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Iron, Copper, Zinc and Selenium Concentrations and their Interaction in Organs of White Nose Disease of Raccoon Dogs

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ABSTRACT

While the etiology of White Nose Disease (WND) is still uncertain, trace elements have been postulated to play a role. The purpose of this study is to determine the concentrations of Fe, Cu, Zn and Se in the liver, heart, lung, spleen, kidney and muscle of WND raccoon dogs and clarify whether changes of those trace elements are related to the disease. We measured concentrations of these trace elements in various organs in both WND and WND-free (control) groups, using flame atomic absorption spectroscopy for Fe, Cu and Zn and fluorometric method with 2,3-diaminonaphthalene for Se. Compared with the controls, the concentrations of liver, lung and muscle Fe were significantly lower in the WND group ($p<0.05$). Copper concentration in the lung was also significantly lower ($p<0.05$) in the WND group, but the levels in the spleen and muscle were significantly higher ($p<0.05$). While the spleen and lung levels of Zn were significantly lower ($p<0.05$) in the of WND group, the heart level was significantly higher ($p<0.05$). In comparison with controls, the lung, kidney and muscle levels of Se in the WND group were significantly lower. The correlations of the different trace element concentrations were statistically analyzed between each pair of tissues. The most frequent correlations were found for Cu-Zn within some tissues, especially in the lung. Interestingly, no correlation exists between metal concentrations was found in the liver. Abnormalities in the contents of the selected trace elements were observed, which revealed some imbalances in the inter-element relations in some organs of the WND infected raccoon dogs. These results suggest that t trace element contents and their homeostasis are disturbed in WND infected raccoon dogs. The WND raccoon dogs were either deficient in iron or in selenium, or deficient in both.

Key words: Raccoon dog, trace element, white nose disease, concentrations

INTRODUCTION

Raccoon dog (*Nyctereutes procyonoides* Gray, 1834) is a species of family Canidae, order Carnivora. The species is native to China, Korea, Japan and northeastern Russia(Zhao *et al.*, 1994). *Nyctereutes procyonoides ussuriensis*, one of the six subspecies of raccoon dogs, is distributed and raised for fur in the Northeast and eastern coast area of China (Liu *et al.*, 2012). The pathogenesis of WND in raccoon dogs is still not clearly known. In the last ten years, a number of studies have been carried out to understand the etiology of WND. These studies have provided evidences on the involvement of disturbances of certain trace elements (Hou *et al*., 2010a).

Maintaining optimum biological levels of trace elements is essential for normal metabolic and physiological processes. The elements function were either as activators of enzyme systems or as constituents of organic compounds and they also are also constituents of some proteins involved in immune defense systems (Kilicalp *et al*., 2009; Abdallah *et al*., 2009; Ogboko, 2011). Imbalances of these elements may adversely impact certain biological processes and lead to many pathological consequences and metabolic disorders (Mertz, 1981). Iron is an important component of hemoglobin and several enzymatic systems and anemia is usually a result of iron deficiency (Munoz *et al*., 2009). Copper has an important function as a component of enzymes such as tyrosinase, cytosinase oxidase and monoamine oxidase. Copper deficiency can result in depressed white cell levels, impaired bone formation, depigmentation of the hair and certain types of anemia (Thornburg, 2000). Zinc has been identified as a cofactor in more than 200 enzyme-catalyzed processes (Kazi *et al*., 2001). Zn is also involved as a component of transcript factors known as Zn fingers that bind to DNA and activate the transcription of a message and impart stability to cell membrane. Zn deficiency is associated with failure of growth, hypogonadism and impaired wound healing (Huang *et al*., 1999). Selenium is a trace mineral that plays an important role in the body's enzyme function and various cellular components. Selenium is also considered an antioxidant and may work with other antioxidants such as vitamins C and E to protect the body's cells against free radicals, which are produced as natural byproducts of oxygen metabolism that are suspected of contributing to many chronic inflammatory diseases. Se deficiency in mammals can result in spontaneous abortion, liver injury and heart impairment (Zachara *et al*., 2001; Navarro-Alarcon *et al*., 2002; Sharadamma *et al*., 2011; Abdallah *et al*., 2009). The iron, zinc and selenium are essential for thyroid hormone, one of important hormones that exerts a broad range of effects on development (Eftekhari *et al*., 2007).

