Original article

Interdependence of productive effort and in vitro vegetal extract treatment on specific cell-mediated immunity in horses

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Abstract

The research aimed at identifying and rendering alcoholic extracts of various plants based on their in vitro potential in stimulating cell mediated immunity and in alleviating stress effects subsequent to workout type in horses.

Experimental horses were selected from different working environments: (a) draft, agricultural works – n = 16, average age 8 years, (b) inconstant effort, leisure, n = 15, average age 7.5 years and (c) constant training, endurance, average age 3.5 years. Blood samples were collected in heparinized vials (50 U/ml) before and after the workout. Alcoholic extracts (2 µl/well, duplicate) of Taraxacum officinale, Symphytum officinale, (L) Weber, Chelidonium majus, Hypericum perforatum, Equisetum palustre, Avena sativa, Capsella bursa pastoris, Viola tricolor were investigated by blast transformation test and statistically interpreted (Student’s t test).

In vitro responses were the highest to all tested extracts in endurance horses and the lowest in leisure horses. C. bursa pastoris was the most efficient in draft and endurance categories before the workout (draft 45.13 ± 8.05, in leisure 48.09 ± 24.13 and 53.44 ± 9.32 in endurance horses), but not after the workout, where C. majus performed better (41.86 ± 22.47 in leisure and 48.1 ± 6.82 in endurance horses), probably due to its complex, protein-rich latex structure. The effects of plant extracts depended on the type of effort and the level of constant training rather than on the taxonomy of the plant.

Key words: Alcoholic plant extracts, blast transformation, effort stress, horses, leukocytes

1. Introduction

Equine represent important partners of humans as help for work, participant to sporting activities or simply as companions. As one of the most sensitive indicators of health, the immune system, provides information on well-being and disease resistance in these animals. The use of horses for various types of exercise could result in stress induced conditions that directly or indirectly prejudice their working performance (Wong et al., 1992; Bartolome et al., 2016). Within this framework, the study of immune effectors’ variations under differentiated workout conditions can be a measure of the predictability of individual welfare and health (Buschmann et al., 1991). The economy of horse keeping is subject to a negative interdependence of productive effort and (Pearson et al., 2012; Subramoniam, 2014; Biradar, 2015).

As in human medicine, in veterinary therapy immunologically active compounds are used for which plants could represent an inexhaustible source. Scientific data support the healing activity of plants (Pearson et al., 2012; Subramoniam, 2014; Biradar, 2015). Due to their feeding peculiarities, equine are exposed to grazing and/or hay consumption and to highly variable nutritional value of the consumed plants. Amongst those, some plants from spontaneous flora also benefit of medicinal qualities and consumed by horses can positively influence their immunity to diseases.

The aim of the research was to render several alcoholic vegetal extracts of various sources in alleviating stress effects subsequent to effort on cell mediated immunity in horses, while monitoring the potential differences in response to these extracts between workout groups of horses.

2. Material and Methods

2.1 Animals

Experimental horses were selected from different working environments: (a) draft, semi-constant effort, agricultural works – n=16, average age 8 years, (b) inconstant effort, leisure, n=15, average age 7.5 years and (c) constant training, endurance, n=10, average age 3.5 years. Blood was sampled on heparin (50 U/ml).
before and after the workout specific for each category, with the same given duration. The blood samples were collected within the context of the national surveillance program for equine infectious anemia, thus being subject to serological testing approved by the national competent authority (National Veterinary and Food Safety Authority) and also with the approval of the owners.

2.2 Plant extracts
To monitor the potential immune modulating effects of various plant extracts on the adaptive cell-mediated immune response, several species of plants were chosen; some of them as such (grass, hay or grain) enterring the horses’ regular diet. Thus, commercial alcoholic extracts for human use, obtained from *Taraxacum officinale* (L.) Weber, *Symphytum officinale* L., *Equisetum palustre* L., *Viola tricolor* L., *Avena sativa* L., *Capsella bursa pastoris* (L.) Medik., *Hypericum perforatum* L., *Chelidonium majus* L. were used. The extracts were prepared by percolation method by Plant extract, Romania, according to the provisions of German Homeopathic Pharmacopœia from plants originating in Romania. Their content in active principles was standardised by the producer.

