

SZENT ISTVÁN EGYETEM

Állatorvos-tudományi Doktori Iskola

**Examinations on the antioxidant system in
different species under physiological and
pathological conditions**

Structured summary of the Ph.D. thesis

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Budapest

2003

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Állatorvos-tudományi Doktori Iskola

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Készült 8 példányban. Ez a sz. példány

Introduction

Free radical (FR) induced and mediated processes are present in a great variety of physiological and pathological pathways. It is well known that FRs have role in aging, exercise, ischemia-reperfusion, diabetes and different inflammatory conditions.

Age-related changes of the antioxidant system have been studied in many species previously. Many observations were taken on the embryonic development of the antioxidant system of chicks. It was established that antioxidant defence mechanisms might vary in the organs due to different concentrations of the major antioxidants. Other investigations revealed many nutritional diseases of poultry that are connected with disturbances of the antioxidant system.

Response to exercise in horses has been studied from several aspects. There is growing evidence supporting the theory that increased oxygen consumption during exercise creates oxidative stress to the animals. Exercise caused elevation of plasma lipid peroxide (LP) level, and decreased red blood cell (RBC) glutathione peroxidase (GPX) activity. Direct proof was produced that during exercise muscle- and RBC-phospholipids are peroxidised and a metastable end product of lipid peroxidation, malondialdehyde accumulates in the muscle fibres and in the erythrocytes. Besides it is well documented that physical stress alters many biochemical and haematological parameters of the blood as well.

Colic sensu stricto -intestinal strangulation/obstruction- in horses is one of the major causes of death in this species therefore it is of outstanding importance. Though surgical techniques and intensive therapy improved in a huge extent over the last decades there is still high mortality among patients that are finally operated on for colic.

In these animals intestinal ischemia-reperfusion (I/R)

induces a series of processes that lead to severe gut wall injury finally resulting in tissue necrosis and shock. There are many pathways involved in the process of I/R that can generate free radicals . FR-caused injury forces endothelial cells to release various kinds of mediators that trigger neutrophil granulocyte migration towards the damaged mucosa. Neutrophils via their myeloperoxidase activity contribute to the production of free radicals. At the same time there is growing evidence that supports that nitric oxide (NO) and its derivatives play important role in the pathogenesis of intestinal I/R. Intestinal I/R can also damage the function of distant organs like the lung and heart via the release of tumour necrosis factor, platelet activating factor, leukotriens and prostaglandins.

There were many trials to prevent these effects with different drugs like enzyme inhibitors, free radical scavengers, antioxidant enzymes, anti-inflammatory compounds, anti-neutrophil agents and metal-chelators. Nevertheless no single drug was found to be completely effective in the prevention of intestinal I/R injury in horses.

Purpose of the experiments involved in the thesis

To evaluate LP in the organs and antioxidant status of plasma of newly hatched chicks from the 1st to the 21st day of life.

To determine biochemical and antioxidant parameter changes in plasma and RBCs in horses competing in pentathlon contests as well as to introduce the recently developed ferric reducing ability of plasma (FRAP) for the measurement of horse plasma antioxidant capacity.

To investigate if LP-changes in the intestines undergoing I/R could be reflected in plasma and red blood cell parameters as well. We also aimed to evaluate effects of iron chelation with deferoxamine and NO synthesis enhancement by L-arginin on the aforementioned processes in a rat model.

To reveal alterations of acid base and blood gas parameters in the perioperative period in horses operated on for colic, as well as to detect changes in the antioxidant indices of plasma and RBCs.

Materials and methods

First experiment

Experimental protocol

Twenty-one one-day-old Ross cockerel chicks were kept together in controlled environment with free access to food and water.

Sample collection and handling

At each sampling time (1, 10, and 21 days of life) 7 chicks were slaughtered, brain, liver and heparinized blood samples were collected. After blood sampling plasma was separated and approximately 0.5 gram of each tissue sample was homogenised with physiological saline solution and stored frozen until analysed.

Second experiment

Experimental protocol

Altogether fourteen horses of various breed, sex and age were used in this trial. Each horse had to complete 20 minutes of warming up followed by a 1-minute contest run consisting of 12 jumps of 120 cm height, involving one double and one triple obstacle as well, then after 20 minutes (while the horses were doing the same work as in the warming up) the contest run was repeated by the same pentathlet riding the same horse. Average speed in the contest runs was approximately 7 m/s.

Sample collection and handling

Blood samples were taken from the jugular vein into heparinized collecting tubes 24 hours prior to the competition,

immediately after the 2nd contest run and 24 hours after the competition. After sampling, plasma was separated and RBC hemolysates were prepared and stored frozen until the analyses were completed.