In this study, the possible association between disturbances of Fe, Cu, Zn and Se in various organs were examined. The main objectives of this study were to find the differences between WND and healthy raccoon dogs in concentrations of selected trace elements and to find the correlations among them.

MATERIALS AND METHODS

Tissues samples: A total of 9 WND and 20 healthy raccoon dogs about 8 (7.92 ± 0.58) months old were selected from a farm in Harbin (125°42'-130°10'E, 44°04'-46°40'N), Heilongjiang Province, China. (If the raccoon dogs have such symptoms, we selected them as WND ones: (1) They were retarded and achromotrichia. (2) The nosewings of them changed into white. (3) The pads of them were incrassation and chapped and claws were curve and prolongable.) The research animals were housed individually in wire cages situated in the same environment in open air and under the roof. The diet for the research raccoon dogs, offered by a feed company, conformed with recommended nutritional standards (Liu *et al*., 2012). Access to tap water was provided ad libitum. All requirements of local animal care committees were adhered to.

The raccoon dogs in both groups were euthanized and tissue samples were collected. Fatty tissues, blood vessels and membranes were removed from the samples. After being squeezed and drained to remove blood, the samples were sealed in clean polythene bags and stored at -20°C for no more than one month before analysis. Kidney samples, including cortex, medulla and diaphragm, from the upper pole, liver samples from the right lobe (gall bladder region), heart samples from the tip, lung samples from the right lobe and spleen samples from the cranial were taken and the gastrocnemius was taken as muscle samples.

Chemical analysis: All reagents used were of ultrahigh purity (certified >99.99%) (Kermel Chemical Reagent Co., Ltd., China). The ultrapure water used in this study was made with ultra pure Milli Q water (18MO resistance) in dust free environment. Laboratory glassware, digestion beakers and covers and tubes for mineralization water storage were soaked in 10% (v/v) solution of nitric acid overnight and then washed thoroughly with de-ionized ultrapure water.

After thawing, samples weighing about 2 g were washed with ultrapure water to remove any remaining blood and then squeezed and drained with clean filter paper. Because of differences in metal contents in the cortex and medulla, kidney samples were homogenized prior to digestion to ensure that the subsamples analyzed were representative of the whole organ. After being accurately weighed, the samples were transferred into glass beakers and treated with 20 mL mixed acid of concentrated nitric acid and perchloric acid (4/1 by vol), kept at room temperature overnight and then heated to soft boil until dense white fumes appeared. The samples were then cooled to room temperature and diluted to 20 mL with ultrapure water, of which 5 mL were used for selenium measurement and 15 were for copper, zinc and iron measurement.

An analytical quality control program was employed during the study. The blank was prepared in the exact same way but without sample. After digestion, copper, zinc and iron concentrations were determined using flame atomic absorption spectrophotometer (Varian, 240-FS) with Zeeman compensation. Selenium concentration was determined using the fluorometric method with 2,3-diaminonaphthalene (DAN) by fluorescence spectrophotometer (HITACHI, F-4600) as described previously.

Statistical analysis: All element concentrations (wet weight) are presented as mg/kg except selenium, which is in ng/kg. Statistical comparisons were carried out using unpaired Student's t-test when variables met parametric conditions and the Mann-Whitney test when they did not. The Kolmogorov-Smirnov test and the Bartlett's test were used to test normality of variable distribution and homogeneity of variances, respectively. The average values for the data are given as means, geometric means and medians. Correlations between trace element levels within the same and between different tissues were determined using non-parametric Spearman correlation analysis. The SPSS 13.0 Software Package for Windows was used in statistical analysis (Liu *et al*., 2012).

