OXIDATIVE-STRESS INDUCED CHANGES IN CORRELATION WITH SEROCONVERSION IN EIA SEROPOSITIVE HORSES

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Summary

The objective of the present experiment was to verify the hypothesis that oxidative stress characteristic changes are correlated with the presence of the equine infectious anemia virus (EIAV) in infected horses. The research was based on the results obtained at the agar gel immunodiffusion test (Coggins test), which represents the prescribed test for international trade at the present time (OIE). From the investigated horses (n=23) blood samples were collected in order to quantify the activity of the main antioxidant enzymes – superoxid-dismutase (SOD), cathalase and the general peroxidaze activity - in parallel with the lipidic oxidative level, the concentration of the total blood lipids and uric acid. Results were statistically analyzed using the "r test" (estimation of the correlation quotient) giving scores for the positivity regarding the agar gel immunodiffusion test - Coggins (value 1) respectively for the negativity (value 0). The obtained results show that the enzymatic activity of superoxid-dismutase (SOD) and the general peroxidaze activity markedly decrease in the EIAV infected horses compared to the healthy ones, while the cathalase activity decreases moderately in these animals. The cathalase activity in the healthy horses is positively correlated with the Coggins test results, with a level of signification smaller then 0.01, while the other enzymes do not have statistically representative changes. The level of lipid peroxidation in the infected horses is the only indicator of oxidative stress which has a positive correlation with the presence of EIAV (highly significant increase – $0.001<p<0.01$). Lower values of the uric acid in the seropositive horses can confirm his antioxidant role in vivo in horses.

In the present, the horse still has an important role in Romania, both in the small private farms, as well as an animal for leisure and sport. Equine infectious anemia can be considered as the most important and difficult to control infectious disease of the horse, because of the high percentages of morbidity and mortality.

Equine infectious anemia virus (EIAV), a member of the genus Lentivirus of the family Retroviridae, affects all members of the family Equidae and is distributed worldwide (Issel and Coggins, 1979; Cook et al., 2001). EIAV is unique among the lentiviruses in that the initial acute febrile response and associated viremia are followed by recurrent cycles of the disease and, finally, a prolonged asymptomatic period (Montelaro et al., 1993). Some animals die during either the acute or chronic stage of the disease. If the animal survives, the disease episodes progressively decrease in frequency and intensity over about a year, after which time the animal enters the inapparent carrier stage (Cook et al., 1996).

In the inapparent carrier stage, clinical signs are absent and viremia is usually undetectable by conventional methods for virus isolation. However, asymptomatic animals may be detected by using a more sensitive technique, such as polymerase chain reaction (PCR), to determine the viral nucleic acids in their blood or tissues (Oaks et al., 1998; Harrold et al., 2000), or by subjecting them to an equine infectious anemia (EIA) serologic test (Issel and Coggins, 1979).

Oxidative stress is caused by an imbalance between the production of reactive oxygen and a biological system's ability to readily detoxify the reactive intermediates or easily repair the resulting damage. All forms of life maintain a reducing environment within their cells. The cellular redox environment is preserved by enzymes that maintain the reduced state through a constant input of metabolic energy. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA (www.wikipedia.org).

In the passed years, a lot of studies have been accumulated to prove the direct relation between accumulation of oxygen reagent species, oxidative stress and a number of diseases. Thus, the oxidative stress state is quoted to be the cause in the appearance and evolution of some degenerative diseases, cardiovascular diseases, in cancer, and even in viral infections.

Although oxidative stress has been studied a lot on laboratory animals, the potential pathogenic role of it has not been studied a lot in veterinary medicine. The horse represents the species in which oxidative stress has been mostly studied, due to the horse’s physical performances which are used in a lot of fields and generate a considerable prooxidant state; the deep impact over the health and performance of the animal is attracting more and more attention and it is started to be described.

Until now in equine pathology the oxidative stress has been correlated with some equine diseases, but there is no literature about his implication in some viral disease.

