EFFECT OF FULVIC AND HUMIC ACIDS ON COPPER AND ZINC HOMEOSTASIS IN RATS

István HULLÁR[1*], András Valentin VUCSKITS[2], Erzsébet BERTA[1],
Emese ANDRÁSOFSZKY[1], András BERSÉNYI[1] and József SZABÓ[1]

[1]Department of Animal Breeding, Nutrition and Laboratory Animal Science,
University of Veterinary Medicine, P. O. Box 2, H-1400 Budapest,
Hungary; [2]Europharmavet Ltd., Budapest, Hungary

The objective of this study was to investigate the effects of fulvic acid (FA) and humic acid (HA), the two main compounds of humic substances (HSs), on copper (Cu) and zinc (Zn) homeostasis. Seventy-two male Wistar rats were randomly divided into nine experimental groups. The control diet (AIN-93G formula) and the diets supplemented with 0.1%, 0.2%, 0.4% and 0.8% FA or HA were fed for 26 days. Cu and Zn concentrations of the large intestinal content (LIC), liver, kidney, femur and hair were determined. FA and HA did not influence significantly the Cu or Zn contents of the experimental diets, the rats' feed intake, weight gain and the feed to gain ratio. Both FA and HA decreased the Cu concentrations of the LIC significantly and in a dose-related manner; however the absorption-stimulating effect of HA was more pronounced. FA increased the Cu content of the liver, but neither FA nor HA had a dose-dependent effect on it. FA or HA supplementations had no significant effect on the Cu concentration of the kidney. At the concentrations used, dietary FA or HA supplementations are not promising growth promoters. FA influences the Cu homeostasis unlike HA, because FA not only stimulates Cu absorption, but the extra quantity of absorbed Cu is retained in the organism. The stimulatory effect of HA on Zn absorption may not be manifested in Cu and Zn homeostasis, because of the tight connection of these microelements to FA and HA, which prevents the transmission of Zn from the ZnHA complex to the organs. As regards the effect of FA and HA on Cu and Zn homeostasis, both FA and HA stimulated the absorption of these microelements, but only FA increased the retention of Cu (in the liver) and Zn (in the kidney).

Key words: Fulvic acid, humic acid, microelement, metabolism

After the ban of antibiotic growth promoters (AGPs), researchers are looking for suitable replacements. Among several other products, humic substances

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*Corresponding author; E-mail: hullar.istvan@univet.hu; Phone: 0036 (1) 478-4122

(HSs) have been tested and found to have some positive effects in broilers (Bailey et al., 1996; Eren et al., 2000; Kocabagli et al., 2002) and turkeys (Parks, 1998). HSs are formed from decayed plant matter with the aid of living bacteria in the soil. Their composition includes humus, humic acid (HA), fulvic acid (FA), ulmic acid and microelements necessary for plant development (Stevenson, 1994; Herzig et al., 2000; Andersen et al., 2005; Herzig et al., 2009). Their most active fractions are HA and FA. HSs are soluble in dilute alkaline solutions but HA can be precipitated in acidic medium. Its molecular weight is between 5000 and 100,000 daltons (Da). FA is soluble under all pH conditions and has the lowest molecular weight, around 2000 Da (Islam et al., 2005).

An important feature of HSs is that they can combine with metal ions, oxides and clay to form water-soluble or insoluble complexes, and interact with organic compounds (Islam et al., 2005). They can affect the macro- and micro-mineral metabolism of living organisms.

In spite of the fact that HSs can bind metal cations, and an almost infinite number of humic-metallic complexes can be formed (Boyd et al., 1981; Senesi et al., 1985; König et al., 1986; Kang et al., 1991; Livens, 1991), only few studies have dealt with the specific effects of the main components (FA and HA) of HSs on the intestinal absorption of microelements and their concentrations in different animal organs.

Hence, the objective of this study was to investigate the dose-related effects of FA and HA as the two main HSs separately on the production parameters of rats, as well as on the concentration of Cu and Zn in the large intestinal content (which may indirectly reflect the absorption of these microelements), and also on Cu and Zn concentrations in the liver, kidney, femur and hair of rats. This study is an organic continuation of our previous work (Szabó et al., 2017) which investigated the effects of FA and HA on iron and manganese homeostasis in rats.