RESULTS

Table 1-4 show the contents of iron, copper, zinc and selenium in the liver, heart, spleen, lung, kidney and muscle of WND raccoon dogs and controls. Compared with corresponding measurements of the controls, the iron levels in liver, lung and muscle of WND raccoon dogs were significantly lower ($p = 0.042$, $p<0.001$ and $p<0.001$, respectively). The copper levels in the lung of WND raccoon dogs were also significantly lower ($p = 0.03$) but in the spleen and muscle they were significantly

Table 1: Iron concentrations in different tissues of WND raccoon dogs and controls

Tissue (μg g^{-1})	Geometric mean	Media	Mean±SD	95% CI of mean	p-value
Liver					
WND	63.80	69.23	73.23±44.76	38.83~107.64	0.042
Control	99.62	98.34	103.70±30.85	89.26~118.14	
Total	-	-	-	-	
Heart					
WND	58.26	56.84	60.39±17.07	47.27~73.51	0.166
Control	52.06	50.38	53.07±10.48	48.17~57.98	
Total	53.91	55.64	55.34 ±13.03	50.39~60.30	
Spleen					
WND	88.03	69.56	127.89±121.00	87.09~220.90	0.123
Control	178.03	179.3	221.88±153.97	145.32~298.45	
Total	140.78	169.3	190.55±148.47	131.81~249.29	
Lung					
WND	49.99	52.33	51.30±11.90	42.16~60.45	<0.001
Control	84.79	84.85	86.23±16.57	78.48~93.99	
Total	-	-	-	-	
Kidney					
WND	29.41	27.65	30.85±10.07	23.12~38.59	0.346*
Control*	34.71	32.82	37.53±20.38	27.99~47.07	
Total	32.97	32.21	36.46±17.91	28.65~42.27	
Muscle					
WND	79.25	79.78	79.52±6.88	74.23~84.81	<0.001
Control	114.79	116.8	116.63±21.01	106.70~126.42	
Total	-	-	-	-	

*Mann-Whitney statistic

Table 2: Copper concentrations in different tissues of WND raccoon dogs and controls

Tissue (μg g^{-1})	Geometric mean	Median	Mean±SD	95% CI of mean	p-value
Liver					
WND	9.92	10.17	11.39±7.15	5.40~17.37	0.134*
Control	13.46	13.60	14.93±7.49	11.20~18.65	
Total	12.25	11.87	13.84±7.43	10.84~16.84	
Heart					
WND	2.25	2.91	2.99±1.28	2.00~3.97	0.397
Control	3.19	3.36	3.37±1.03	2.89~3.86	
Total	2.86	3.21	3.251.11	2.83~3.68	
Spleen					
WND	4.37	3.98	5.12±3.32	2.57~7.76	<0.001*
Control	1.59	1.52	1.77±0.88	1.33~2.21	
Total	-	-	-	-	
Lung					
WND	1.24	1.22	1.25±0.19	1.10~1.40	0.03*
Control	1.50	1.41	1.58±0.62	1.29~1.87	
Total	-	-	-	-	
Kidney					
WND	4.79	4.63	5.04±1.62	3.79~6.29	0.174
Control	4.16	4.49	4.30±1.16	3.77~64.85	
Total	4.35	4.51	4.54±1.33	4.03~5.04	
Muscle					
WND	1.96	1.91	2.00±0.46	1.65~2.36	0.002
Control	1.19	1.11	1.31±0.54	1.05~1.56	
Total	-	-	-	-	

Table 3: Zinc concentrations in different tissues of WND raccoon dogs and controls