Third experiment

Experimental protocol

Altogether 56 White Wistar rats were utilized in this study. Before the experiment the animals were divided into 4 groups as follows:

The experimental ischemia reperfusion (I/R) group: The animals were anaesthetized by intraperitoneal injection of sodium pentobarbital. The jugular vein was accessed surgically and blood samples were collected into heparinized tubes. Afterwards, midline laparotomy was performed and the cranial mesenteric artery was ligated. After 30 minutes 1 ml of vehicle solution was injected into the jugular vein of the animals. Another 15 minutes later blood sample was taken from the jugular vein again and full thickness specimens were harvested from the ischemic jejunum from 7 animals. In the other half of the animals in this group, blood flow was restored in the cranial mesenteric artery and blood sampling was repeated and jejunal sampling was completed after 45 minutes. After collection of the intestinal samples the animals were euthanized by overdosing sodium pentobarbital.

L-arginin (A) and deferoxamine (D) treated groups: The animals underwent the same procedure as the ones in group I/R, except that L-arginin (300 mg/kg bw.) or deferoxamine (50 mg/kg bw.) was injected to the animals 15 minutes prior to reperfusion.

In the sham operated (SOP) group four rats were anaesthetized and jejunal samples were collected immediately after anaesthesia. Samples were collected from the rest of the animals according to the same scheme as in group I/R except

no ligature of the cranial mesenteric artery was performed.

Sample handling

After sampling blood samples were immediately centrifuged, plasma was removed and red blood cell haemolysates were prepared. Jejunal specimens were flushed with physiological saline solution then homogenized with physiological saline solution. All samples were stored frozen at -20°C to await the biochemical analysis.

Fourth experiment

Experimental protocol

The study was completed in a clinical setting. Altogether 14 colic horses of various breed, sex and age were used. Intra-operative diagnoses consisted of the following: left dorsal displacement of the large colon ($n=3$), -accompanied by 180° torsion ($n=2$), retroflexion of the pelvic flexure ($n=2$), right dorsal displacement ($n=1$), ileocaecal intussusception (1), scrotal herniation of the ileum (1), strangulation of the jejunum (4). Nine horses undergoing orthopaedic surgery with the same anaesthetic and recumbency protocol were used as controls for the colic patients.

Sample collection and handling

Blood samples were harvested from the jugular vein of the animals into heparinized collecting tubes at the following times: Before the induction of anaesthesia (xylazin-ketamin-diazepam), right after anaesthesia but before the opening of the abdomen, 30 minutes, 1, 2, 12 and 24 hours after the opening of the abdomen. Blood flow was restored in the displaced intestinal segments 15-45 minutes after the opening of the abdomen. Immediately after sampling blood gas analysis was performed on a Radiometer ABL 330 analyser to determine pH, actual base excess (ABE), standard bicarbonate concentration (HCO_3^-), total carbon dioxide concentration (TCO_2), partial carbon dioxide tension (pCO_2), partial oxygen

tension (pO_2) and oxygen saturation of haemoglobin (SAT). After blood gas analysis plasma was separated and RBC haemolysates were prepared. All samples were frozen and stored at -20°C to await the biochemical analysis.

Analytical procedures

The following biochemical and antioxidant parameters of plasma, intestinal tissue and erythrocytes were determined spectrophotometrically in the corresponding experiments: total protein (TP) uric acid (UA) lactate, nitric oxide (NO), thiobarbituric acid reactive substances (TBARS) reduced- (GSH) and oxidized- (GSSG) glutathione concentration, overall concentration of lipid peroxidation end products malondialdehyde and 4-hydroxi-nonenal (M4HN), total antioxidant status (TAS), ferric reducing ability of plasma (FRAP) as well as lactate-dehydrogenase (LDH), creatine-kinase (CK), glutathione-peroxidase (GPX) and superoxide-dismutase (SOD) activities

Statistical analysis

The statistical analysis of the laboratory data was completed by using the Microsoft Excel 5.0 and the Statgraphics 6.0 software programs. One way and multifactor analysis of variance (ANOVA) two samples and paired t-tests and correlation analysis were performed. Significance level was set to $p < 0.05$.

Results

First experiment

The concentration of TBARS was almost three times higher in the brain of the newly hatched chicks than in their liver. At day 10 the TBARS level was almost twice higher in the liver than in the previous samples and there was no

significant difference between the two organs. Another ten days later the brain TBARS concentration showed a marked increase while the level of this substance in the liver remained almost constant throughout the rest of the investigated period. The TAS value of plasma of 10 days-old-chicks was less than half of those measured in the other samples.

Second experiment

The FRAP method was applied successfully for the measurement of horse plasma antioxidant capacity. Plasma UA and FRAP values showed good correlation in a linear model. After the exercise horses showed elevated levels of plasma TP, lactate, CK, LDH and FRAP compared to the pre-exercise values and all these returned approximately to the initial values after 24 hours rest. Similar tendencies were observed in the change of plasma TAS and RBC GPX values. GSH and TBARS concentrations did not show any change immediately after the exercise but decreased TBARS and increased GSH concentrations were observed after 24 hours rest.