Materials and methods

Animals

Seventy-two weaned, Wistar CRL:(WI) BR male SPF rats were used in this experiment. Animals were housed in individual cages at 24 °C ambient temperature. After an adaptation period of 4 days, the animals were randomly divided into nine dietary treatment groups on the basis of their body weight.

Diets

The control diet was prepared according to the AIN-93G formula of the American Institute of Nutrition (Reeves, 1997). The experimental diets were prepared by supplementing the control diet with 0.1%, 0.2%, 0.4% or 0.8% of FA or HA to replace starch. The diets were fed for 26 days. Water and feed were provided *ad libitum* during the experiment.

HSs fractionation

Both FA and HA were extracted from leonardite (Szabó et al., 2017).

Measurements and analysis

The individual body weight and feed consumption of rats were measured three times a week. On the 26th day of the trial rats were euthanised (90 mg/kg BW CP ketamine and 0.5 mg/kg BW medetomidine) and exsanguinated. Samples of large intestinal content, liver, kidney, femur and hair were collected and stored until assayed for Cu and Zn concentration. The Cu and Zn contents of the samples were determined by a Carl Zeiss Jena AAS3 atomic absorption spectrometer. As regards the hair samples, identical quantities were collected from each animal and pooled in treatment groups because of the low quantities collectible per animal.

The results are expressed as means ± SD. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) with Tukey's *post hoc* multiple comparison test. Response curves were fitted by linear and quadratic regression procedures using MS Excel 2007 software.

The experimental protocol met the standard criteria of the Scientific Ethics Committee of Animal Experiments of the University of Veterinary Medicine, Budapest, Hungary.

Results and discussion

Effects of FA and HA on the Cu and Zn concentrations of diets

Table 1 presents the Cu and Zn contents of FA, HA and that of the experimental diets. Because of the negligible Cu and Zn concentrations of FA and HA, the supplementation levels applied did not change the Cu and Zn contents of the experimental diets significantly (Table 1 and Fig. 1). The relative changes of these minerals in the FA- or HA-supplemented diets were maximum 1.81%. On this basis we may assume that the changes in Cu or Zn content of the supplemented diets were minimal, and only the chelating ability of FA and HA can be responsible for the presumed effects of FA or HA on Cu and Zn homeostasis of the animals.

Effects of FA and HA on production

The effects of the FA- or HA-supplemented diets on the feed intake, weight gain and feed to gain ratio of rats are summarised in Table 2.

FA or HA supplementation had no significant influence on the feed intake, weight gain and feed to gain ratio of the rats. These results are in accordance with the findings of Mevlüt et al. (2004) and Kaya and Tuncer (2009) in broilers, and Schuhmacher and Gropp (2000) in weaning pigs.

Table 1

Copper (Cu) and zinc (Zn) concentrations of fulvic acid (FA) and humic acid (HA) in the experimental diets (mg/kg)

	Cu	Zn
FA	4.38	128.00
HA	6.91	8.10

	Diets				
FA supplementation (% of diet)	0.0	0.1	0.2	0.4	0.8
Cu	7.50	7.50	7.51	7.52	7.54
Relative change of Cu content (%)	0.00	0.05	0.12	0.24	0.47
Zn	56.1	56.2	56.4	56.6	57.1
Relative change of Zn content (%)	0.00	0.23	0.46	0.90	1.81
HA supplementation (% of diet)	0.0	0.1	0.2	0.4	0.8
Cu	7.50	7.51	7.51	7.53	7.56
Relative change of Cu content (%)	0.00	0.09	0.18	0.37	0.67
Zn	56.1	56.1	56.1	56.1	56.2
Relative change of Zn content (%)	0.00	0.02	0.04	0.05	0.11