Tissue (μg g^{-1})	Geometric mean	Median	Mean±SD	95% CI of mean	p-value
Liver					
WND	18.86	18.96	19.05±2.78	16.91~21.19	0.131
Control	20.78	20.92	21.03±3.32	19.48~22.58	
Total	20.16	20.43	20.42±3.25	19.18~21.65	
Heart					
WND	18.72	20.93	19.65±5.07	15.75~23.54	0.002*
Control	15.77	16.18	15.99±2.32	14.91~17.08	
Total	-	-	-	-	
Spleen					
WND	13.59	12.93	13.67±1.62	12.42~14.92	0.001*
Control	17.09	16.33	17.25±2.50	16.00~18.50	
Total	-	-	-	-	
Lung					
WND	10.27	11.61	10.58±2.45	8.70~12.47	<0.001
Control	14.27	14.20	14.85±2.20	13.83~15.88	
Total	-	-	-	-	
Kidney					
WND	14.86	14.74	14.90±1.21	13.97~15.83	0.605*
Control	15.00	15.11	15.17±2.31	14.06~16.28	
Total	14.95	15.01	15.00±2.00	14.31~15.86	
Muscle					
WND	11.84	11.36	12.04±2.61	10.03~14.05	<0.001*
Control	34.88	34.16	35.44±6.56	32.36~38.51	
Total	-	-	-	-	

Table 4: Selenium concentrations in different tissues of WND raccoon dogs and controls

Tissue (ng g^{-1})	Geometric mean	Median	Mean±SD	95% CI of mean	p-value
Liver					
WND	186.2	246.9	226.4±123.5	131.5~321.4	0.248
Control	241.5	326.9	293. 8±149.3	223.9~363.6	
Total	222.8	298.5	272.8±143.1	218. 5~327.3	
Heart					
WND	160.3	154.5	159.3±123.83	139.39~179.24	0.468
Control	165.0	161.2	166.53±22.70	155.24~179.24	
Total	163.5	159.1	164.31±22.83	155.09~173.53	
Spleen					
WND	218.9	235.8	231.9±82.7	168.3~295.4	0.169*
Control	264.4	259.6	265.4±23.2	253.4~277.3	
Total	247.7	258.8	253.8±52.9	232.4~275.1	
Lung					
WND	165.7	176.8	170.59±39.64	140.11 ~201.06	0.011*
Control	194.4	227.8	209.19±58.73	181.70 ~236.68	
Total	-	-	-	-	
Kidney					
WND	612.9	573.3	629.3±162.4	504.6~754.1	<0.001
Control	881.1	875.5	888.4±115.5	834.3942.4	
Total	-	-	-	-	
Muscle					
WND	138.1	140.1	139.3±19.7	124.2~154.5	<0.001
Control	181.2	180.1	182.6±23.6	171.5~193.6	
Total	-	-	-	-	

higher ($p<0.001$ and $p = 0.002$, respectively). The zinc levels in spleen, lung and muscle of WND raccoon dogs were significantly lower ($p = 0.001$, $p<0.001$ and $p<0.001$, respectively), whereas in the heart it was significantly higher ($p = 0.002$). The selenium levels in the lung, kidney and muscle of WND raccoon dogs were significantly lower ($p = 0.011$, $p<0.001$ and $p<0.001$, respectively).

Table 5 shows the correlation coefficients between several pairs of trace elements within certain tissues. In the control group, Cu-Zn is positively correlated in the heart, lung and kidney and Fe-Cu is negatively correlated in the spleen and muscle. However, neither Cu-Zn nor Fe-Cu is correlated in the WND raccoon dogs. While both Fe-Zn and Fe-Se are negatively correlated in the lung of the WND group, Zn-Se is positively correlated in the heart in the controls and in the lung of the WND raccoon dogs. No correlation is found between any pair of the trace elements in the liver.

Significant correlations in trace elements in different tissues are presented in Table 6. Most of the correlations are found between kidney and muscle. Some correlations exist only in controls but not WND animals, while others are only found in the WND group. Cu-Zn and Cu-Se are positively correlated and Fe-Se are negatively correlated either between or within some tissues in both groups. Zn-Zn correlation exists only between liver and heart in the controls. Zn is directly correlated with Fe between kidney and muscle in the control group but these two elements show a tendency of inverse relationship in WND group. Strong positive correlations in Se concentrations are found between heart and spleen as well as between kidney and muscle but it is negatively correlated between the liver and heart in the controls, while no correlation is found between or within tissues in the WND group. When Cu-Cu do show any correlation between two tissues, it is always negative except between the liver and lung and liver and kidney in the WND group, where the correlation is positive.

