

Epidemiology

Hair multi-bioelement profile of Kashin-Beck disease in the endemic regions of China

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ABSTRACT

Background: Kashin-Beck disease (KBD) is a serious chronic osteochondral disease that is endemic in humans. Selenium (Se) has been considered in hypotheses of the aetiology of KBD, but few studies have explored the relationship between other elements and KBD.

Objective: This study explored the relationship between 39 elements and KBD. **Methods:** In this retrospective study, 150 boys aged 6 to 14 years were randomly selected from a total population of 1,214 children. Subjects from endemic areas were divided into five groups: (KBD -Bin, N = 30; KBD + Se-Yongshou, N = 30; Control -Bin, N = 30; Control + Se -Yongshou, N = 30; and Control External -Chang'an, N = 30). Samples of occipital hair were collected from each subject, and thirty-nine elements, including 15 toxic elements, 19 nutrient elements, and 5 other elements were assayed by inductively coupled plasma mass spectrometry (ICP-MS). Correlation analysis of the elements in each group was performed by ggcorrplot (visualization of a correlation matrix using 'ggplot2') and PerformanceAnalytics packages in the program R Project.

Results: Among the 15 toxic elements, the levels of aluminum (Al) and bismuth (Bi) in the KBD -Bin were significantly higher than in the other groups, and the levels of silver (Ag), beryllium (Be), platinum (Pt), antimony (Sb), tin (Sn) and lead (Pb) in the KBD -Bin were significantly lower than in the other groups. Among the 19 nutrient elements, the levels of selenium (Se), iodine (I), sulfur (S), and boron (B) in the KBD -Bin were significantly lower than in the other groups. The levels of calcium (Ca), iron (Fe), manganese (Mn), chromium (Cr), strontium (Sr) and phosphorus (P) in the KBD -Bin were significantly higher than in the other groups. Correlations between various elements were remarkably different among the groups. There were positive correlations between As and Pb, Cd and Sb, Pb and Sb, Sb and U in the Control External -Chang'an, between Al and Ni, Cd and Pb, Tl and Ni, Ti and U in the Se-S KBD, and between B and I, B and Mo, Mn and V in the Control External -Chang'an.

Conclusions: These findings indicate that the interactions between elements do not represent a simple reciprocal relationship in the occurrence of KBD. In fact, KBD was associated with an imbalance in multiple elements that play a dynamic and interactive role in the development of the disease.

1. Introduction

Kashin-Beck disease (KBD), resulting from lesions in articular cartilage and epiphyseal plate cartilage, is a severe osteochondropathy causing disability; it is similar to osteoarthritis (OA) in certain clinical manifestations, such as pathological cartilage degeneration including extracellular matrix (ECM) degradation and deep zone chondrocyte necrosis and apoptosis [1,2]. China is the largest endemic area and has

the most patients worldwide. There are 378 endemic counties with more than 104 million residents at risk including 567,600 patients with first degree disease and 12,730 juvenile patients under 13 years old. In 2014, the prevalence of KBD was 44.44% in residents from 26 villages of the Changdu region in Tibet. In 2015, the prevalence of KBD in children was 25.27% in Tibet [3].

The aetiology and pathogenesis of KBD are still unknown. Biogeochemical hypotheses suggests that KBD is caused by a

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significantly lower or excess level of one or several elements [4]. Trace elements, a component of many enzymes in organisms, are involved in important metabolic processes in vivo. An imbalance of trace elements in vivo can lead to many diseases [5]. Environmental Se deficiency has been identified as the main risk factor for KBD, and Se supplementation has been verified to be effective in preventing and repairing KBD [4]. Preliminary data have reported consistency in the distributions of selenium and KBD with regard to location, time and population [3]. However, the incidence of KBD has declined in non-Se-supplemented KBD areas, and few new cases have been detected in recent years. The reasons for the decrease in the incidence of KBD are still unknown. However, research in recent years has been focused on the relationship between Se deficiency and KBD, and the potential correlations between KBD and other elements including toxic elements and nutrient elements have not been investigated.

In this study, we explored the relationships among diverse elements in patients with KBD. Thirty-nine elements, including 15 toxic elements, 19 nutrient elements and 5 other elements obtained from the hair of patients with KBD and from controls with or without Se-supplement in 1998 were analyzed retrospectively to explore the potential relationships among different elements in the study groups.

2. Subjects and Methods

2.1. Study Design and Selection of Endemic areas

This case-control study was conducted in Bin County and Yongshou County, two endemic areas for KBD with a 19.20%-41.94% prevalence rate and in Qidu Town in Chang'an County, a non-endemic area for KBD in 1998. The economic level, geographical conditions, and residents' living habits and customs were similar between Bin and Yongshou County (Fig. 1), both of which were areas with a high prevalence of KBD and a high number of residents exposed to natural Se deficiency. Using a stratified random sampling method, 1,214 children aged from 6 to 14 years in Bin County, Yongshou County and Qidu town in Chang'an County were screened to identify 150 children, who were divided into five groups; thirty children with KBD (KBD -Bin, N = 30) without any preventive measures and 30 healthy children (Control -Bin, N = 30)

Table 1

The age distribution of different groups in this study.

	N	Mean age	Minimum	Maximum
Control External Ca	30	9.50	6	13
Control -Bin	30	10.60	8	13
KBD -Bin	30	12.00	10	14
Control + Se - Ys	30	9.30	7	12
KBD + Se-Ys	30	11.33	7	13
Total	150	10.56	6	14

Note: Ca: Chang'an; Ys: Yongshou.

without any preventive measures from Bin County were randomly selected; 30 KBD children who took sodium selenite 1-2 mg orally every week (KBD + Se-Yongshou, N = 30), thirty healthy children with 1/60000 Se + salt (the proportion of sodium selenite in edible salt) from Yongshou County were randomly selected (Control + Se -Yongshou, N = 30); and thirty healthy children from Qidu town in Chang'an County were randomly selected (Control External -Chang'an, N = 30). Each subject was diagnosed by X-ray of the right hand according to the national diagnostic criteria for KBD in China (WS/T207-2010).

All study subjects or their parent or guardian gave informed consent for the collection of hair samples. The study was approved by the Ethical Committee of Xi'an Jiaotong University (Table 1).

2.2. Sample Collection and Preparation

After obtaining informed consent from all of the subjects and their guardians, 0.5 cm to 1.0 cm of occipital hair from the scalp of the children was collected by hired barbers. Hair has been broadly considered a material that can be used to examine the relationship between different elements and human health [6–8]. Samples were coded, randomized in a double-blind fashion, and sent to Biological Trace Element Research Institute, San Diego, CA, USA. The samples were reduced using a sterile surgical scalpel into smaller fragments to facilitate the subsequent washing procedure recommended by the International Atomic Energy Agency (IAEA), which consists of the sequence acetone-water-water-water-acetone [9,10]. More precisely, the samples were immersed in 20 ml of acetone or water and each time stirred an

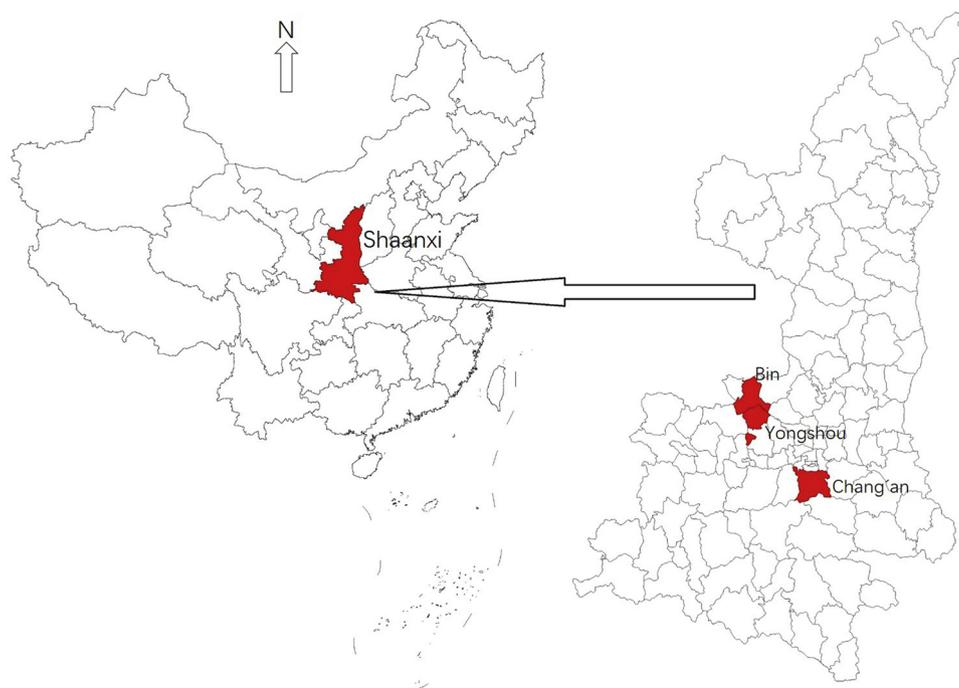


Fig. 1. The location of Shaanxi Province in China and the KBD endemic counties and non-endemic county in Shaanxi Province in the study.

