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The Effect of Humic Acid on the Content of Trace Element in Mitochondria

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Abstract: Based on our previous *in vitro* studies which pointed to the antioxidant properties of humic acids especially concerning radical scavenging activities and ability to maintain the redox system in the cell, an addition of 0.6% humic acid into the diet of farm chicken for 42 days was evaluated. The administration of humic acids have a positive effect on the antioxidant status of plasma and mitochondria of important synthetic, metabolic and organs directly involved in the elimination of oxidative stress conditions-the liver, kidney. With regard to the described chelating properties of humic acid, the current levels of iron, copper, manganese, zinc and selenium considering their functions as cofactors in mitochondria was investigated, too. Our finding incline to an expectation that the administration of humic acid does not affect the binding abilities to metals but rather competition, leading to a decline in selenium use and compensatory responses which should be considered especially when administered over 42 days.

Keywords: Humic Acids, Chicken, Chelating, Mitochondria, Liver, Kidney

Introduction

Humic Acids (HAs) are known for their antidiarrheal, analgesic, immunostimulant and antimicrobial properties (Rath *et al*., 2006). They are actively used in prophylaxis and as therapeutic drugs in veterinary practice in Europe with all kinds of animal species, solely for peroral administration (EMEA, 1999). However, they are constantly studied in relation to health and livestock production. It was found that humates administered in food or water promotes growth in poultry. A positive effect of humic substances was found on feed conversion in broiler poultry (Kocabaǧli *et al*., 2002) and also on growth, meat quality, carcass characteristics, selected parameters determined in the blood and in the gastrointestinal tract (Ozturk *et al*., 2010). Ozturk *et al*. (2012) have also demonstrated an increase in live and carcass weight and a reduction in blood cholesterol levels. Even though the use of HAs preparations in veterinary practice and the food production process can undoubtedly produce the desired positive effects, it was also observed that positive or even negative effects of HS and HAs depend on their source of origin and the physiological condition of the animal (Steinberg *et al*., 2003). Other studies indicate that HS can modulate the toxicity of pollutants, xenobiotics and the bioavailability of metals (Paquin *et al*., 2002; Glover and Wood, 2004; Timofeyev *et al*., 2006) and can alter pH, ionic concentration and enzymatic activity (Timofeyev *et al*., 2006). In Slovakia, there is a well-known and available formulation of HAs from natural oxihumolite, HUMAC®. *in vitro* studies of antioxidant properties of HAs at the recommended prophylactic dosage of 0.1% (Vašková *et al*., 2011) in rat liver mitochondria, the organ with major metabolic functions and responsibility for the metabolism of xenobiotics pointed out that the electrophilic properties of HAs markedly balance the mitochondria redox status. Moreover, strong scavenging properties against hydroxyl radicals and to a lesser extent against superoxide radicals were found.

These data are sufficient to lead us to our long-term

of monitoring the organism's adaptive antioxidant response to conditions induced in the presence of HAs as there is a basic assumption that the dose and length of use is important to achieve the desired positive effect. Antioxidant enzymes commonly use the metal cofactors for catalytic activity. The question arises of how the proposed chelating properties of HAs can affect the antioxidant status and current levels of these elements. We investigated mitochondria from liver, kidney and plasma from chickens, having received HAs in food (0.6%) for 42 days.