DISCUSSION

This study has found a normal range of Fe, Cu, Zn and Se concentrations in various organs of raccoon dogs and this is useful for future studies on raccoon dogs.

Hoekstra *et al.* (2003) reported that Fe and Zn levels in the livers of arctic fox and wolverine were 285 and 29 and 356 and 35 mg kg^{-1}, respectively. They postulated that these values were similar to those in other canine species and were within the normal range, even though some differences existed. Compared with other canine species including dog, arctic fox, wolverine and silver fox (Hoekstra *et al.*, 2003; Cybulski *et al.*, 2009; Lopez-Alonso *et al.*, 2007; Adamama-Moraitou *et al.*, 2001), most of the Fe, Cu, Zn and Se levels in the liver and kidney of raccoon dogs in our study were likely lower, although there were lacking a data analysis.

Table 5: Spearman rank correlations between concentrations of different trace elements within the same tissue

	Liver		Heart		Spleen		Lung		Kidney		Muscle	
	Element	Corr.	Element	Corr.	Element	Corr.	Element	Corr.	Element	Corr.	Element	Corr.
WND	-	-	-	-	-	-	Fe-Zn	-0.85**	-	-	-	-
	-	-	-	-	-	-	Fe-Se	-0.73*	-	-	-	-
	-	-	-	-	-	-	Zn-Se	0.87**	-	-	-	-
Control	-	-	Cu-Zn	0.47*	Fe-Cu	-0.56*	Cu-Zn	0.50*	Cu-Zn	0.63**	Fe-Cu	-0.47*
	-	-	Zn-Se	0.49*	-	-	-	-	-	-	-	-

-: Nonsignificant correlation exists. *Significantly different at $\alpha = 0.05$, **Significantly different at $\alpha = 0.01$

Table 6: Spearman rank correlations between concentrations of different trace elements between the deferent tissues

	WND		Control	
Tissues	Element	Correlation (sig.)	Element	Correlation
Liver-heart	Cu-Zn	-	Cu-Zn	0.47*
	Zn-Zn	-	Zn-Zn	0.53*
	Se-Se	-	Se-Se	-0.56*
Liver-spleen	Fe-Fe	0.85**	Fe-Fe	-
Liver-lung	Cu-Cu	0.86*	Cu-Cu	-
	Zn-Cu	0.75*	Zn-Cu	-
Liver-kidney	Cu-Cu	0.76*	Cu-Cu	-
Liver-muscle	Cu-Fe	-0.74*	Cu-Fe	-
	Se-Zn	-0.70*	Se-Zn	-
Heart-spleen	Fe-Zn	0.80*	Fe-Zn	-
	Fe-Cu	-	Fe-Cu	-0.55*
	Se-Se	-	Se-Se	0.70**
Heart-lung	Cu-Cu	-	Cu-Cu	-0.47*
Heart-kidney	Fe-Cu	-	Fe-Cu	-0.49*
Spleen-lung	Fe-Zn	-0.78*	Fe-Zn	-
	Zn-Fe	-	Zn-Fe	-0.56*
Spleen-kidney	Fe-Se	-0.87**	Fe-Se	-0.51*
	Cu-Se	-	Cu-Se	0.61**
Spleen-muscle	Fe-Cu	-0.87**	Fe-Cu	-
	Cu-Fe	-	Cu-Fe	0.48*
	Zn-Fe	-	Zn-Fe	-0.57*
Lung-kidney	Fe-Zn	-	Fe-Zn	-0.49*
	Cu-Se	-	Cu-Se	0.50*
Lung-muscle	Se-Cu	0.67*	Se-Cu	-
		-	Cu-Cu	-0.46*
Kidney-muscle	Fe -Se	0.68*	Fe -Se	-
	Cu-Fe	-	Cu-Fe	0.61**
	Cu-Cu	-0.68*	Cu-Cu	-0.54*
	Zn-Fe	-0.67*	Zn-Fe	0.49*
	Se-Se	-	Se-Se	0.67**
	Se-Cu	0.82**	Se-Cu	-
	Se-Zn	0.67 *	Se-Zn	-