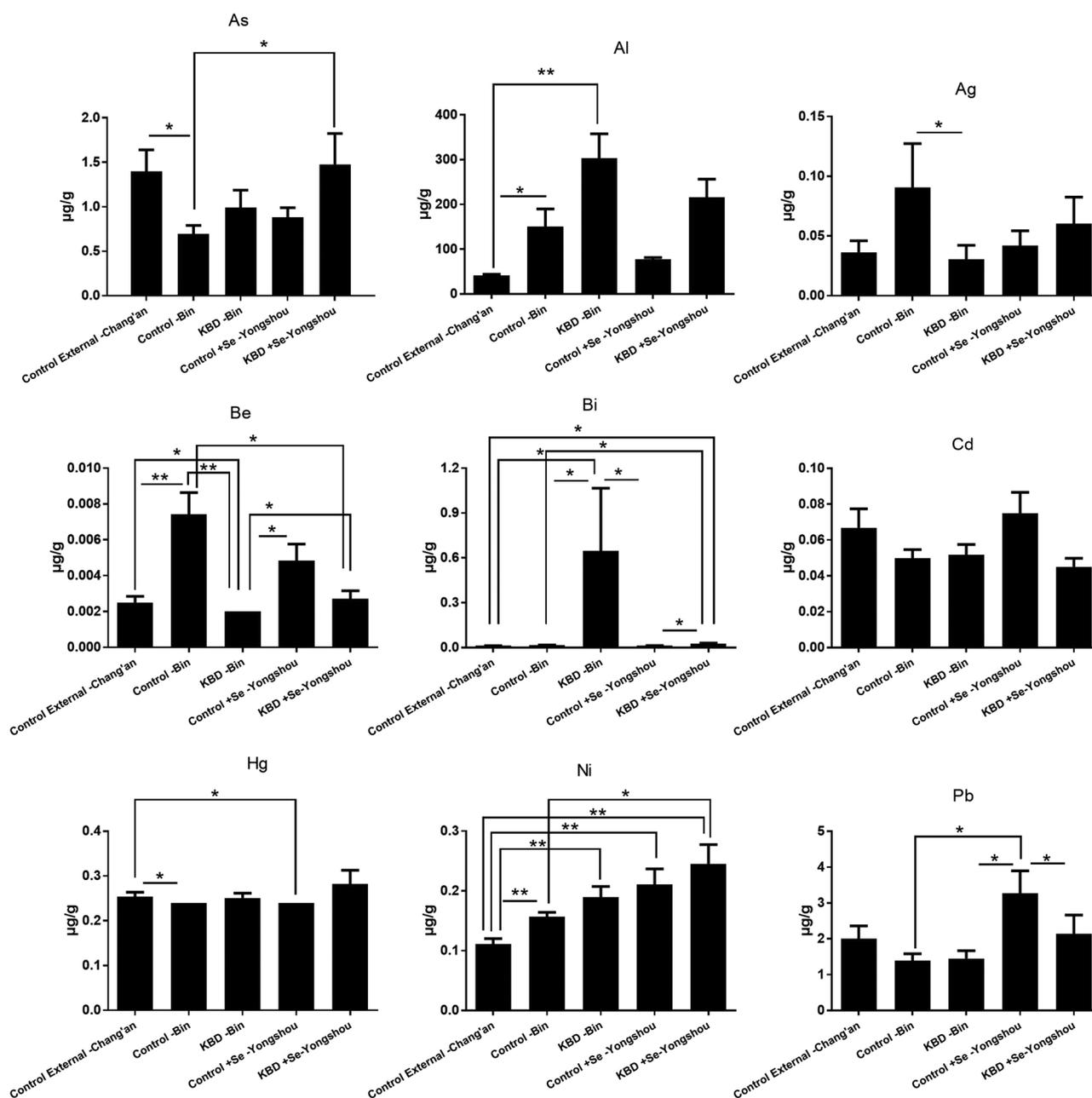


Fig. 2. The average content of 15 toxic elements in different groups. * indicates $P < 0.05$, ** indicates $P < 0.01$. The y-axis scales are different from each other.

ultrasonic bath for 15 minutes. After washing, the samples were placed in beakers and dried at a low temperature (40 °C) for 24 h and then weighed. Then, 3 ml of HNO_3 (Suprapur, Merck, Germany) was added to approximately 150 mg of washed hair sample and digested for 24 h. Digestion was then completed by adding 500 µL of H_2O_2 (Suprapur, Merck, Germany) for a further period of 24 h. Finally, the solutions were diluted by the addition of 18 MΩ cm demineralized water to reach a volume of 25 mL.

2.3. Elemental Determination

Hair element content is a good indicator of element status in the whole body over an extended time period. Hair samples can be collected easily, and they retain a high, stable concentration of elements [6,11–13]. Quantification of the following 39 elements, including 15 toxic elements, was performed: aluminum (Al), antimony (Sb), arsenic (As), beryllium (Be), bismuth (Bi), cadmium (Cd), lead (Pb), mercury

(Hg), nickel (Ni), platinum (Pt), silver (Ag), thallium (Tl), thorium (Th), tin (Sn) and uranium (U), 19 nutrient elements: boron (B), calcium (Ca), chromium (Cr), cobalt (Co), copper (Cu), iodine (I), iron (Fe), lithium (Li), magnesium (Mg), manganese (Mn), molybdenum (Mo), sodium (Na), phosphorus (P), potassium (K), selenium (Se), strontium (Sr) and sulfur (S), vanadium (V), zinc (Zn), 5 other elements: barium (Ba), germanium (Ge), rubidium (Rb), titanium (Ti), zirconium (Zr). The quantification was performed by inductively coupled mass spectrometry (ICP-MS; Sciex Elan 6000, Perkin-Elmer; Biological Trace Element Research Institute, University of California, USA) after the addition of Re–Sc–Y as internal standards, using the technique of the additions to minimize the matrix effect. All standard solutions were prepared with 18 MΩ cm demineralized water. The analytical precision was estimated in the range 5–10% by running triplicate analyses on several samples. The IDL (instrument detection limit) was determined on the basis of three standard deviations (SD) of five blank measurements.