Materials and Methods

The experiment was carried out on 36000 broilers COBB 500 from poultry farm Vinica in Veľký Krtíš region (Slovakia). Chicks were divided into 2 groups. The control group (15700 pcs) was fed conventional feed mixtures during 42 days. The experimental group (20000 pcs) was fed conventional feed mixtures enriched by 0.6% humic acid (Humac® Natur, Humac Ltd, Košice, Slovakia) from the first day of fattening for 42 days. All chicks were subjected to standard management and health practices. Feed and water was provided *ad libitum* during the whole experimental period (42 days). Randomly selected 10 chickens from both groups were killed by cervical dislocation, followed by tissue harvesting and collection of blood plasma. Liver and kidney mitochondria were isolated by the method described by Fernández-Vizarra *et al*. (2010). The activity of glutathione reductase (GR; E.C.1.6.4.2) was measured according to a modified method previously described by Carlberg and Mannervik (1985), while that of glutathione peroxidase (GPx; E.C. 1.11.1.9) was measured as described by Flohé and Gunzler (1984). Superoxide Dismutase (SOD; E.C. 1.15.1.1) activity was measured by means of the SOD-Assay Kit-WST (Sigma-Aldrich, Switzerland) following the user manual provided. Reduced glutathione (GSH) levels in mitochondria and plasma were measured by a modified method from Floreani *et al*. (1997) using Ellman's reagent. All the measured parameters were calculated per mg or g of mitochondrial protein (mg_{Prot}, g_{prot}) determined using the bicinchoninic acid assay (Smith *et al*., 1985). Determination of total content of iron, zinc by flame atomic absorption spectroscopy and that of copper, manganese, selenium by graphite furnance atomic absorbtion spectrometry were detected (Shimadzu AA7000). The measured parameters were expressed as the mean ± S.E.M. of three independent measurements. The difference between the two groups was determined using an unpaired student's t-Test.

Results and Discussion

One aspect to consider is the fact that, once taken up, Humic Substances (HS) are able to migrate to organs or organelles and may provoke stress response reactions (Steinberg *et al*., 2003). They have both non-specific and specific effects. The non-specific effects are physical and chemical membrane irritation, induction and modulation of biotransformation activity, induction of chemical defense proteins, the development of internal oxidative stress and the induction of ROS defense enzymes. All organisms have the means to rid themselves of chemical burdens (exotic food chemicals, xenobiotics etc.), i.e., they have developed so-called biotransformation pathways. Also HS behave like chemical clues in the biotransformation pathway. Since HS possess a variety of functional groups, we assume that the Phase II enzymes of the biotransformation system (conjugation reactions with glutathione), in particular, are subject to modulation upon HS exposure (Steinberg *et al*., 2003; Wiegand *et al*., 2004). It is interesting to observe the antioxidant enzyme activity in the mitochondria, as they are the second most important organelle involved in the metabolism of xenobiotics and circulating antioxidants. As shown in Fig. 1, there was no demonstrated change in the activity of SOD compared to the control, but the activity of GPx was significantly lower in the group with HAs. The activity of GR was significantly higher in the liver and kidneys and level of GSH significantly decreased in liver in group supplemented with HAs. Taking into account the interdependencies between the activities of enzymes and levels of GSH in comparison between the three bodies, the results are favorable. The redox potential of GSH is not lost in either kidney or circulating plasma, demonstrating the antioxidant effect of HAs.

The second aspect to be considered is the presence of other HA features, such as chelation. So far, we have not thought about the connection between the chelating properties of HAs and their biological activity in an *in vivo* system. We therefore used processed biological material to investigate this further. Experiments by Tao *et al*. (2000) have shown that the availability of Cu was reduced for fish uptake via their gills in the presence of Fulvic Acid (FA) in water. Sanmanee and Areekijseree (2010) have shown that FA treatment reduces the toxicity of Cu in the mammalian cell-porcine oviductal epithelial cells. However, Fe, Zn, Mn and Cu are included in the group of essential trace elements required for maintaining cellular function and are integral components of numerous metal-containing enzymes (Rajkowska and Protasowicki, 2013). Antioxidant enzymes commonly use the oxido-reduction properties of metal cofactors for catalytic activity. It is quite logical here to question whether the absorption ability and complexation of metals by HAs may reduce the adsorption of biologically important elements available to the animal. Thus, we examined the distribution of Cu, Zn, Mn, Fe and also non-metal cofactor Se.

The levels of metals detected by atomic absorption spectrometry pointed to particularly significant changes in the amounts of metal present (Table 1). Cu was higher in the liver and plasma, but lower in the kidneys. The amounts of Zn decreased in the liver and kidneys, as did Mn. The levels of Fe were uniformly significantly high. Se was significantly higher in the kidneys but lower in the plasma. It is also desirable to compare our findings with those of whole bodies.