-: Nonsignificant correlation exists. *Significantly different at $\alpha = 0.05$, **Significantly different at $\alpha = 0.01$

Generally, liver is believed to have higher concentrations of trace elements. However, in our study, the highest concentration of iron is found in spleen followed by muscle, in both normal control group and WND raccoon dogs. For zinc, the highest concentration is found in muscle in normal control group but in liver in WND raccoon dogs. Zongping (2003) reported that camel Fe level was higher in liver, followed by spleen and then other organs. Other studies showed that Zn concentration was higher in muscle than in liver and kidney, whereas concentrations of Cu in liver and Se in kidney were the highest. The results of our study are similar to these findings, indicating that the concentrations of the trace elements in the liver are not always higher than those in other organs as commonly believed and factors such as animal species and diseases can affect their quantities in different organs.

Hou *et al.* (2010b) found that, compared with controls, the serum iron concentration was lower in WND raccoon dogs, which also had a symptom of iron deficiency anemia. However, the blood is a nutrient medium that reflects the transient status resulting from dietary intakes that vary from day to day, whereas organs, especially the liver, can reflect long term conditions (the metabolism of minerals over several days to several months). So the findings in this study based on low Fe concentrations in both organs and blood, offered more solid proof that the WND raccoon dog was in iron deficiency.

Alonso *et al.* (2004) and Kojouri *et al.* (2008) reported that Fe-Cu levels were positively correlated not only in the muscle but also in the liver of the cattle. Pasha *et al.* (2010) found that hair Fe and Cu levels in benign breast lesions patients were negatively correlated, but there was no viable relationship in the healthy group. They believed that the disturbance was due to impairment of lesions. Alonso *et al.* (2004) and Rahil-Khazen *et al.* (2002) reported that cattle and human Cu-Zn levels in the kidney were positively correlated. The similarities between those findings and the results of our study indicate that trace elements disturbances occur in the WND raccoon dogs.

Comparison results of the trace elements in organs of WND raccoon dogs and control subjects in the present study show that disturbances in element homeostasis and inter-element relations occur in various organs of WND raccoon dogs, indicating that the effects of an increase or decrease in the concentration of a single element is not restricted to this element alone and the total element distribution pattern in the system will be affected as a result. Therefore, there is a need to understand the primary factor triggering the element imbalance in the body and its consequences.

Although, the disturbance in the inter-relationships have been found among the trace elements in WND raccoon dogs, the molecular mechanisms of most of these metal interactions and the direct causation of the disease are not understood. Recent studies suggest that the basis of these relationships could be common transporters that control the metal uptake and trafficking. One of the best studied is the transporter DMT1 (divalent metal transporter 1) that clearly plays a major part in Fe, Zn and Cu metabolism (Alonso *et al.*, 2004; Garrick *et al.*, 2003). Loss of function of DMT1 or other transporters might have caused the disturbances correlations between Cu, Zn and Fe in the WND raccoon dogs.

In general, the minerals deficiency is a result of low dietary minerals or malabsorption (e.g., iron deficiency anemia) (Munoz *et al.*, 2009), while an abnormally high concentration of minerals is due to defective pathways (For example, an elevated copper concentration is secondary to gallbladder lesions that interfere with normal excretion of excess copper into bile) (Schultheiss *et al.*, 2002). It is interesting that the Cu and Zn concentrations in WND raccoon dog were higher in some organs but lower in other organs compared to the controls at the same conditions.

CONCLUSION

Abnormalities in the contents of the selected trace elements were observed, which revealed an imbalance in the inter-element relations in some organs of the WND raccoon dogs. These results suggest that there is a disturbance in trace element contents and their homeostasis in WND raccoon dogs. And the raccoon dog was either deficiency in selenium or in iron, or deficiency in both can be confirmed directly by this study.

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