enzyme and a neutrophil faintly positive.*
B*: two neutrophils with strongly citoplasmatic positivity to Perox enzyme.*
C*: a monocyte faintly positive to Perox anzyme.*

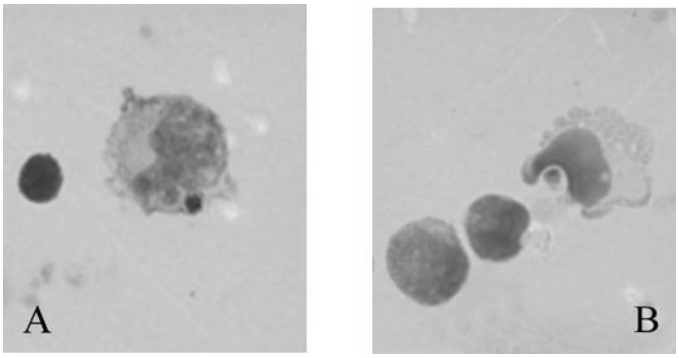


Figure 4. *Cytocentrifuged on slide of horse blood cells (MGG, 1000X).*
A*: binding of monocyte-macrophages with Candida albicans particles and a little lymphocyte.*
B*: engulfment of monocyte-macrophages with Candida albicans particles and two large lymphocytes.*

In Tab. 1 was also showed the increase of ß-D-glucan after the race can be seen both as a temporary reduction in the capacity of this molecule.

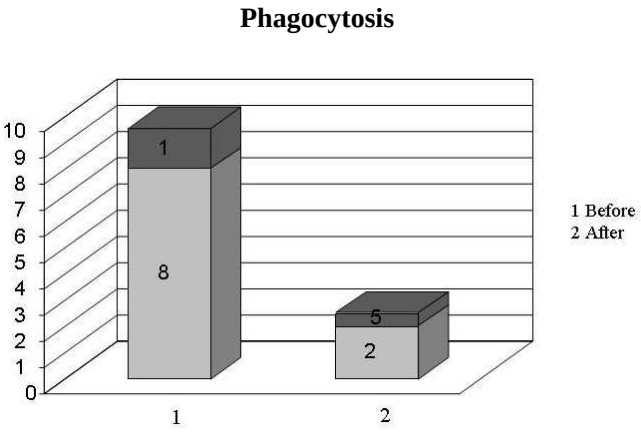
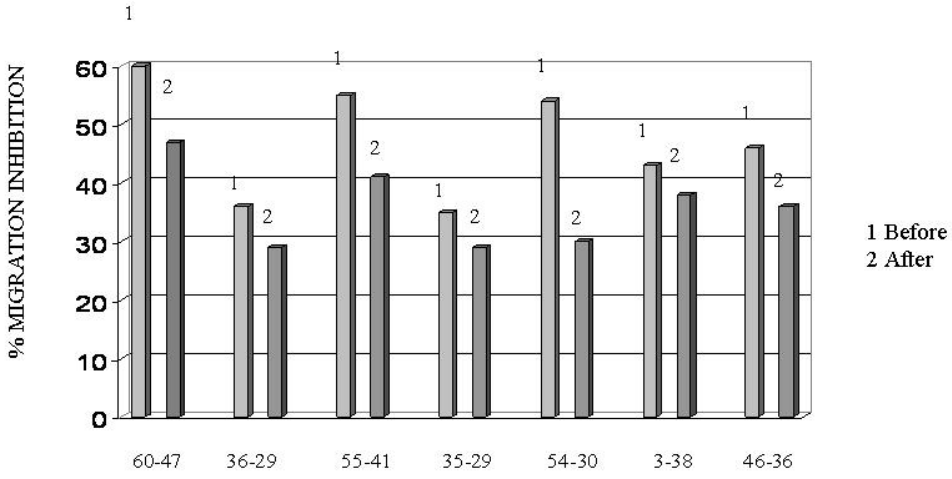


Figure 5*. Phagocytosis of CA by macrophages before (column l) and after (column 2) a race. The columns indicate mean percentages ± standard errors.Statistical difference was observed by p < 0,005*



Figur 6. *MIF assay in 7 trotters before (1) and after (2) race. Percent of migration inhibition was calculated by reading migration areas under agarose and determining the formula described in the methods section.*

Discussion

The improvement of animals' welfare gives animals a better quality of life, which means also better health status by avoiding chronic stress reactions that lower the organism's coping ability. Such stress reactions may induce the development of pathologies due to e.g. the impairment of the immune system.

Our results have shown how extreme and intensive physical training induces a chronic prolonged stress and, consequently, discomfort to horses.

Our findings regards about lymphocyte and monocyte increases are in contrast with data of others [29], who demonstrated, following acute stress, a rapid and reversible decrease of T cells, B cells, natural killer cells and monocytes in the blood. Such a transient decrease

in WBC numbers has been considered as a sort of redistribution or redeployment of leukocytes from the blood to other compartments of the body where an enhanced immune function is required [30]. In our case, we hypothesize that the short duration of the race (2 min. about) does not allow us to take the exact moment of leukocyte migration to other sites. It is likely that collection of our blood samples, immediately after race, coincides with the time of lymphocyte and monocyte extravasation before their redeployment from the blood to other districts.

The quantitative analysis of the elements figured blood, red cells and white blood cells, has always shown an increase in the number of cells after the race, statistically significant, which led to *a inspessitio sanguinis* shown by the values of ESR. The increase of red blood cells is determined by a greater demand for oxygen. The increase in white blood cells is typical of a state of stress.

In particular, this increase occurred borne by lymphocytes and monocytes, confirming the hypothesis that stress to which they were subjected horses were chronic in nature and not sharp, or acute stress condition.

Noteworthy the increase of ß-D-glucan after the race could be as a result of an immune-response inhibited, both as a non-receptor function of macrophages. So we can confirm our hypothesis that the immune inhibiting of MØ observed was a result of repeated stress, is closely related to chronic stress.

We believe that the training represent therefore a potential welfare risk that must not be ignored.
It is necessary to underline that the welfare of trotters depends on its ability to sustain fitness and avoid suffering. In fact, improved keeping conditions as well as performance-adapted training methods will help to avoid defects.

Any person responsible for the welfare of horse should acquire maximum possible expertise, because the well-being and usefulness of horse depend on the skill and attitude of the individuals who manage them. Assistance or advice on management of horse can be obtained from veterinarians.

Veterinarians have many opportunities to improve the welfare of horses, but owners will not always be happy with their veterinarian's comments on these issues. However, veterinarians have the duty to insure the horses health and welfare. Nowadays, many horse owners lack the basic knowledge of horse care and they are not aware of the welfare needs of their horses, nor are they capable of safeguarding their horses under all foreseeable conditions.

We would propose that the following key concepts should be considered:

1. Decisions regarding care and management in equine husbandry and associated activities based on sound scientific principles;
2. Oversight and regulation of the care and man agement should be clearly defined.
3. An industry's "buying in" to the process will likely be most productive. To move beyond the issue of whether or not horses should participate at all, industries must demonstrate the ability to safeguard the well-being of the horse.

In conclusion, given the current lack of laws, the Authors recommend, in accordance with the FEI Code of Conduct, at the Italian legislator the adoption of a law to supervise and ensure the horse's wellness during the horse training, practice and the planning to compete in a horse race.

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