Third experiment

The only significant change found in the SOP group was an increase of the intestinal SOD activity during the first 45 minutes of anaesthesia. There was no change of intestinal TBARS concentration in group D whilst some 2-3 fold higher levels were seen in groups I/R and A during reperfusion. The concentration of intestinal M4HN showed no change in groups SOP, A and D, however there was 77-87% higher values observed in group I/R after 45 minutes of ischemia staying approximately at the same level after reperfusion.

Higher FRAP concentrations were measured in groups I/R, A and D in samples taken after the reperfusion period than in those harvested after the ischemic insult. In addition FRAP concentration increased already during the first 45 minutes in

Group D. Plasma NO concentration showed a significant increase upon reperfusion in Group D. Erythrocyte TBARS concentration was higher than the basal value after reperfusion in Group I/R; however no significant change was observed during ischemia. During reperfusion RBC GPX activity decreased in Group D. The RBC SOD activity increased significantly during ischemia then returned to the initial values in Group I/R.

Fourth experiment

No significant change over time was observed in any parameter in the control group. Colic horses showed significant changes over time of venous blood pH, ABE, pCO₂, pO₂, HCO₃⁻, TCO₂, and SAT, as well as plasma uric acid and NO, and RBC TBARS concentration. Significant interaction occurred between sampling time and the type of groups in case of pH, pCO₂, ABE, uric acid and NO concentration. Results of the two group (regardless of sampling time) were significantly different in case of all measured parameters except SAT, TCO₂, GSH and GSSG concentration.

Horses affected with colic were presented with metabolic acidosis. Upon anaesthesia colic horses exhibited marked mixed type acidosis. In spite of that oxygen tension and saturation of the mixed venous blood showed significant increase during anaesthesia with a peak two hours after the incision. Plasma uric acid concentrations were some 1,5 fold higher in the control group during anaesthesia. At the same time colic horses exhibited approximately twice higher FRAP values than the control ones. Plasma NO concentration was significantly higher in the colic horses before the operation and showed a constant decrease thereafter. The highest TBARS concentration was found 1 hour after incision (ie.: 15-45 minutes after reperfusion). There were significantly higher RBC SOD activities observed in the first samples compared to

the 2nd, 3rd and 7th ones in colic horses.

Conclusions and recommendations

It is stated that newly hatched chicks have similar plasma antioxidant capacity to other species. Rate of LP is comparatively low in the liver and high in the brain of the 1-day-old broiler chicks. Increased LP was observed in the liver tissue on the 10th and in the brain tissue on the 21st day of life, the former was accompanied by concomitant decrease of plasma antioxidant capacity. These findings also put light on the role that liver might play in maintaining the plasma antioxidant capacity.

All the measured routine plasma biochemical parameters showed significant increase due to the contest exercise in pentathlon horses indicating first of all the increased load on muscles leading to enzyme leakage, lactate and uric acid accumulation in plasma. FRAP values were also elevated after physical stress. On the other hand TAS values showed opposite picture to FRAP obviously due to the differences of the two methods. The change RBC antioxidant parameters reflects that physical stress alters RBC antioxidant mechanisms as well though we cannot exclude that these findings are part of a longer term process. It is suggested that antioxidant parameters of horses competing in pentathlon contest be monitored. However measurement of multiple parameters rather than one overall index seems to be more informative.

Intestinal I/R induced LP is reflected in the plasma and red blood cell parameters as well in a rat model. Therefore plasma and RBC parameters can serve as indirect measures of LP effects of intestinal I/R. Deferoxamine treatment was proven to be beneficial in the prevention of I/R induced LP, but

the role of L-arginine and nitric oxide remains controversial and necessitates further investigation.

Horses operated on for colic were presented with moderate uncompensated metabolic acidosis. After the induction of anaesthesia the difference between acid-base and blood gas parameters of the control and colic horses became more marked. Though both group of horses were under controlled ventilation and given iv. fluids and dobutamine during the anaesthesia these could not prevent further loss of bicarbonate, and accumulation carbon dioxide and obviously the accumulation of other organic acids in horses having colic. Furthermore changes in the blood LP parameters can serve as an indication for antioxidant therapy during the operation of horses with ileus. It is documented that NO accumulates in plasma of horses having intestinal I/R but the exact role the molecule plays in the pathogenesis has to be clarified. Based on these findings it is suggested that blood antioxidant parameters in the colic horse be monitored and antioxidant therapy be used.