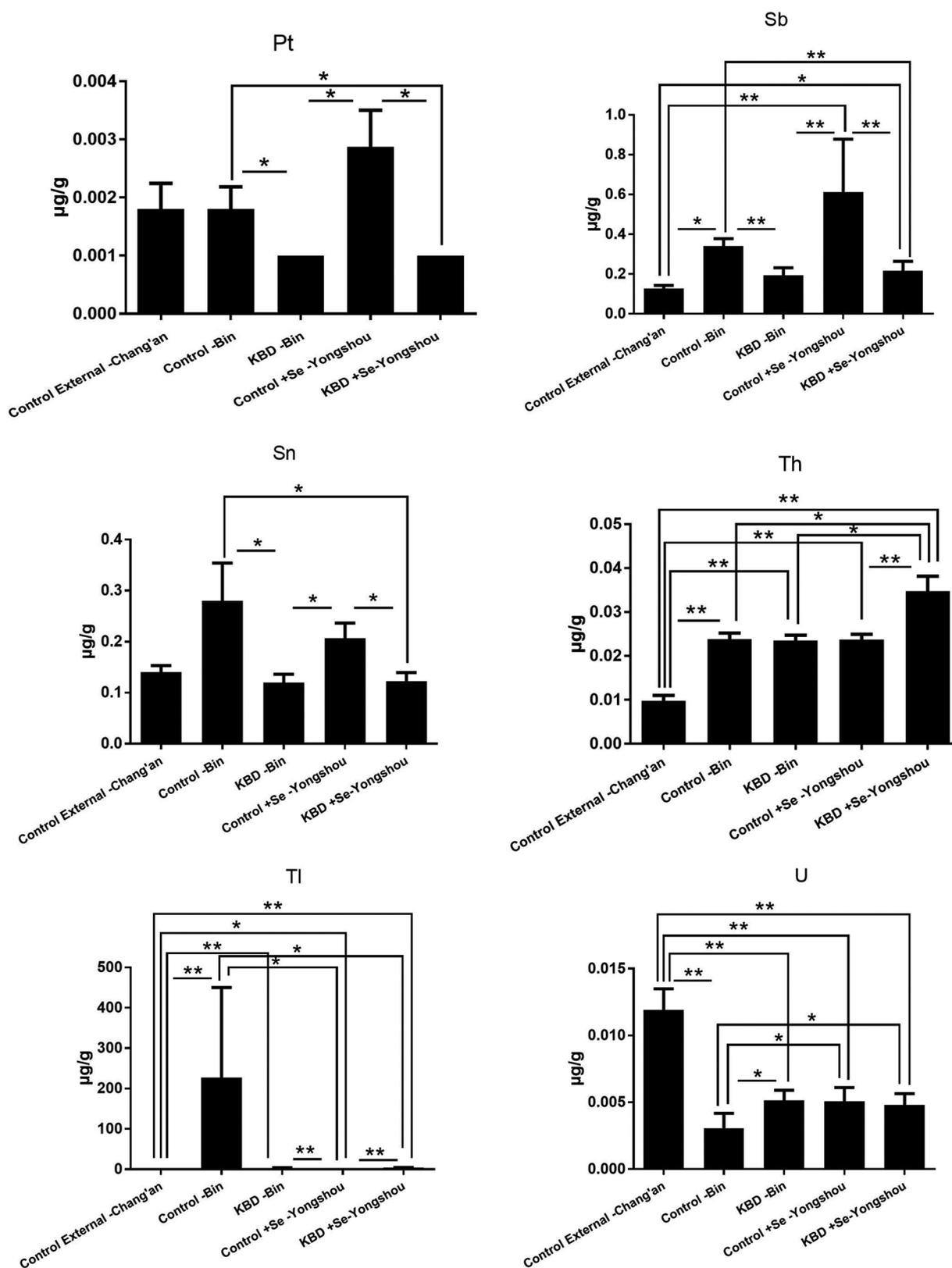


Fig. 3. The average content of 15 toxic elements in different groups. * indicates $P < 0.05$, ** indicates $P < 0.01$. The y-axis scales are different from each other.

2.4. Quality Control

For quality control, the accuracy was guaranteed by using certified reference materials (CRMs). The element concentrations were obtained

from reference material GBW09101 from the Shanghai Institute of Nuclear Research (China) and NCS ZC 81002B from the National Analysis Center for Iron and Steel (China). Analytical precision was estimated from triplicate analyses of every tenth sample and was in the

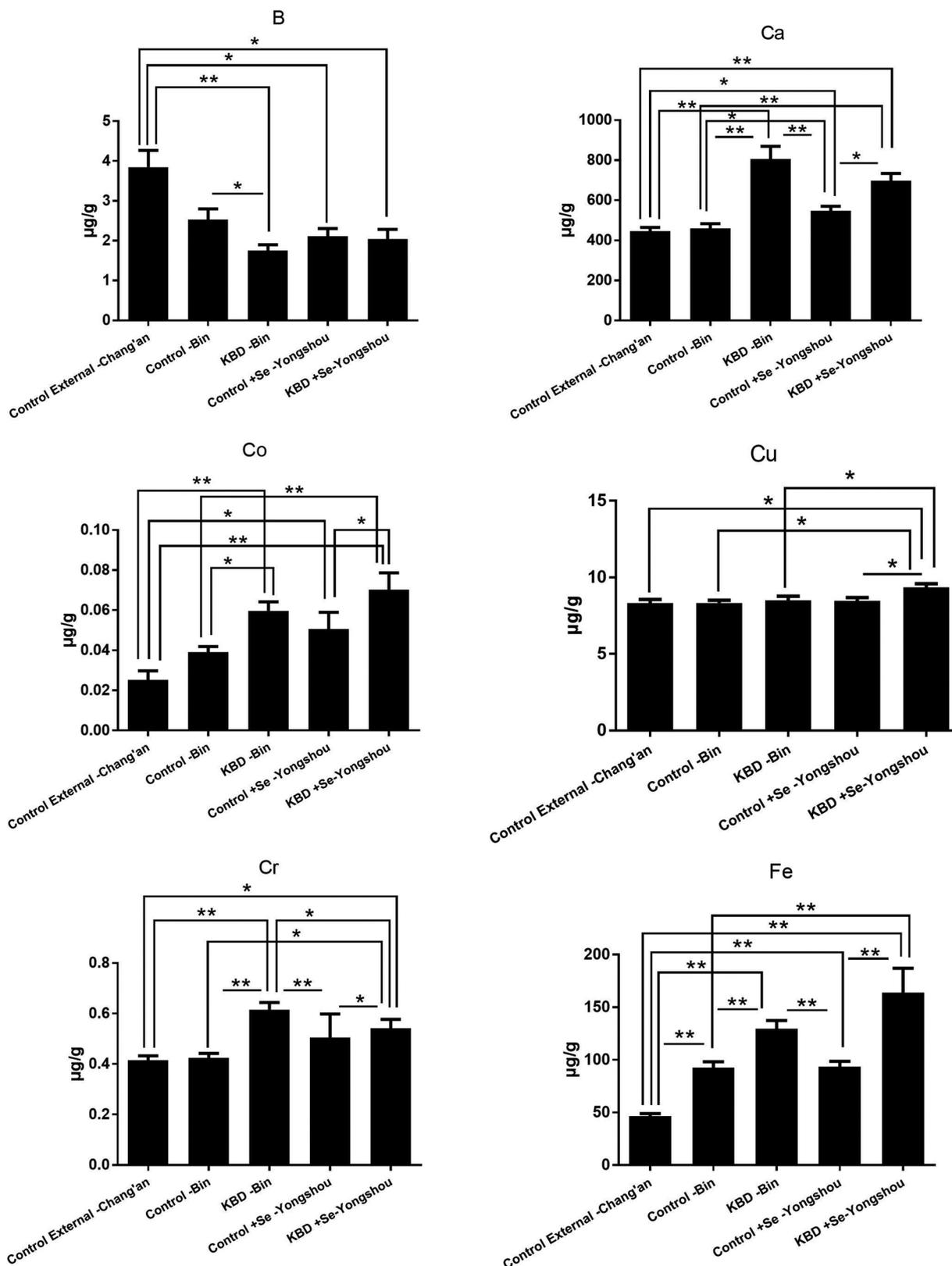


Fig. 4. The average content of 19 nutrient elements in different groups. * indicates P < 0.05, ** indicates P < 0.01. The y-axis scales are different from each other.

range of 5% to 10% for all elements analysed. In addition, the laboratory participates in the international external quality control program (interlaboratory testing) organized by the Biological Trace Element Research Institute, San Diego, CA (USA).