The relation between oxidative stress and lower airway disease, i.e. heaves, has been investigated. It has been demonstrated that heaves-affected horses suffer from pulmonary oxidative stress, which is correlated with lung dysfunction and airway inflammation. Furthermore, there are indices of systemic oxidative stress detectable in blood of heaves-affected horses. There are studies are indicating that exercise-induced pulmonary haemorrhage (EIPH) and exercise-induced rhabdomyolysis are favoured by pro-oxidants. Oxidative stress is also believed to play a deleterious role in muscle ischemia-reperfusion injury occurring during/after anaesthesia. The role of oxidative stress in the motor neuron disease is currently investigated (Nathalie Kirschvink, P. Lekeux, 2005).

In this report, we tried to verify the hypothesis that oxidative stress characteristic changes are correlated with the presence of the equine infectious anemia virus (EIAV) in infected horses. One of the aspects of originality of this

study would be the quantification of the oxidative stress state of the horses in a viral disease comparatively with healthy animals.

Materials and methods

Animals and experimental design: The investigated horses (n=23) belonged to private farms from the territory of the Bistrita-Nasaud county, Feldru village; Six horses (three males and three females) were seronegative for EIAV antibodies by the agar gel immunodiffusion (AGID) test, 2-10 years old and were used as controls. Seventeen horses (ten females and seven males) were positive at the Coggins test, with the age between 2 and 12 years.

Agar gel immunodiffusion (AGID) tests (Coggins et al. 1972) are accurate, reliable tests for the detection of EIA in horses, except for animals in the early stages of infection and foals of infected dams. In other rare circumstances, misleading results may occur when the level of virus circulating in the blood during an acute episode of the disease is sufficient to bind available antibody, and if initial antibody levels never raise high enough to be detectable (Toma B., 1980). The AGID test has the advantage of distinguishing between EIA and non-EIA antigen-antibody reactions by lines of identity (www.oie.int). For our experiment, the AGID test was performed in the laboratory for animal health (LSVSA) Bistrita according to the manufacturer's instructions.

The techniques in the experiment were used to demonstrate the influence of EIA on the oxidative stress. Post infectious oxidative stress was revealed by determining superoxid-dismutase (SOD), cathalase and the general peroxidaze activity. These were analyzed parallel with the level of lipid oxidation and the concentration of the total lipids from the blood and the uric acid levels.

The results were statistically correlated using the *r test* (calculation of the correlation quotient), giving scores for the positivism to the AGID test (value 1), respectively for the negativity (value 0).

Samples: Blood samples were collected by puncturing the jugular vein. The samples were taken on heparin tubes (50 IU/ml) for the quantification of the activity of the main antioxidant enzymes and without an anticoagulant for the serum (d-vac blood collecting system). The samples were transported in an interval of four hours after sampling to the Department of Biochemistry and the Department of Clinical Laboratory from the Faculty of Veterinary Medicine Cluj-Napoca.

The assessment of the superoxid-dismutase: The quantification was based on the auto oxidation of the pyrogalol in the presence of the nitrotethrasolium blue with the formation of a colored compound photometrical dosed at λmax=540 nm (Sanda Andrei et al., 2004).

The assessment of the blood cathalase (Bach Zubkova method): The assessment is based on its main biochemical action, which is to catalyze the decompose of the hydrogen peroxide. This method assesses the quantity of the hydrogen peroxide before and after the action of the enzyme present in the blood.

The cathalase activity is expressed by the cathalazic cipher, which indicates the quantity (milligrams) of hydrogen peroxide decomposed by 0.1 ml of blood.

The assessment of the blood peroxidaze activity: The determination is based on the oxidation reaction of the guaiacol as a hydrogen donor in decomposing of the hydrogen peroxide to peroxide, with the forming of a colored compound that was photometrical dosed at λmax= 470nm (Sanda Andrei et al., 2002).

The assessment of the total lipids (Nobis): Fatty acids from the serum lipids release themselves and sulfonate by treating them with concentrated sulfuric acid, at warm. Then, through a reaction with vanillin and phosphoric acid a violet colored compound is formed (phosphovanilinsulfonic complex). After an incubation of 40-50 minutes at room temperature the compound is photometrical dosed at λmax=546 nm.