Remark: Cu and Zn content of the basal diet, FA and HA were measured and, based on these values, Cu and Zn levels of the supplemented diets were calculated
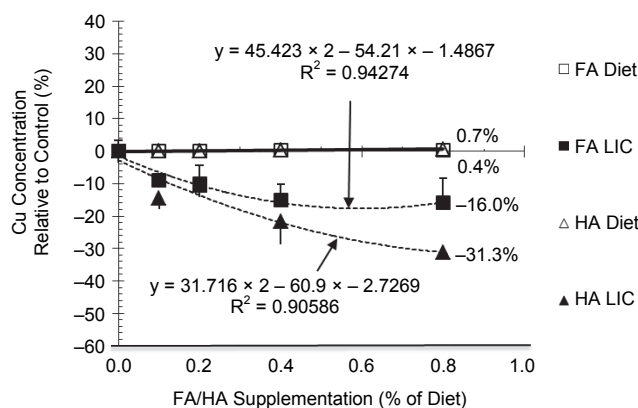

Fig. 1. Effect of fulvic acid (FA) or humic acid (HA) supplementation on the copper (Cu) concentration of diets and that of the large intestinal content (LIC)

In contrast to these results, some published data prove the growth-promoting effect of HSs in broilers (Bailey et al., 1996; Eren et al., 2000; Kocabagli et al., 2002) and turkey toms (Parks, 1998).

One of the factors responsible for the discrepancies between our data and the results of other researchers regarding the growth-promoting effects of HSs might be the different composition (FA/HA ratio) of humic products on the market (Kocabagli et al., 2002).

Table 2

Effect of fulvic acid (FA) or humic acid (HA) supplementation of diets on feed intake, body weight gain (BWG) and feed to gain ration (mean ± SD, n = 8)

	FA				
FA (% of diet)	0.0	0.1	0.2	0.4	0.8
Feed intake (g/100 g BW/day)	6.54 ± 0.19	6.58 ± 0.40	6.50 ± 0.35	6.35 ± 0.52	6.68 ± 0.43
BWG (g/100 g BW/day)	2.16 ± 0.19	2.10 ± 0.18	2.17 ± 0.12	2.10 ± 0.12	2.03 ± 0.11
Feed/gain (g/g)	3.02 ± 0.32	3.14 ± 0.38	3.00 ± 0.28	3.04 ± 0.38	3.30 ± 0.31
	HA				
HA (% of diet)	0.0	0.1	0.2	0.4	0.8
Feed intake (g/100 g BW/day)	6.54 ± 0.19	6.71 ± 0.34	6.49 ± 0.41	6.53 ± 0.24	6.67 ± 0.31
BWG (g/100 g BW/day)	2.16 ± 0.19	2.08 ± 0.14	2.18 ± 0.16	2.21 ± 0.11	2.18 ± 0.18
Feed/gain (g/g)	3.02 ± 0.32	3.23 ± 0.32	2.98 ± 0.37	2.95 ± 0.25	3.06 ± 0.29

Effects of FA and HA on the Cu and Zn concentrations of large intestinal content, liver, kidney, femur and hair

Assuming the effects of FA and HA supplementations on microelement homeostasis, two factors must be taken into consideration, namely the effect of their micro-mineral content and their effects as biologically active substances. We propose that the dose-related relative differences between the Cu and Zn concentration changes of the FA- or HA-supplemented diets and the concentrations found in the large intestinal content and the different organs may give direct (microelement concentrations of organs) and indirect (absorption) information about the specific effects of FA and HA on Cu and Zn homeostasis.

For this assumption to be true, there should be no significant differences in the digestibility of organic matter of the experimental diets between the control and treatment groups. As in this study the organic components of the diets were identical and the feed intake, body weight gain and feed to gain ratio did not differ significantly from the control values (Table 2), we can state that this experiment complies with these requirements.

Copper

Table 3 and Fig. 1 summarise the Cu concentration changes in the large intestinal content and in the different organs. Compared to the control values, both FA and HA supplementations significantly decreased the Cu concentration of the LIC (Table 3 and Fig. 1).

At 0.8% dietary FA or HA level these decrements were 16.0 and 31.3%, while the increase of Cu concentrations in the diets was only 0.7 and 0.4%, respectively (Fig. 1).