2.5. Statistical Analyses

Statistical analysis was performed using Statistical Package for the Social Sciences for Windows version 18.0 (SPSS, IBM company, New York, USA) Individual samples were divided into triplicates for the

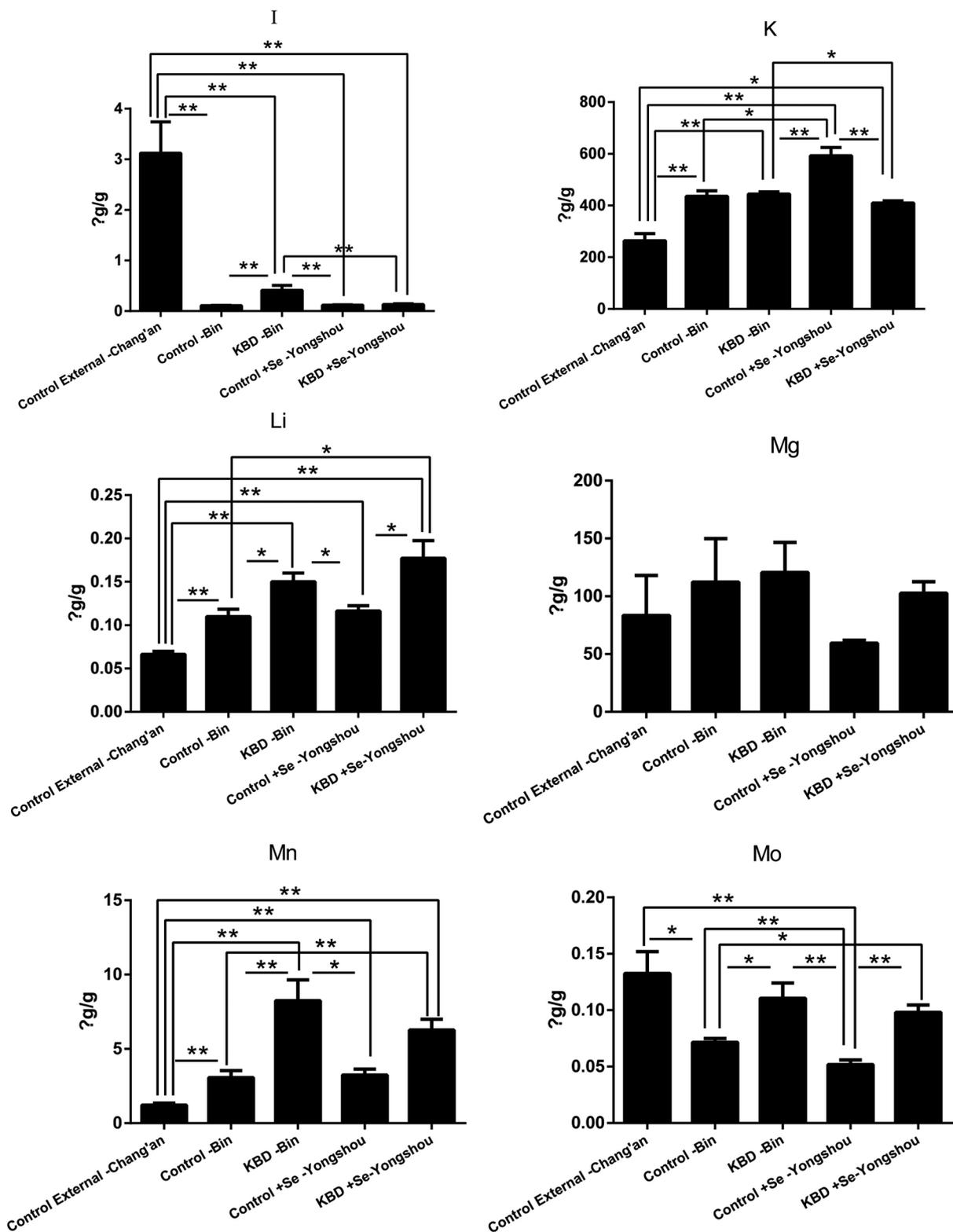


Fig. 5. The average content of 19 nutrient elements in different groups. * indicates $P < 0.05$, ** indicates $P < 0.01$. The y-axis scales are different from each other.

study. The normality and homogeneity of variance of the data were tested before any further analyses. A non-parametric test was performed if the conditions for normality and homogeneity properties were not fulfilled. A Kruskal–Wallis H test of one-way analysis of variance (ANOVA) was performed to compare 39 elements in the hair of children the different regional groups. The Mann–Whitney U test was used to compare the differences in hair elements between any two

groups. Correlation analysis of different types of elements among groups was performed by gcorrplot and PerformanceAnalytics packages (“R & R” from the Statistics Department of the University of Auckland, Auckland City, New Zealand) in R Project. P values less than 0.05 were considered significant.

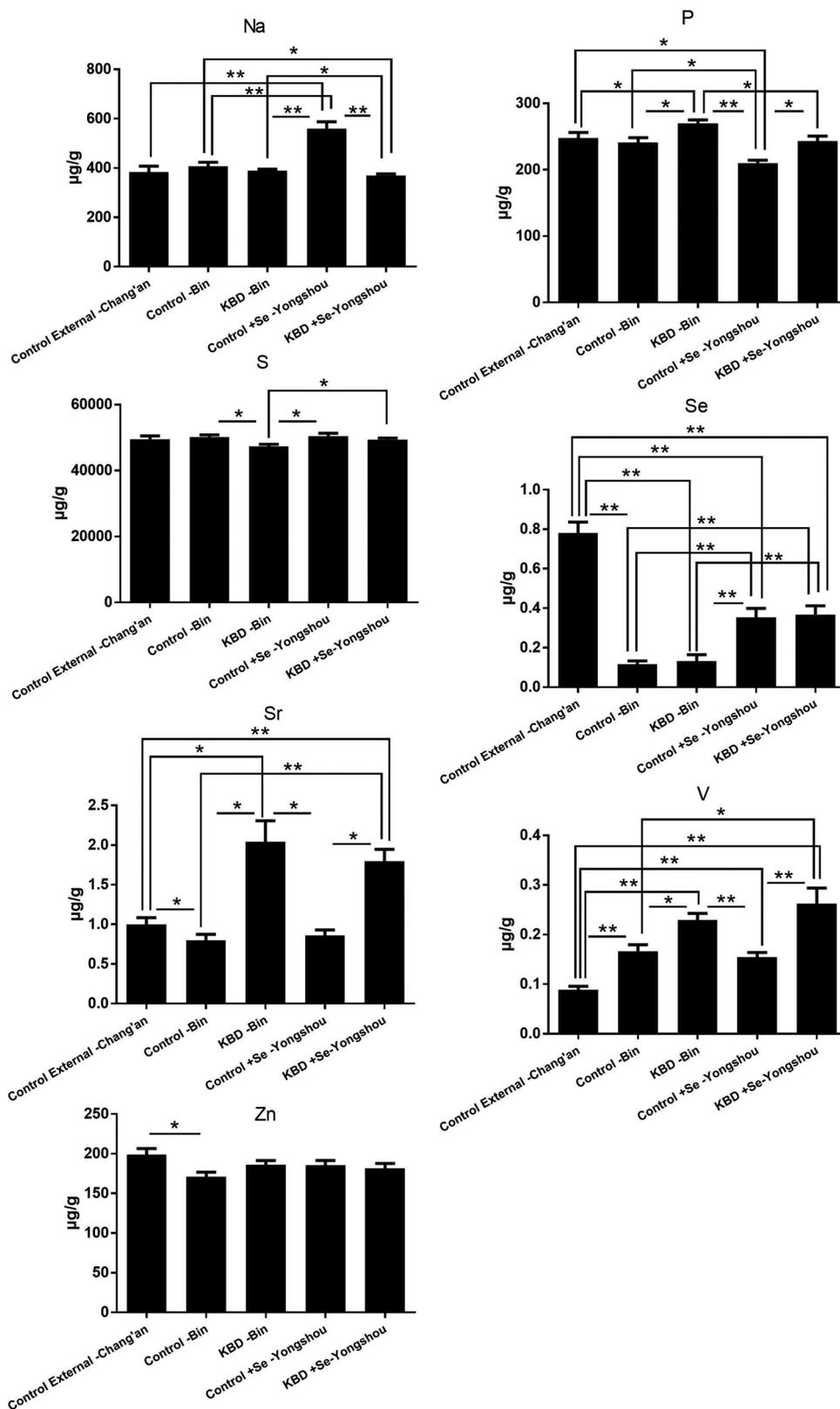


Fig. 6. The average content of 19 nutrient elements in different groups. * indicates $P < 0.05$, ** indicates $P < 0.01$. The y-axis scales are different from each other.