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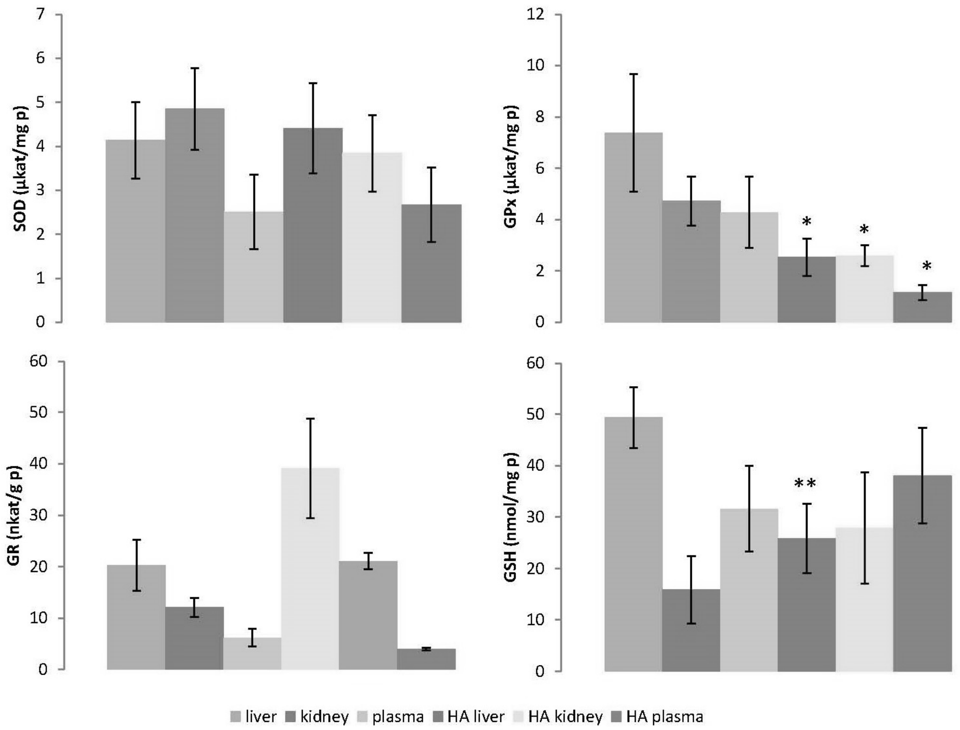

Fig. 1. Activities of the antioxidant enzymes superoxide dismutase, glutathione peroxidase, glutathione reductase and reduced glutathione in plasma and mitochondria isolated from liver and kidney. Comparison of groups receiving humic acid Vs. without humic acid (*p<0.05; **p<0.01versus control)

Table 1. Distribution of mean concentration of Zn, Cu, Mn, Fe and Se in plasma and mitochondria isolated from liver and kidney (*p<0.05; ***p<0.001versus control)

		Without HA	HA
Zn (µg/g)	Plasma	59.62±2.97	60.16±4.36
r=0.9996	Kidney	49.67±3.10	29.188±2.01***
Det. lim 0.001 mg/L	Liver	63.86±4.50	50.35±3.33*
Cu (ng/g)	Plasma	50.99±0.01	56.27±0.03***
r=0.9976	Kidney	73.16±0.04	55.21±0.08***
Det. lim 0.0004 mg/L	Liver	33.34±0.03	112.58±0.04***
Mn (ng/g)	Plasma	44.14±0.27	71.03±0.33***
r=0.9860	Kidney	127.28±0.65	46.54±0.56***
Det. lim 0.0002 mg/L	Liver	124.79±0.11	50.04±0.31***
Fe (µg/g)	Plasma	1.52±0.03	3.84±0.05***
r=0.9998	Kidney	0.79±0.01	2.98±0.02***
Det. lim 0.004 mg/L	Liver	2.12±0.08	3.51±0.02***
Se (ng/g)	Plasma	535.13±0.04	199.24±0.03***
r=0.9971	Kidney	107.17±0.05	1163.27±0.04***
Det. lim 0.0009 mg/L	Liver	Under limit	51.95±0.01