Overview of the new scientific results

1. Newly hatched chicks have similar plasma antioxidant capacity to other species. Rate of LP is comparatively low in the liver and high in the brain of the 1-day-old broiler chicks. Increased LP was occurs in the liver tissue on the 10th and in the brain tissue on the 21st day of life, the former is accompanied by concomitant decrease of plasma antioxidant capacity.
2. The FRAP method can be used as a measure of plasma antioxidant capacity in horses. There is strong linear correlation between plasma uric acid and FRAP concentrations in horse plasma. Exercise usual for

pentathlon contests causes increased activity of muscle-originated enzymes, in blood plasma together with elevated TP, lactate and uric acid concentration. These biochemical changes are accompanied by the significant increase of plasma FRAP concentration and decreased TAS level.

3. Intestinal I/R induced changes in LP parameters are reflected in the plasma and red blood cell parameters in a rat model. Deferoxamine treatment was proven to be beneficial in the prevention of I/R induced LP, but the role of L-arginine and nitric oxide remains controversial and necessitates further investigation.
4. Horses affected with colic were presented with metabolic acidosis that became more pronounced upon anaesthesia. There were significant changes over time in the blood antioxidant indices of horses having colic as well as compared to the control ones basically due to increased LP. There are high plasma NO levels in colic horses that return to the physiological levels gradually.

List of publications related to the thesis

Original articles

1. **Balogh, N.,** Gaál, T., Husvéth, F., Vajdovich, P. (2001): Rate of lipid peroxidation in brain and liver tissues and the total antioxidant status of blood plasma in developing chicks. *Acta Vet Hung*; 9 (2): 197-202.
2. **Balogh, N.,** Gaál, T., Ribiczeyné, P. S., Petri, Á. (2001): Biochemical and antioxidant changes in plasma and erythrocytes of pentathlon horses before and after exercise. *Vet Clin Path*: 30 (4): 214-218.
3. **Balogh, N.,** Krausz, F., Lévai, P., Ribiczeyné, P. S., Vajdovich, P., Gaál, T. (2002): Effect of deferoxamine and L-arginin treatment on lipid peroxidation in an intestinal

ischemia reperfusion model in rats Acta Vet Hung; 50 (3): 343-356

4. **Balogh, N.**, Gaál, T., Vajdovich, P. Gastrointestinalis ischemia reperfúzió, Kórélettani alapok, terápiai lehetőségek *submitted for publication* Magyar Állatorvosok Lapja

Abstracts

1. **Balogh, N.**, Vajdovich, P., Takáts, A., Szlezák, Sz.: The effect of hypoxo-reperfusion on the lipid peroxidation parameters of red blood cells and on the endotoxin levels of plasma during the operation of colic horses - Proceedings, ESVCP Annual Meeting, 1999. 06.04.-06. Verona, Italy Abstract: Comparative Clinical Pathology, 1999, 219-234
2. **Balogh, N.**, Föhrécz, A., Gaál, T.: Az antioxidáns védelmi rendszer egyes elemeinek összehasonlítása hús- és tojótípusú csirkékben – MTA Állatorvosi Bizottsága, Akadémia Beszámolók 1999.
3. **Balogh, N.**, Gaál, T., Ribiczeyné, P. S., Petri, Á.: The effect of exercise on some plasma and red blood cell biochemical and lipid peroxidation parameters in pentathlon horses-ESVIM Congress, 1999.10.14-16. Perugia, Italy
4. **Balogh, N.**, Krausz, F., Lévai, P., Ribiczeyné, Sz. P., Vajdovich, P., Gaál, T.: Effect of deferoxamin and L-arginin treatment on the change of lipid peroxidation parameters in an experimental intestinal ischaemia-reperfusion model in rats- Proceedings ESVCP Annual meeting 26th June Edinburgh, U.K. Absrtact: J Vet Clin Path 30 (3) 2001 p158
5. **Balogh, N.**, Garami, J., Ribiczeyné, Sz. P., Mézes M., Bakos, Z., Bodó, G., Péntek G., Gaál, T., Lukács, Z.: Changes of some plasma and erythrocyte lipid peroxidation indices during the operation of colic horses - preliminary results Proceedings ESVCP Annual meeting 26th June Edinburgh, U.K. Absrtact: J Vet Clin Path 30 (3) 2001 p159

6. **Balogh, N.**, Gaál, T., Ribiczeyné, P. S., Kovács, M.: Changes of some antioxidant parameters in foals during the first three weeks of life Proceedings ESVCP Annual Meeting.: 2000. 06. 21. Toulouse France, Abstract: J Vet Clin Path 29 (4) 2000 p140.
7. **Balogh, N.**, Garami, J., Ribiczeyné, P. Sz., Mézes, M., Bakos, Z., Bodó, G., Péntek, G., Gaál, T., Lukács, Z., Evaluation of acid base and antioxidant indices in horses operated on for colic Proceedings ESVCP Annual meeting, München 2002 09.27. Abstract: : J Vet Clin Path 31 (4) 2002 p202