3. Results

3.1. Element content analysis in each group

The Kruskal-Wallis test showed that all 37 types of elements were

significantly different among the groups except Cd and Mg. For the 15 toxic elements, the hair Al level in the KBD -Bin was 75% and 29% higher as compared to the Control + Se -Yongshou and KBD + Se-Yongshou. The hair Bi level in the KBD -Bin was 98% and 96 % higher compared to the Control + Se -Yongshou and KBD + Se-Yongshou,

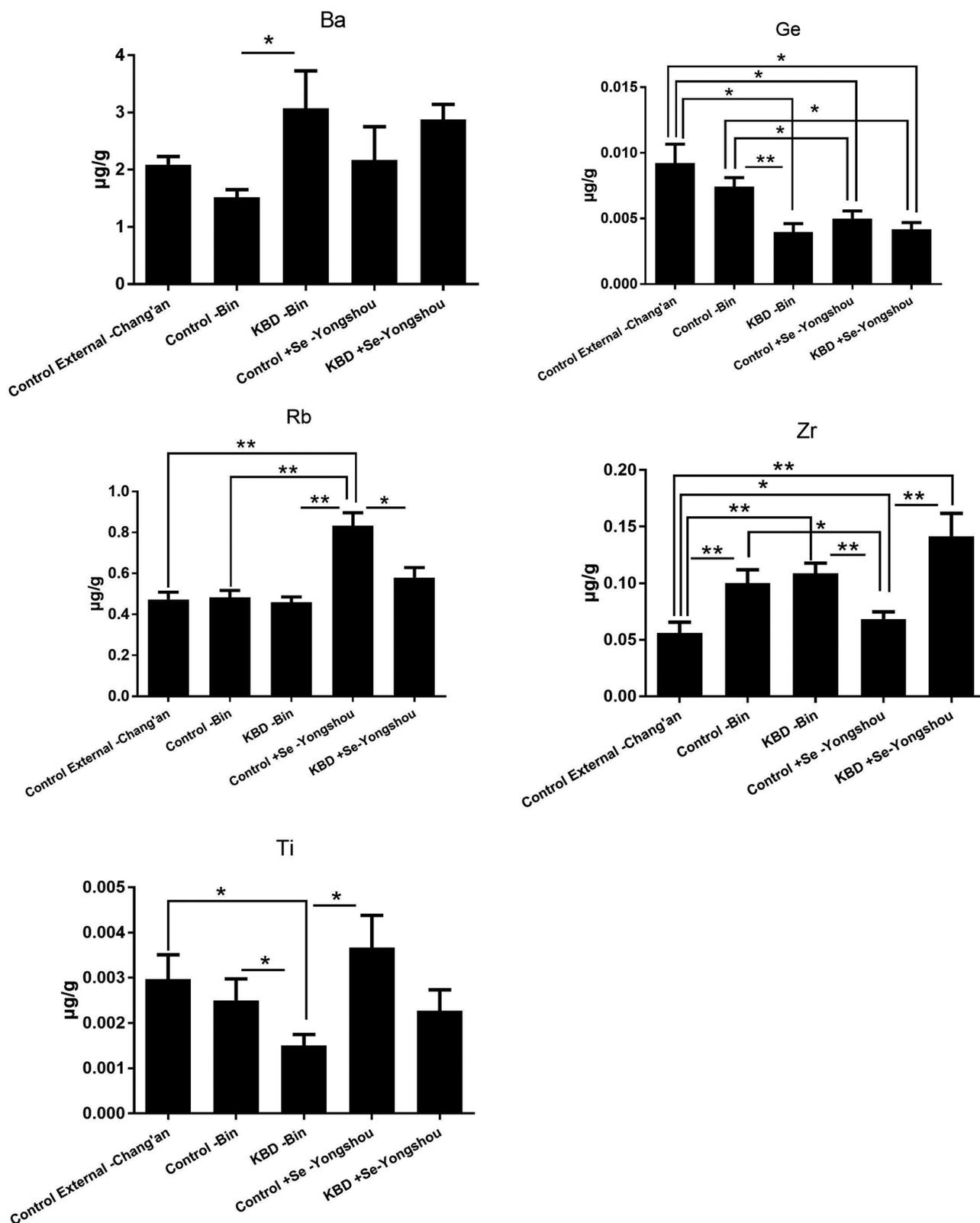


Fig. 7. The average content of 5 other elements in different groups. * indicates $P < 0.05$, ** indicates $P < 0.01$. The y-axis scales are different from each other.

respectively. The hair Be level in the KBD -Bin was 59% and 26% lower compared to the Control + Se -Yongshou and KBD + Se-Yongshou, respectively. The hair Pt level in the KBD -Bin was 56% and 32% lower compared to the Control + Se -Yongshou and KBD + Se-Yongshou, respectively. The hair Sn level in the KBD -Bin was 42% and 2% lower compared to the Control + Se -Yongshou and KBD + Se-Yongshou,

respectively. The details information of other toxic elements can be found in Figs. 2 and 3. For the 19 nutrient elements, the hair Se level in the KBD -Bin was 63% and 64% lower compared to Control + Se -Yongshou and KBD + Se-Yongshou, respectively. The hair B levels in the KBD -Bin were 17% and 14% lower than those in the Control + Se -Yongshou and KBD + Se-Yongshou, respectively. The hair Ca levels in

Table 2
Bioelements in the hair of the tested groups of children (µg-g⁻¹).