Table 3

Effect of fulvic acid (FA) or humic acid (HA) on the copper (Cu) concentration of the large intestinal content (LIC), liver, kidney, femur and hair of rats (mean ± SD, n = 8)

FA					
FA (%)	0.0	0.1	0.2	0.4	0.8
Cu concentration (mg/kg dry matter)					
LIC	143 ± 4.92[a]	130 ± 2.12[b]	128 ± 8.38[bc]	121 ± 7.09[cd]	120 ± 10.8[bcd]
Liver	13.5 ± 0.51[a]	14.0 ± 1.49[ab]	15.5 ± 1.32[b]	14.9 ± 1.81[ab]	15.2 ± 0.83[b]
Kidney	22.3 ± 2.33	21.8 ± 5.11	21.5 ± 1.27	21.8 ± 1.97	23.3 ± 2.70
Femur	8.95 ± 0.68	8.58 ± 0.72	8.73 ± 0.31	8.98 ± 0.53	9.25 ± 0.38
Hair[**]	12.3	12.5	12.3	12.6	12.4
HA					
HA (%)	0.0	0.1	0.2	0.4	0.8
Cu concentration (mg/kg dry matter)					
LIC	143 ± 4.92[a]	122 ± 4.87[b*]	128 ± 5.22[b]	112 ± 9.96[c]	98.1 ± 2.09[d*]
Liver	13.5 ± 0.51[a]	14.8 ± 0.96[b]	12.4 ± 0.23[c]	14.1 ± 1.47[ab]	13.7 ± 1.21[ab]
Kidney	22.3 ± 2.33	22.6 ± 1.28	22.5 ± 0.46	23.4 ± 0.76	24.5 ± 0.87
Femur	8.95 ± 0.68	8.88 ± 0.34	8.98 ± 0.21	8.80 ± 0.27	8.77 ± 0.09
Hair[**]	12.3	12.3	12.4	12.5	12.1

[*]significant difference between FA and HA groups; [**]identical quantities were collected from each animal and pooled in treatment groups

The Cu concentration changes in the LIC were dose dependent in both the FA- and HA-supplemented rats. The relationships between the dietary and LIC concentration changes of Cu were polynomial and significant ($P < 0.05$; $R^2 = 0.943$ and 0.906, respectively, Fig. 1). In HA-supplemented rats the Cu concentrations in the LIC were consistently lower than in the FA-supplemented animals, but the differences between the FA and HA groups of rats were not significant up to the highest investigated doses.

Compared to the control, FA increased the Cu concentration of the liver at all supplementation levels; however, these increases were significant only at the 0.2 and 0.8% dietary FA levels, suggesting that the Cu-storing capacity of the liver was probably saturated at the 0.2% dietary FA level.

Because of the very firm bonding strength between Cu and HA (Rashida, 1974), and of its large molecular weight (Islam et al., 2005; Zraly et al., 2008), we hypothesised that HA would inhibit Cu absorption. Contrary to this assumption, HA supplementation of the diets significantly stimulated the intestinal absorption of Cu.

The effects of HA on the Cu content of the liver were inconsistent: 0.1% increased, 0.2% decreased it significantly, while 0.4 and 0.8% HA supplementation levels had no significant effect on liver Cu content (Table 3). Neither FA nor HA had a dose-related effect on the Cu content of the liver.

FA or HA supplementation had no significant effect on the Cu concentration of the kidney, femur and hair (Table 3).

The balance between Cu intake and excretion determines the amount of Cu in the organism. The intestinal absorption of Cu is dependent on the high-affinity copper transporter 1 (CTR1) and ATP7A, but other factors such as the divalent metal transporter 1 (DMT1) and the low-affinity copper transporter 2 (CTR2) are also involved in the course of Cu absorption from the intestinal tract (Peter et al., 2009); however, the entire mechanism is not clearly understood.

Copper is eliminated from the body by the excretion of poorly reabsorbable biliary Cu (Owen, 1964) and via the urine (Yu and Beynen, 1995).

Summarising these results, we may conclude that both FA and HA dose-dependently stimulated Cu absorption from the intestinal tract and, as a tendency, HA was more efficient than FA. Unlike HA, FA can increase the Cu content of the liver.