2.3 Methods

2.3.1 Neutrophile/lymphocyte (N/L index) evaluation
Blood smears were performed according to the usual technique and stained with Dia Quick Panoptik. Leukocyte subpopulations were counted and N/L ratios were evaluated as an indicator of the stress level caused by the workout type (Davis et al., 2008).

2.3.2 Leukocyte blast transformation test
The leukocyte blast transformation test measures the *in vitro* reactivity of lymphocytes and monocytes to sensitizing antigens. The blood samples were diluted (1:4) with RPMI 1640 (with 5% FCS and antibiotics, at pH 7.4) (Sigma-Aldrich, USA) and distributed in duplicate in sterile 96-well plates (200 µl per well). Twelve *in vitro* experimental variants were tested for each individual animal, namely: (1) untreated control variant, (2) phytohaemagglutinin-M (PHA) (1 µl per well), (3) LPS, (4) 70°C alcohol and (5-12) commercial alcoholic extracts of *T. officinale, S. officinale, E. palustre, V. tricolor, A. sativa, C. bursa pastoris, H. perforatum, C. majus* (Plant extract, Romania) (1.5 µl/well). Subsequent to an incubation of 60 h at 37°C in a 5% CO₂ atmosphere, glucose consumption was evaluated. For this, 12.5 µl of the cultural supernatant was transferred to 0.5 ml of orto-toluidine reagent, boiled for 8 min and read in a spectrophotometer at 610 nm wavelength (SUMAL PE2, Karl Zeiss, Jena, Germany). The stimulation/inhibition index (SI) was calculated as: SI % = ([IG–GR]/IG)×100, where SI = blast transformation index, IG = the initial glucose concentration in the culture medium and GR = glucose residue in the sample after incubation (Spinu et al., 2016).

2.3.3 Statistical analyses
Average values and standard error were calculated by use of Excel program. Student’s-t test was applied to evaluate the statistical significance of the differences.

3. Results and Discussion
Phytomedicine, in spite of being known for millennia, is nowadays gaining more importance due to the scientific recognition of the therapeutic power of natural molecules from plants with increased bioavailability and lesser side effects than synthetic compounds (Pushpangadan, 2013). Veterinary medical use of plant extracts represents one of the broad windows opened to scientific research due to numerous potential subject species and also different perception of the active plant compounds by each and every one of those.

The medicinal plants that represented the subject of the present study were chosen due to their high prevalence, thus the high likeness to be ingested by the horses on the pasture. Nevertheless, researches indicated immunological effects for all including anti-inflammatory effects (C. bursa pastoris, C. majus) (Choi et al., 2014; Nawrot et al., 2017), inhibition of activated lymphocyte proliferation (*E. palustre, V. tricolor*) (Gründemann et al., 2014; Hellinger et al., 2014), anticomplementary activity (*S. officinale*) (Staiger, 2012), modulation of antibody levels (*A. sativa, T. officinale*) (Yoon, 2008; Chatuevedi et al., 2011).

Antimicrobial defence in mammals is mainly based on adaptive immunity, involving the tri-cellular cooperation among antigen presenting cells, T and B lymphocytes. These mechanisms are subject to external influence including stress of various origins. Several studies indicated that the type of exercise is important in conditioning the types of changes that occur at the immune system level, including an augmentation of lymphokine activated killer (LAK) cell function (Horohov et al., 1996).

It has been shown that during a single strenuous exercise, in spite of the significant decrease in chemotactic index and peak chemo-luminescence, one day after exercise, both IgA levels and the mitogen-induced blastogenesis of lymphocytes stayed unchanged (Wong et al., 1992; Souza et al., 2010). Similarly, a prolonged bout of exercise lead to a long-term suppression of the non-specific immune response which was thought to partially stand for certain infectious episodes in trained horses (Robson et al., 2003).

Regular, moderate stress was reported as beneficial for enhancing the function of neutrophils (Korhonen et al., 2000). The N/L was increased in horses after transportation, due to increased stress (Davies et al., 2018). As indicated in recent studies (Krumrých et al., 2018), and also supported by the N/L index in this study (Figure 1); the stress levels in various workout categories of horses investigated differed by effort type. It depended less on the constant effort, such as in draft or endurance horses, than on the interrupted, inconstant exercise as in leisure equine.