Elements	Groups	Mean(N = 30) ± SD	Median	95% CI	
Al	KBD-Bin	302.7 ^d ± 55.14	129.5	189.9	415.5
	KBD + Se-Ys	215.7 ± 40.88	107.5	132.1	299.3
	Control -Bin	150.1 ^d ± 40.09	76.50	68.14	232.1
	Control + Se - Ys	77.13 ± 4.267	73.00	68.41	85.86
Ag	Control External - Cha	41.53 ^c ± 2.780	41.00	35.85	47.22
	Control -Bin	0.090 ^d ± 0.036	0.030	0.015	0.165
	Control External - Cha	0.036 ± 0.009	0.020	0.016	0.056
	Control + Se - Ys	0.042 ± 0.012	0.020	0.016	0.067
As	KBD + Se-Ys	0.060 ± 0.022	0.020	0.014	0.105
	KBD -Bin	0.030 ^d ± 0.011	0.010	0.006	0.054
	Control External - Cha	1.396 ^d ± 0.244	1.050	0.897	1.896
	KBD + Se-Ys	1.476 ^d ± 0.346	1.050	0.767	2.186
B	KBD -Bin	0.990 ± 0.197	0.720	0.587	1.394
	Control + Se - Ys	0.883 ± 0.105	0.710	0.666	1.099
	Control -Bin	0.693 ^c ± 0.097	0.050	0.494	0.891
	Control External - Cha	3.849 ^b ± 0.417	3.920	2.995	4.703
Ba	Control -Bin	2.529 ^d ± 0.267	2.330	1.982	3.076
	Control + Se - Ys	2.110 ^d ± 0.196	2.060	1.708	2.512
	KBD + Se-Ys	2.041 ^d ± 0.243	1.680	1.542	2.540
	KBD -Bin	1.754 ^c ± 0.144	1.480	1.458	2.049
Be	KBD -Bin	3.072 ^d ± 0.657	2.420	1.727	4.416
	KBD + Se-Ys	2.877 ± 0.263	2.310	2.339	3.416
	Control External - Cha	2.087 ± 0.143	1.880	1.794	2.381
	Control + Se - Ys	2.172 ± 0.581	1.560	0.983	3.360
Bi	Control -Bin	1.517 ^d ± 0.135	1.450	1.241	1.794
	Control -Bin	0.007 ^b ± 0.001	0.004	0.004	0.009
	Control External - Cha	0.002 ^c ± 0.0003	0.002	0.001	0.003
	KBD -Bin	0.002 ^a ± 0.000	0.002	0.002	0.002
Ca	Control + Se - Ys	0.004 ^d ± 0.001	0.002	0.002	0.006
	KBD + Se-Ys	0.002 ^c ± 0.0004	0.002	0.001	0.003
	KBD -Bin	0.645 ^b ± 0.420	0.020	-0.213	1.505
	KBD + Se-Ys	0.026 ^b ± 0.004	0.015	0.017	0.034
Cd	Control External - Cha	0.011 ^c ± 0.001	0.010	0.009	0.014
	Control -Bin	0.014 ^c ± 0.003	0.010	0.007	0.020
	Control + Se - Ys	0.012 ^c ± 0.001	0.010	0.009	0.015
	KBD -Bin	805.7 ^b ± 62.84	680.5	677.2	934.2
Co	KBD + Se-Ys	698.1 ^b ± 35.27	654.5	626.0	770.3
	Control + Se - Ys	546.9 ^a ± 23.49	539.0	498.8	594.9
	Control -Bin	458.6 ^b ± 24.37	451.0	408.7	508.4
	Control External - Cha	446.0 ^b ± 18.67	441.0	407.8	484.1
Cr	Control + Se - Ys	0.074 ± 0.011	0.058	0.050	0.099
	Control External - Cha	0.066 ± 0.010	0.053	0.045	0.088
	Control -Bin	0.049 ± 0.004	0.046	0.040	0.059
	KBD -Bin	0.051 ± 0.005	0.039	0.040	0.063
Cu	KBD + Se-Ys	0.045 ± 0.004	0.038	0.035	0.054
	KBD + Se-Ys	0.070 ^b ± 0.008	0.061	0.052	0.087
	KBD -Bin	0.059 ^c ± 0.004	0.059	0.050	0.068
	Control + Se - Ys	0.050 ^c ± 0.008	0.038	0.033	0.067
Fe	Control -Bin	0.039 ^c ± 0.002	0.036	0.033	0.044
	Control External - Cha	0.025 ^b ± 0.004	0.019	0.016	0.034
	KBD -Bin	0.616 ^a ± 0.027	0.575	0.559	0.672
	KBD + Se-Ys	0.540 ^a ± 0.036	0.495	0.465	0.615
Ge	Control -Bin	0.425 ^c ± 0.017	0.435	0.389	0.460
	Control External - Cha	0.415 ^c ± 0.017	0.420	0.378	0.451
	Control + Se - Ys	0.505 ^c ± 0.092	0.390	0.315	0.695
	KBD + Se-Ys	9.330 ^a ± 0.251	9.000	8.820	9.850
Hg	Control External - Cha	8.300 ^d ± 0.254	8.000	7.780	8.820
	Control -Bin	8.330 ^d ± 0.182	8.000	7.960	8.700
	KBD -Bin	8.500 ^d ± 0.270	8.000	7.950	9.050
	Control + Se - Ys	8.470 ^d ± 0.208	8.000	8.040	8.890
K	KBD -Bin	129.5 ^b ± 7.838	124.0	113.4	145.5
	KBD + Se-Ys	163.7 ^b ± 23.37	121.5	115.8	211.5
	Control + Se - Ys	93.50 ^b ± 5.085	86.00	83.10	103.9
	Control -Bin	92.67 ^b ± 5.653	83.50	81.10	104.2
Mn	Control External - Cha	46.63 ^a ± 2.206	44.50	42.12	51.15
	Control External - Cha	0.009 ^b ± 0.001	0.008	0.006	0.012
	Control -Bin	0.007 ^b ± 0.0006	0.006	0.006	0.008
	Control + Se - Ys	0.005 ^c ± 0.0005	0.005	0.003	0.006
Ni	KBD + Se-Ys	0.0041 ^c ± 0.0005	0.003	0.003	0.005
	KBD -Bin	0.003 ^c ± 0.0006	0.002	0.002	0.005
	Control External - Cha	0.254 ^c ± 0.009	0.240	0.234	0.273
	Control -Bin	0.240 ^d ± 0.000	0.240	0.240	0.240
Pb	KBD -Bin	0.250 ± 0.010	0.240	0.228	0.272
	Control + Se - Ys	0.240 ^d ± 0.000	0.240	0.240	0.240

(continued on next page)

Table 2 (continued)

Elements	Groups	Mean(N = 30) ± SD	Median	95% CI
.	KBD + Se-Ys	0.281 ± 0.031	0.240	0.218
I	Control External - Cha	3.122 ^a ± 0.618	1.500	1.842
.	KBD -Bin	0.413 ^a ± 0.095	0.300	0.218
.	Control -Bin	0.106 ^c ± 0.006	0.100	0.093
.	Control + Se - Ys	0.116 ^c ± 0.008	0.100	0.099
.	KBD + Se-Ys	0.130 ^c ± 0.016	0.100	0.096
K	Control + Se - Ys	592.1 ^a ± 32.23	570.5	526.2
.	KBD -Bin	444.4 ^b ± 8.382	450.5	427.2
.	Control -Bin	435.7 ^c ± 21.43	439.5	391.8
.	KBD + Se-Ys	409.6 ^b ± 8.927	408.0	391.3
.	Control External - Cha	263.7 ^a ± 28.06	208.5	206.3
Li	KBD -Bin	0.150 ^b ± 0.010	0.143	0.129
.	KBD + Se-Ys	0.177 ^b ± 0.020	0.142	0.135
.	Control + Se - Ys	0.116 ^b ± 0.006	0.116	0.103
.	Control -Bin	0.109 ^b ± 0.008	0.104	0.092
.	Control External - Cha	0.066 ^a ± 0.003	0.066	0.059
Mg	KBD -Bin	120.5 ± 26.11	90.00	67.09
.	KBD + Se-Ys	102.5 ± 10.13	83.50	81.81
.	Control -Bin	112.2 ± 37.79	61.50	34.91
.	Control + Se - Ys	59.47 ± 2.443	59.00	54.47
.	Control External - Cha	83.47 ± 34.54	49.00	12.82
Mn	KBD -Bin	8.246 ^b ± 1.396	5.275	5.390
.	KBD + Se-Ys	6.271 ^c ± 0.719	4.830	4.799
.	Control + Se - Ys	3.240 ^c ± 0.403	2.685	2.415
.	Control -Bin	3.065 ^b ± 0.482	2.135	2.078
.	Control External - Cha	1.222 ^a ± 0.128	1.105	.9592
Mo	Control External - Cha	0.132 ^c ± 0.019	0.099	0.092
.	KBD + Se-Ys	0.098 ^c ± 0.006	0.096	0.084
.	KBD -Bin	0.110 ^c ± 0.013	0.086	0.082
.	Control -Bin	0.071 ^b ± 0.003	0.076	0.064
.	Control + Se - Ys	0.051 ^a ± 0.004	0.048	0.042
Na	Control + Se - Ys	560.1 ^a ± 27.31	520.5	504.2
.	Control External - Cha	383.8 ^d ± 24.13	421.5	334.4
.	Control -Bin	406.8 ^c ± 16.87	396.0	372.2
.	KBD -Bin	389.6 ^c ± 5.842	393.5	377.6
.	KBD + Se-Ys	370.1 ^b ± 6.056	359.5	357.7
Ni	KBD + Se-Ys	0.245 ^c ± 0.032	0.195	0.179
.	Control + Se - Ys	0.211 ^d ± 0.025	0.165	0.158
.	Control -Bin	0.156 ^c ± 0.007	0.160	0.141
.	KBD -Bin	0.189 ^d ± 0.018	0.160	0.152
.	Control External - Cha	0.111 ^a ± 0.009	0.095	0.092
P	KBD -Bin	269.7 ^a ± 5.509	271.5	258.4
.	KBD + Se-Ys	242.8 ^c ± 7.782	248.5	226.9
.	Control External - Cha	247.6 ^c ± 8.487	243.5	230.2
.	Control -Bin	241.0 ^c ± 7.364	233.5	225.9
.	Control + Se - Ys	209.6 ^a ± 4.843	210.5	199.7
Pb	Control + Se - Ys	3.267 ^b ± 0.629	2.250	1.979
.	Control External - Cha	2.003 ± 0.358	1.400	1.269
.	KBD + Se-Ys	2.143 ^d ± 0.522	1.300	1.076
.	KBD -Bin	1.453 ^d ± 0.217	1.200	1.008
.	Control -Bin	1.393 ^d ± 0.192	1.100	1.001
Pt	Control External - Cha	0.002 ± 0.0004	0.001	0.0008
.	Control -Bin	0.002 ^c ± 0.0003	0.001	0.001
.	KBD -Bin	0.001 ^c ± 0.000	0.001	0.001
.	Control + Se - Ys	0.002 ^c ± 0.0006	0.001	0.001
.	KBD + Se-Ys	0.001 ^c ± 0.000	0.001	0.001
Rb	Control + Se - Ys	0.833 ^a ± 0.062	0.812	0.704
.	KBD + Se-Ys	0.579 ^d ± 0.048	0.523	0.480
.	Control -Bin	0.482 ^d ± 0.034	0.462	0.411
.	Control External - Cha	0.473 ^d ± 0.035	0.456	0.399
.	KBD -Bin	0.458 ^d ± 0.026	0.423	0.404
S	Control + Se - Ys	50479 ^d ± 865.6	51789	48709
.	Control -Bin	50206 ^d ± 595.3	50579	48988
.	KBD + Se-Ys	49388 ^d ± 511.2	50527	48342
.	Control External - Cha	49537 ± 982.3	50159	47528
.	KBD -Bin	47378 ^b ± 589.1	47782	46173
Sb	Control + Se - Ys	0.613 ^b ± 0.264	0.299	0.071
.	Control -Bin	0.340 ^b ± 0.037	0.279	0.263
.	KBD + Se-Ys	0.216 ^b ± 0.047	0.141	0.119
.	KBD -Bin	0.192 ^c ± 0.038	0.134	0.114
.	Control External - Cha	0.127 ^b ± 0.015	0.104	0.095
Se	Control External - Cha	0.780 ^a ± 0.055	0.703	0.666
.	Control + Se - Ys	0.354 ^b ± 0.044	0.368	0.264
.	KBD + Se-Ys	0.368 ^b ± 0.043	0.336	0.278
.	Control -Bin	0.117 ^b ± 0.015	0.094	0.086