Assessing the peroxidating level of the lipids: The peroxidating level of the lipids was determined from blood sera and is based on the property of the malonidialdehide to react with the tiobarbituric acid forming a colored reaction product dosed photometrical at λmax=535nm (Andrei and col., 2002). The concentration of the malonilaldehide was calculated using the extinction coefficient of 1.56 x 105 M 1 cm 1/24 and was expressed in μmoli / ml blood sera.

Uric acid dosing: The uric acid quantification is based on the oxidation of the product to alantoin in the presence of uricasis. Hydrogen peroxide is produced from this reaction, which reacts with TOOS and 4-aminoantipiryn in the presence of the peroxidase, forming a violet compound. This compound is proportional with the concentration of uric acid in the serum and is photometrical dosed at λ between 510 and 560 nm (Victoria Ciurdaru et al., 2001).

Results and discussions

From the biochemical point of view few dates are known that present the implication of oxidative stress in the infectious pathology of the horses and none regarding viral diseases of the horse.

Table 1 represents the obtained values in the assessment of the main antioxidant enzyme activity and picture 1 the variation of the enzymes.

Table 1

Mean and standard deviation for the main antioxidant enzymes in EIAV infected horses and in healthy horses

	SOD activity (u/ml)		**Cathalaze activity (c-Cat $KMNO_4$)**		**Peroxidase activity (Px/ml)**	
	Mean	*St Dev.*	*Mean*	*St Dev.*	*Mean*	*St Dev..*
AIE pozitive	30.78	5.98	8.57	1.9	51.92	7.38
Whitness	37.85	9.3	10.53	1.76	62.08	4.53

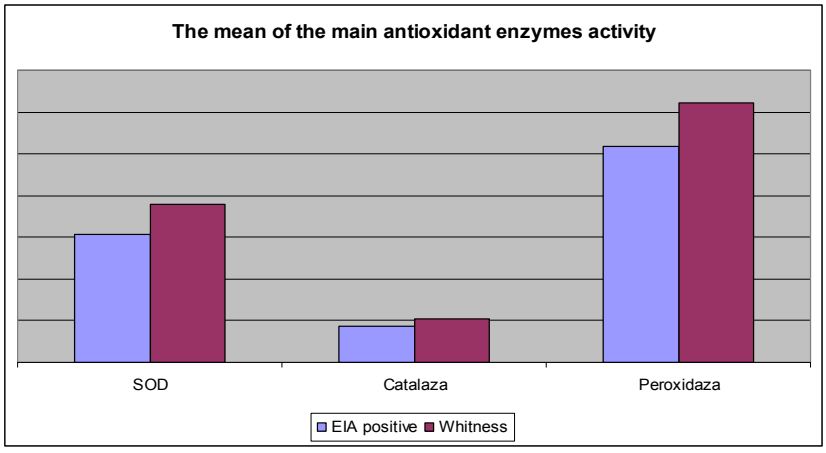

Fig. 1: The activity of the main antioxidant enzymes

Because the oxidation of the lipids is easier to prove, the peroxidative derivatives are continued to be the most used indexed of the oxidative stress.

Lipoperoxidation is often the first parameter used in the proving the involvement of free radicals in the cellular lesions. For highlighting the pro-oxidative status, the degree of the lipid's oxidative level was assessed within the blood sera. The tiobarbituric acid test is the most popular and the easiest method to prove the concentration of MDA and presence of lipoperoxidation. This test was used in the present study too to assess the manner in which the appeared variation in the antioxidating enzymatic activity leads or not to the change of the lipid peroxidase's level. The mean values are presented in table 2. The same table presents the obtained values in the assessment of the total sera lipids and of the uric acid.