As an indirect conclusion, increased urinary elimination of Cu absorbed from the intestine of rats fed the HA-supplemented diet can be assumed. The antagonistic effects of FA and HA on Cu homeostasis can be beneficial in cases of unexplained anaemia in which a U-shaped relationship between serum Cu levels and unexplained anaemia was reported (Knovich et al., 2008), indicating that both high and low serum Cu levels are associated with unexplained anaemia in adults.

Zinc

Compared to the control, FA or HA supplementation had no significant effect on the Zn level of the diet (Table 1).

HA supplementation significantly decreased the Zn concentration of the LIC ($P < 0.05$; Table 4, Fig. 2), and there was a strong dose-related relationship ($R^2 = 0.985$; $P < 0.05$) between the HA dose and the Zn concentration change in the LIC of rats.

Table 4

Effect of fulvic acid (FA) or humic acid (HA) on the zink (Zn) concentration of the large intestinal content (LIC), liver, kidney, femur and hair of rats (mean ± SD, n = 8)

	FA				
FA (%)	0.0	0.1	0.2	0.4	0.8
	Zn concentration (mg/kg dry matter)				
LIC	796 ± 39.2	745 ± 42.4	753 ± 16.7	722 ± 66.0	732 ± 66.8
Liver	105 ± 11.4	101 ± 5.28	107 ± 9.32	103 ± 13.5	104 ± 10.2
Kidney	96.7 ± 6.21^a	89.4 ± 0.93^b	91.9 ± 2.03^{ac}	90.1 ± 1.50^c	92.2 ± 4.72^c
Femur	474 ± 11.3^a	456 ± 32.0^{ab}	462 ± 32.9^{ab}	469 ± 19.0^a	444 ± 14.4
Hair[**]	289	290	286	297	293

	HA				
HA (%)	0.0	0.1	0.2	0.4	0.8
	Zn concentration (mg/kg dry matter)				
LIC	796 ± 39.2^a	742 ± 29.3^b	736 ± 34.4^b	667 ± 41.9^c	597 ± 91.1^{c*}
Liver	105 ± 11.4	103 ± 11.0	97.7 ± 1.52	106 ± 9.12	99.6 ± 4.39
Kidney	96.7 ± 6.21^a	94.4 ± 5.21^a	93.3 ± 3.28^a	91.6 ± 4.60^a	92.1 ± 4.18^a
Femur	474 ± 11.3^a	455 ± 7.94^{bc}	462 ± 9.81^{abc}	451 ± 10.9^b	466 ± 8.52^{ac}
Hair[**]	289	284	290	293	288

[*]significant difference between FA and HA groups; [**]identical quantities were collected from each animal and pooled in treatment groups
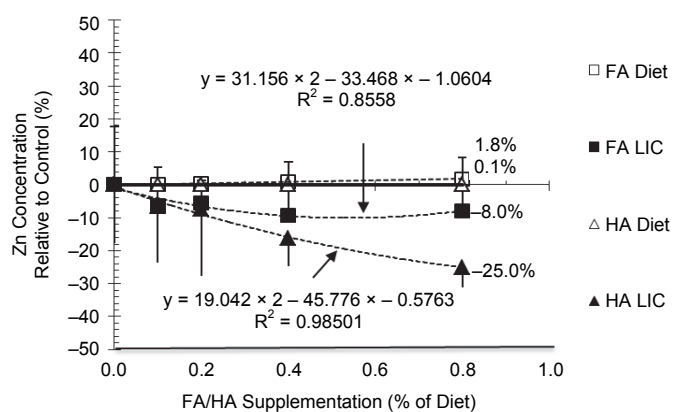

Fig. 2. Effect of fulvic acid (FA) or humic acid (HA) supplementation on the zinc (Zn) concentration of diets and that of the large intestinal content (LIC)

Neither FA nor HA induced Zn accumulation in the liver, suggesting that the liver did not receive Zn from the ZnFA or ZnHA complexes, probably because of the strong binding between Zn and FA or HA.