(continued on next page)

Table 2 (continued)

Elements	Groups	Mean(N = 30) ± SD	Median	95% CI
.	KBD -Bin	0.131 ^b ± 0.033	0.040	0.062
Sn	Control External - Cha	0.140 ± 0.013	0.100	0.113
.	Control -Bin	0.280 ^c ± 0.074	0.100	0.128
.	KBD -Bin	0.120 ^c ± 0.016	0.100	0.087
.	Control + Se - Ys	0.207 ^c ± 0.029	0.100	0.146
.	KBD + Se-Ys	0.123 ^c ± 0.016	0.100	0.090
Sr	KBD + Se-Ys	1.798 ^b ± 0.148	1.625	1.494
.	KBD -Bin	2.042 ^b ± 0.265	1.570	1.498
.	Control External - Cha	0.997 ^b ± 0.086	0.905	0.819
.	Control + Se - Ys	0.859 ^c ± 0.070	0.825	0.716
.	Control -Bin ^e	0.801 ^b ± 0.073	0.630	0.649
Th	KBD + Se-Ys	0.034 ^a ± 0.003	0.031	0.027
.	Control -Bin	0.023 ^c ± 0.001	0.023	0.020
.	Control + Se - Ys	0.023 ^c ± 0.001	0.023	0.021
.	KBD -Bin	0.023 ^c ± 0.001	0.021	0.020
.	Control External - Cha	0.009 ^a ± 0.001	0.008	0.007
Ti	Control External - Cha	0.002 ^d ± 0.0005	0.001	0.001
.	Control -Bin	0.002 ^d ± 0.0004	0.001	0.001
.	KBD -Bin	0.001 ^c ± 0.0002	0.001	0.0009
.	Control + Se - Ys	0.003 ^d ± 0.0007	0.001	0.002
.	KBD + Se-Ys	0.002 ± 0.0004	0.001	0.001
Tl	KBD + Se-Ys	4.097 ^b ± 0.354	3.438	3.372
.	KBD -Bin	3.744 ^c ± 0.307	3.378	3.116
.	Control -Bin	226.8 ^b ± 223.2	2.679	-229.6
.	Control + Se - Ys	2.320 ^a ± 0.109	2.222	2.096
.	Control External - Cha	1.824 ^a ± 0.104	1.824	1.610
U	Control External - Cha	0.011 ^a ± 0.001	0.010	0.008
.	KBD -Bin	0.005 ^c ± 0.0007	0.003	0.003
.	KBD + Se-Ys	0.004 ^c ± 0.0008	0.003	0.003
.	Control + Se - Ys	0.005 ^c ± 0.001	0.002	0.002
.	Control -Bin	0.003 ^b ± 0.001	0.001	0.0007
V	KBD -Bin	0.229 ^b ± 0.013	0.234	0.201
.	KBD + Se-Ys	0.262 ^b ± 0.031	0.220	0.199
.	Control -Bin	0.166 ^b ± 0.012	0.153	0.140
.	Control + Se - Ys	0.154 ^b ± 0.009	0.152	0.135
.	Control External - Cha	0.088 ^a ± 0.007	0.085	0.074
Zn	Control External - Cha	198.6 ^d ± 7.972	211.5	182.3
.	KBD -Bin	185.8 ± 5.388	191.5	174.8
.	KBD + Se-Ys	181.8 ± 6.106	187.5	169.3
.	Control + Se - Ys	185.2 ± 6.273	185.5	172.4
.	Control -Bin	171.2 ± 5.455	176.0	160.0
Zr	KBD + Se-Ys	0.141 ^c ± 0.020	0.110	0.100
.	KBD -Bin	0.109 ^b ± 0.009	0.103	0.089
.	Control -Bin	0.099 ^b ± 0.011	0.079	0.075
.	Control + Se - Ys	0.068 ^a ± 0.006	0.066	0.055
.	Control External - Cha	0.055 ^a ± 0.009	0.039	0.036

Note: a,b,c,d means bear different superscripts for the same bioelement differ significantly ($p < 0.05$) Ys: Yongshou; Cha: Chang'an.

the KBD -Bin were 32% and 13% higher than those in the Control + Se -Yongshou and KBD + Se-Yongshou, respectively. Detailed information for the other nutrient elements can be found Figs. 4–6. For the 5 other elements, the hair Ba levels in the KBD -Bin were 29% and 6% higher compared to the Control + Se -Yongshou and KBD + Se-Yongshou, respectively. The Ge level in the KBD -Bin was 21% and 5% lower than those in Control + Se -Yongshou and KBD + Se-Yongshou, respectively (Fig. 7). Detailed information for the other elements can be found in Fig. 7. All the results for the average content, standard deviation and 95% confidence interval for each element are shown in Table 2.