Table 2

The concentration of total lipids, the level of lipid peroxidation and uric acid in the blood sera (mean and standard deviation)

	Lipoperoxidation level (nmoli MDA/l		**Uric acid (mg/dl)**		**Total lipids (mg/dl)**	
	Mean	*St Dev.*	*Mean*	*St Dev.*	*Mean*	*St Dev..*
AIE pozitive	56.52	18.4	1.1	0.48	203.4	57.57
Whitness	52.33	19.84	1.33	0.65	154	39

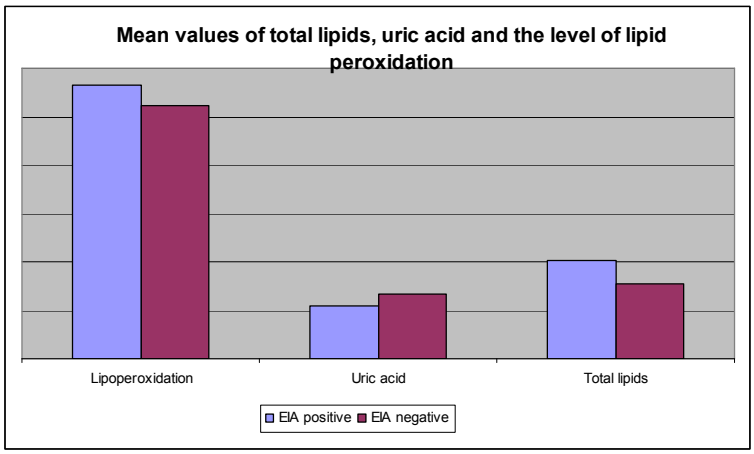

Fig.2: The mean values of lipoperoxidation, uric acid and total lipids

The SOD enzymatic activity decreases considerably in the diseased animals compared to the healthy ones. In the healthy animals SOD average activity is 37.85 U/ml while in the group infected with EIAV this average is 30.78 U/ml. The obtained values were statistically correlated using the r test with the results from the Coggins test. The results obtain at the *r test* were not statistically significant. The activity of SOD alone has not a great value that is why it is necessary to analyze the other antioxidant enzymes.

The activity of cathalase in the EIAV infected horses the activity of the enzyme has values between 4.9 and 11.9 cathalase units, while at the healthy animals the values range between 7.6 and 16.1 units. Media values assessed for the two groups were 10.53 for the group of healthy horses and 8.57 units for the horses with anemia, recording a slight decrease in the cathalase activity in the blood of the infected animals. The correlation quotient at the EIA negative horses $p<0.001$ (level of signification smaller than 0.001), allows us to assert that cathalase activity is positively correlated with the results of the AGID test.

General peroxidase activity is also decreased in the diseased group compared to the control one. The media of the control group is of 62.08 units in one ml of blood (minimum 48.6 and maximum 70.2); in the infected group this media is of 51.92 units (minimum de 39.1 and maximum 62). From the statistical point of view the results of the *r test* (comparing the AGID test results and the general peroxidase activity) were not significant in both infected and healthy horses.

The decrease of the antioxidant enzyme's activity leads to the accumulation of the superoxide anions and of the hydrogen peroxide in the analyzed pathological samples giving a persistent pro-oxidant state at cellular level, which promotes the installation of oxidative stress.

As seen in the obtained results, the change in the antioxidant enzyme activity leads to an increase of the oxidations level of the lipids in the blood sera. Statistically seen, the level of lipid peroxidation is the only indicator of the oxidative stress in EIAV infected horses which correlates positively with the Coggins test results ($0.001<p<0.01$). Thus it can be sustained that in positive horses the level of lipid peroxidation is significantly grown.

Membrane lipids peroxidation leads to the change of the cells' membrane and mitochondrial membrane's properties, in the way of increasing their fluidity and permeability. These changes lead to the disappearance of the osmotic and chemical gradients, resulting in the decrease of cellular excitability, perturbation of metabolic processes and in the appearance of morphological lesions (Dejica, 2000).

The involvement of the uric acid in the oxidative stress is not completely clear. There are more and more proofs, both experimental and clinical, proving the fact that uric acid plays an important antioxidant role in vivo (Glantzounis and col. 2005).