The results obtained by Zralý and Písaříková (2010) confirmed that feeding sodium humate to animals had no significant adverse effect on the Cu or Zn content in the investigated organs and tissues and cited many other authors with the same findings. On the other hand, elevated concentrations of trace elements in pig tissues were reported by López-Alonso *et al*. (2007). The detected trace element levels in blood serum reflected the current level of dietary mineral supply to the animals and were consistent with the finding of Stowe *et al*. (1992), Kim and Mahan (2001) and others. The highest content of trace elements, except Se, was detected in the liver which is a depot organ of a higher diagnostic value than muscular tissue for the assessment of dietary mineral supply to animals. The kidneys, where the highest concentrations of selenium were detected in the present study, are the most important organ involved in selenium disposition (López-Alonso *et al*., 2007). Owing to sodium humate feeding, the levels of Mn and, above all, Se were significantly decreased in blood serum (Zralý and Písaříková, 2010). The observations in the bodies and mitochondria share a

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lot of similarities but there are also differences that are likely to have a fundamental effect on the overall redox status and the use of elements.

Potential antagonistic interactions, such as those between iron and manganese or iron, copper and zinc etc should also be taken into consideration (Creech *et al*., 2004). Mitochondria provide cellular Fe-S clusters and GR is required to maintain oxidant-labile Fe-S enzymes such as aconitase. It is therefore logical to assume that the total Fe concentration will rise. The increased amount of Fe may contribute to oxidative stress alone, but also to increased activity of iron-dependent enzymes for the synthesis of antioxidant enzymes, thus directly relating to the use of Cu, Zn and Mn in the mitochondria. Conditions for the oxidation of SOD to form disulfides are essential for the activity of SOD and Cu transfer into the active site. Recent *in vivo* studies have shown that the copper chaperone for SOD1 controls the formation of this disulphide in an O_2-responsive step (Brown *et al*., 2004). Although there are still many mechanistic alternatives, it is becoming apparent that this metallochaperone, copper chaperone, does far more than deliver Cu: it has both sulphydryl oxidase and protein disullfide isomerase activities that appear to allow for higher order types of physiological regulation in response to oxidative stress (Culotta *et al*., 2006). The binding of Cu is the limiting step for the use of Zn.

The level of mitochondrial Mn, however, is normally 1-2 orders less than Fe (Cobine *et al*., 2004). Presently we cannot say whether there is metal competition for SOD binding due to an increase in Fe and thereby reduced MnSOD synthesis; however, high Mn levels in the plasma may prevent the impairment of arginase activity and nitrosative stress. Finally, GSH levels were offset in the plasma. Regarding the relationship between humic acid and selenium, the availability of selenium was also impaired in humans due to its inhibition by humic substances present in drinking water (Wang *et al*., 1992). This feature of HAs could be responsible for our findings, since GPx activity in the mitochondria was significantly reduced in mitochondria from both organs and the decrease was the most pronounced in the plasma. The case may yet be that, despite the increased levels of Se in the kidney the total level of Se is significantly lower in the control group, as the circulating isoforms of glutathione are formed only in the kidney. This could even be the case when considering the presence of other forms of transport such as selenoprotein P or bound to albumin.

Conclusion

The results of *in vivo* experiments on isolated liver mitochondria are much the same as those found *in vitro* regardless of the differences between the representatives of the classes of vertebrates. There is an expectation that the administration of HAs does not affect the binding ability to metals but rather competition, leading to a decline in Se use, producing a sequence of compensatory responses. Further studies will be focused on clarifying this, as it may be an important factor in the length of safe usage of HAs since the 42-day ingestion appears to limit the beneficial effect.

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Author Contributions

Daniel Žatko: Substantial contribution to acquisition of data, drafting the article.

Janka Vašková: Analysis and interpretation of the data, drafting the article, revising for intellectual content, final approval of the article.

Ladislav Vaško: Substantial contribution to conception and design, revising critically for intellectual content, final approval of the article.

Peter Patlevič: Substantial contribution to acquisition of data, analysis and interpretation of the data.

Ethics

The authors declare no financial/commercial conflicts of interest with the published data.

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