FA supplementation tended to decrease the Zn content of the kidney, and this decrease was significant at 0.1% and 0.4% supplementation levels. There was no significant relationship between the dietary FA dose and kidney Zn concentrations.

Although FA supplementation did not have a significant influence on Zn concentration of the LIC compared to the control group, the Zn levels of the LIC were lower in the FA-supplemented animals than in the control group, and the correlation between the dietary FA dose and the Zn content of the LIC was polynomial and significant ($R^2 = 0.855$, $P < 0.05$; Fig. 2). HA supplementation tended to decrease the Zn concentrations compared to the control, but there was no significant difference from the control value even at the highest dose of HA. In contrast to the effect of FA, there was a highly significant quadratic relationship between the dietary HA doses and the Zn concentration of the kidney tissue ($R^2 = 0.989$; $P < 0.01$; Fig. 3). As an indirect conclusion, we may hypothesise that elimination of the absorbed FA-Zn and HA-Zn complex via the urine may be important in the Zn homeostasis in rats receiving FA- or HA-supplemented diets.
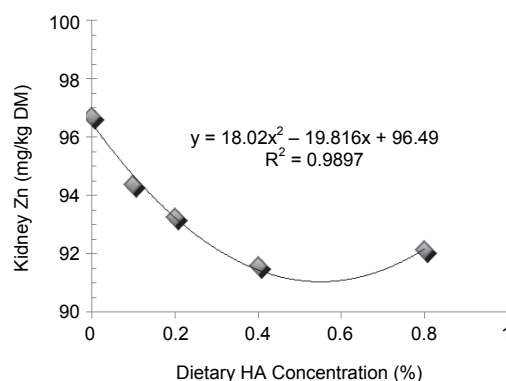

The plot shows Kidney Zn (mg/kg DM) on the y-axis (90 to 100) versus Dietary HA Concentration (%) on the x-axis (0 to 1), with the fitted curve:

$$y = 18.02x^2 - 19.816x + 96.49$$
$$R^2 = 0.9897$$

Fig. 3. Dose-dependent relationship between the humic acid (HA) content of the diets and the zinc (Zn) concentration of the kidney

Based on the pooled data of the FA- or HA-supplemented groups, the Zn content of the femur decreased significantly; however, this decrease was not always significant in the different treatment groups (Table 4).

Neither FA nor HA supplementation influenced the Zn content of hair.

Summarising these results, we may conclude that both FA and HA dose-dependently stimulated Zn absorption from the intestinal tract, and that HA tended to be more efficient than FA.

Data on the Zn content of the femur suggest the possibility that HA has a sufficiently strong Zn binding capacity to receive Zn from the bones and that the ZnFA or ZnHA complexes are probably eliminated via the kidney.

Based upon the Zn content of the kidneys, we can hypothesise that both FA and HA stimulate the urinary elimination of Zn; however, a dose–effect relationship could be detected only in the HA-supplemented rats.

It is generally accepted that zinc homeostasis is controlled by both its small intestinal uptake through a transcellular saturable carrier-mediated mechanism (Lee et al., 1989) and its excretion via the shedding of epithelial cells and in pancreatic and biliary secretions. In rodents, colonic absorption of zinc also occurs (Hara et al., 2000). The ZIP family of transporters regulates Zn transport from the intestinal lumen into the cytosol and ZnT moves Zn in the opposite direction (Cousins et al., 2006). Both transporter families are expressed along the gastrointestinal tract; however, the mechanism involved in zinc absorption is not fully understood yet.

Finally, we can conclude that, in the concentrations used in this study, dietary FA or HA supplementations are not promising growth promoters. Unlike HA, FA influences Cu homeostasis, because FA not only stimulates Cu absorption, but the extra quantity of absorbed Cu is retained in the organism. The stimulatory effect of HA on Zn absorption may not be manifested in Cu and Zn homeostasis because of the tight connection of these microelements to FA and HA which prevents the transmission of Zn from the ZnHA complex to the organs. When evaluating the effect of FA and HA on Cu and Zn homeostasis, we should consider not only their effects on absorption but also on the retention of these elements.

Acknowledgements

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