The higher values of the N/L ratio in the majority of the working horses might indicate their constant exposure to acute bouts of stress and, most probably, also to subliminary chronic stress. The N/L ratio calculated for leisure horses was the highest in this experiment, the animals being subject to inconstant, different intensity workout and, therefore, more prone to workout stress. Some authors consider a N/L ratio around 3 indicating an impairment of the health status that can not be restored without substantial changes in the working schedule or management system. In draft horses, the animals showed the lowest levels of stress, probably because the workout had a relatively constant pace.
Equine athletes are exposed to various levels of effort which might impact on their health status and, therefore, susceptibility to various infectious agents, including ported ones (Hines et al., 1996); a previous study indicated that subsequent to endurance ride, the number of CD4 (helper) T cells decreased, while the number of suppressor cells increased (Heines et al., 1994), decreasing the antimicrobial adaptive immune potential. A decrease in proliferative responses of lymphocytes appears as soon as 12 to 16 hours after a horse participates in a race (Nesse et al., 2002).

Different extracts of plant origin were tested for their therapeutic and disease preventing potential in both humans and animals (Pearson et al., 2012; Ansari, 2016). As products with increased bioavailability and little or no side effects, plant extracts could intervene in restoring the immune response and improving the
welfare and the antimicrobial protective level. In this study, the immune enhancing capacity of some alcoholic plant extracts was tested, while the main cell groups were exposed to these extracts in vitro, in whole blood cultures from horses exposed to various effort levels.

The highest values of stimulation indices were observed in endurance horses, both before and after the workout, associated with intermediate stress levels (Figures 2a and 2b). Although, the encountered SIs were low compared to other species (Spina et al., 2016), they were always in the positive zone. C. majus was the most efficient in all categories (draft 30.973 ± 22.578, in leisure 51.63 ± 25.19 and 41.86 ± 22.48 and endurance 51.59 ± 4.83 and 48.06 ± 6.82, before and after the workout, respectively), probably due to its complex, protein-rich latex structure.

![Figure 2(b): In vitro stimulation indices (%) in horses differentiated before their workout (draft, leisure, endurance) (alcoholic plant extracts, continued).](image-url)

![Figure 3(a): In vitro stimulation indices (%) in horses differentiated after their workout (draft-leisure, endurance) (unstimulated control, alcohol and mitogen controls, Taraxacum and Symphitum alcoholic extracts).](image-url)
Of the other vegetal extracts, *C. bursa pastoris* was the most active in the same workout category, before the effort, suggesting an appropriate mean to boost the adaptive response of the mononuclear equine cells during the workout. In Romania, it represents a very handy cure, the plant being ubiquitous on almost all pastures. *C. majus* performed better after the workout (41.86 ± 22.47 in leisure and 48.1 ± 6.82 in endurance horses), probably due to its complex, protein-rich latex structure (Nawrot et al., 2017).

The SIs values were close for the leisure and endurance horses, with lower figures recorded for the draft group before the exercise, but highly variable after the animals were subject to the workout. Thus, all indices were in the negative zone for the draft horse group after the effort, except for the *A. sativa* (common oats) extract, which proved to be the strongest stimulating extract for this group (Figure 3b).

When compared to the SI for the same extract before the workout (Figure 2b), which were the lowest for the group, we could conclude that the active principles of the plant had modulating effects. *T. officinale, S. officinale* and *V. tricolor* extracts had stimulating effects when compared with the alcohol but not with the untreated control.

The results of the Student t-test indicated significance of the differences between draft versus leisure/endurance horses ($p<0.010$ - $p<0.001$), for most of the variants, between the initial and after workout measurements.

**4. Conclusion**

The effects of various plant extracts depended mostly on the type of effort/degree of stress and the level of constant training rather than on the taxonomy of the plant. Nevertheless, the experiment allowed rendering some plant extracts that could help in alleviating the stress subsequent to various levels of effort in horses.

**Conflict of interest**

We declare that we have no conflict of interest.

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