In this research, the values of the uric acid were lightly decreased in the EIAV infected horses (mean value = 1.1 mg/dl) when compared with the values from the healthy horses (mean value = 1.33 mg/dl). The results of the r test have no statistically valuable conclusion regarding the infecting status and the uric acid.

Conclusions

- Enzyme activity of superoxide dismutase (SOD), peroxidase and of the cathalase decreases markedly in the group of anemia compared to the healthy one; the correlation quotient at the EIA negative horses $p<0.001$ (level of signification smaller than 0.001), allows us to assert that cathalase activity is the only enzyme positively correlated with the results of the AGID test.
- The level of lipid peroxidation is the only indicator of the oxidative stress in EIAV infected horses which correlates positively with the AGID test results ($0.001<p<0.01$). Thus it can be sustained that in positive horses the level of lipid peroxidation is significantly grown.
- Decreased levels of uric acid in the sera of EIAV infected horses correlated with the increase of the level of serum lipids peroxidation could establish its role of in vivo antioxidant in horses.
- The decrease of antioxidant enzymes leads to the accumulation of the superoxide anion radicals and of the hydrogen peroxide, respectively of the products resulted through the oxidative decompose of the lipids, proteins and nucleic acids in the analyzed pathological samples, resulting a persistent pro-oxidative state at cellular level, which promotes oxidative stress.

References

1. **Andrei Sanda Maria**, 2002, Studii morfologice şi biochimice ale țesuturilor tumorale spontane şi experimentale, Teză de doctorat.
2. **Ciurdaru Victoria, Sanda Andrei, Adela Pintea, Bele C.,** 2001, Biochimie Medicala Veterinara – Metode si tehnici de laborator, Editura Academic Pres, Cluj-Napoca,
3. **Coggins L., Norcross N.L. & Nusbaum S.R.** (1972). Diagnosis of equine infectious anemia by immunodiffusion test. Am. J. Vet. Res., 33, 11-18.
4. **Cook, R.F., Issel, C.J., Montelaro, R.C.,** 1996. Equine infectious anemia. In: Studdert, M.J. (Ed.), Virus Infections of Equines. Elsevier, Amsterdam, pp. 297–323.
5. **Cook, S.J., Cook, R.F., Montelaro, R.C., Issel, C.J.,** 2001. Differential responses of Equus caballus and Equus asinus to infection with two pathogenic strains of equine infectious anemia virus. Vet. Microbiol. 79, 93–109.
6. **Dejica D.,** 2000, Stresul oxidativ in bolile interne, Editura Casa Cartii de Stiinta, Cluj Napoca
7. **Harrold, S.M., Cook, S.J., Cook, R.F., Rushlow, K.E., Issel, C.J., Montelaro, R.C.,** 2000. Tissue sites of persistent infection and active replication of equine infectious anemia virus during acute disease and asymptomatic infection in experimentally infected equids. J. Virol. 74, 3112–3121.
8. **Issel, C.J., Coggins, L.,** 1979. Equine infectious anemia: current knowledge. JAVMA 174, 727–733.
9. **Moffarts B., Kirschvink Nathalie, van Erck Emmanuelle, Art Tatiana, Pincemail J., Lekeux P.,** 2005, Oxidative stress in exercising horses : fact or fiction ? Equine and Comparative Exercise Physiology, Volume 2, Number 4, November 2005, pp. 253-261(9)
10. **Montelaro, R.C., Ball, J.M., Rushlow, K.E.,** 1993. Equine retroviruses. In: Levy, J.A. (Eds.), The Retroviridae, vol. 2. Plenum Press, New York, pp. 257–360.
11. **Oaks, J.L., McGuire, T.C., Ulibarri, C., Crawford, T.B.,** 1998. Equine infectious anemia virus is found in tissue macrophages during sub clinical infection. J. Virol. 72, 7263–7269.
12. **Toma B.** (1980). Réponse sérologique négative persistante chez une jument infectée. Rec. Med. Vet., 156, 55-63.
13. www.oie.int
14. www.wikipedia.org