3.2. Correlation analysis of elements in each group

Correlation analysis of the elements in each group was performed by ggcorrplot and PerformanceAnalytics packages in R Project. For the 15 toxic elements, there was a positive correlation between As and Pb, Cd and Sb, Pb and Sb, and Sb and U in the Control External -Chang'an (Fig. 8). Positive correlations were identified between Hg and Ni in the KBD -Bin, but false positive correlations were caused by outliers according to the scatter plot (Fig. A1 in Appendix A). Pb and Sb, and Pt and U positively correlated in the Control + Se -Yongshou, but there was a false positive correlation caused by outliers according to the

scatter plot (Fig. A2 in Appendix A). Al and Ni, Cd and Pb, Tl and Ni, and Ti and U positively correlated in the KBD + Se-Yongshou (Fig. A3 in Appendix A). For the 19 nutrient elements, there was a positive correlation between B and I, B and Mo, and Mn and V in the Control External -Chang'an (Fig. A4 in Appendix A). Co and Fe, Co and Li, Fe and Li, Co and Mn, K and Na, Co and Sr, Mn and Sr, Co and V, Fe and V, Li and V, and Mn and V in the Control -Bin and Ca and Co, Cr and Fe, Co and Li, Fe and Li, K and Na, Ca and Sr, and Co and V in KBD -Bin were positively correlated (Figs. A5 and A6 in Appendix A). We found a positive correlation between Fe and Li, Ca and Mn, Mn and Sr, and Mn and V in the Control + Se -Yongshou and between Co and Fe, Cr and Fe, Co and Li, Fe and Li, K and Na, Ca and Sr, and Co and V in the KBD + Se-Yongshou (Figs. A7 and A8 in Appendix A). For the 5 other elements, a positive correlation between Rb and Ti in the Control External -Chang'an, Ge and Rb, and Rb and Ti in the Control -Bin, Rb and Zr in the KBD and Ba and Zr in the KBD + Se-Yongshou were identified.

4. Discussion

In this study obtained 39 elements in hair samples collected from children who are continuously living in KBD endemic and non-endemic areas and factors related to element concentrations were identified by

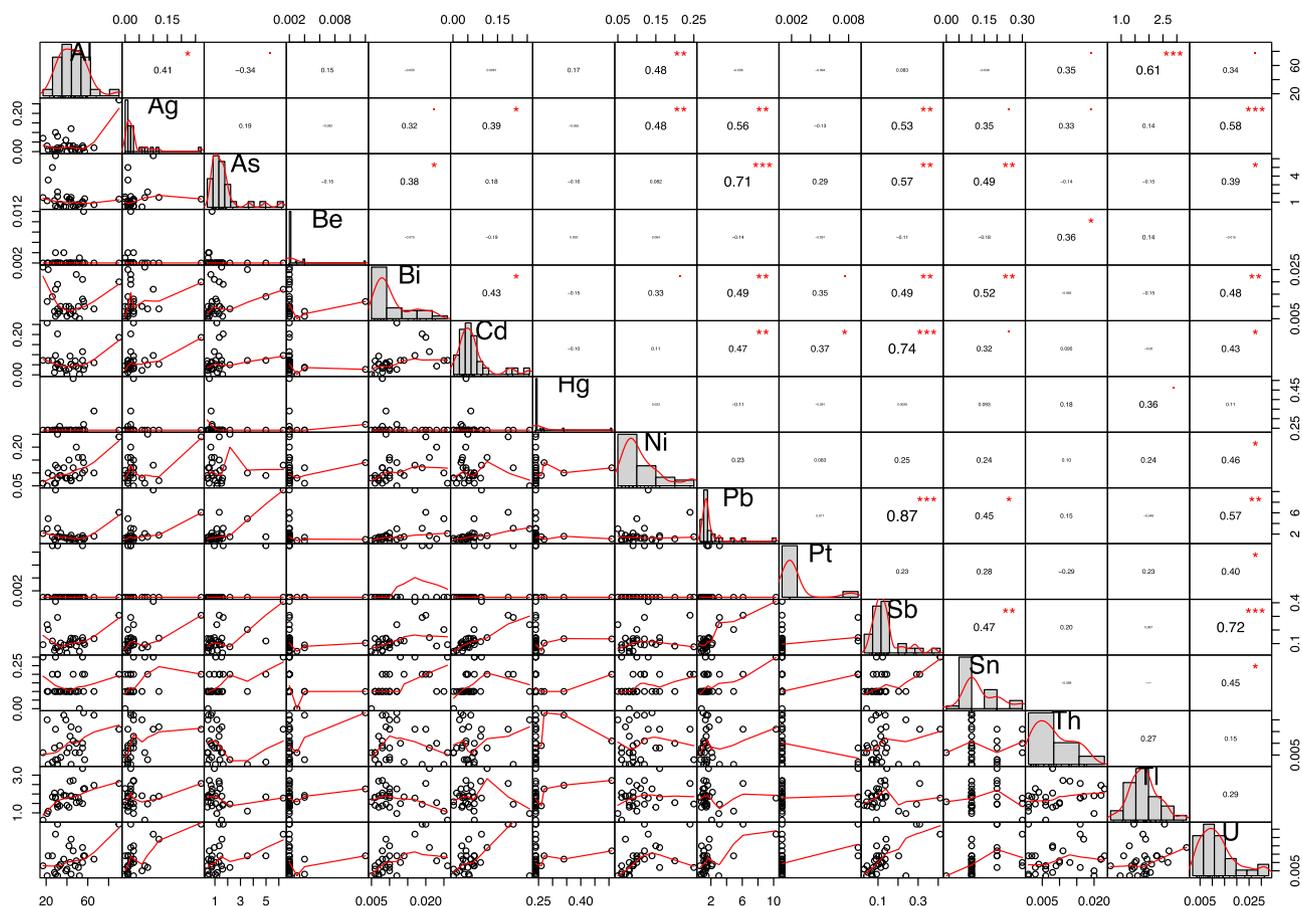


Fig. 8. Correlation analysis of the content ($\mu\text{g/g}$) of 15 toxic elements in Control External -Chang'an. The distribution graph is displayed on the diagonal line. The bottom left shows a bivariate scatter plot with fitted lines. The upper right shows the correlation coefficient and significance level. * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$.

univariate regression analysis and correlation analysis. This study demonstrated that the levels of elements needed for maintaining children's development could be affected by environmental factors in endemic areas, which might be helpful for studying the mechanism of articular damage in KBD.

Se was shown to be an essential component of glutathione peroxidase (GPX), an enzyme that provides antioxidant protection by reducing levels of hydroperoxides in cell [14,15]. Therefore, Se deficiency could reduce resistance against KBD suspicious risk factors such as T-2 toxin, humic acid, and parvovirus B19 [16]. It could also accelerate cell ageing and strengthen lipid peroxidation [17,18]; the latter action plays an important role in articular cartilage damage in KBD [19]. In recent years, Se deficiency is the primary aetiological factor for KBD, and the correlation between Se and KBD has been suggested based on the stable relationship between the occurrence of KBD and Se concentrations in various samples, including soil, drinking water, food, and human biological samples [20,21]. In addition, Se supplementation has been proven effective in preventing KBD in children by meta-analysis [22]. However, no KBD cases were found in other countries with low soil Se content, such as New Zealand, this may be because Se intakes are substantially higher because of the wide availability of non-local and imported food, or this may indicate that Se might not be the only risk factor for KBD [23]. This investigation found that the hair Se content in Control External -Chang'an was significantly higher than that in the other groups, and the content in KBD -Bin was significantly lower than that in the KBD + Se-Yongshou and Control + Se -Yongshou. Furthermore, the levels of some elements in KBD patients were decreased after Se supplementation, such as Cr, I, K, Na and P. Additionally, the levels of some elements in KBD patients were increased after Se

supplementation, such as Be, Th, Cu and S. This further proved that there is correlation between Se levels and the levels of some other elements in the bodies; this indicates that multi-element metabolic disorders occurred in KBD patients and that only Se supplementation may affect the process of KBD by regulating the intake of other elements.

Zn, which is closely involved in the synthesis of enzymes, nucleic acids and proteins, is an essential microelement in vivo [24]. A conundrum still exists in research on the relationship between Zn and KBD due to inconsistent results, but it has been confirmed that Zn combined with Se can remarkably help repair metaphyseal lesions in patients with KBD [25], indicating that Zn might play a key role in the pathogenesis of KBD, although the mechanism is unknown. In our previous meta-analysis, the serum Zn level was lower in KBD patients than in healthy controls, whereas the hair Zn level was higher in KBD than in healthy controls [26]. However, in this study, the hair Zn level in the Control External -Chang'an was higher than that in the KBD + Se-Yongshou and KBD -Bin, but the difference was not significant. There was no significant difference between the KBD -Bin and KBD + Se-Yongshou, which suggests that Se cannot improve the level of Zn in vivo.

I deficiency can cause developmental disorders of the central nervous system and endocrine system and can also cause growth retardation, and damage to the skeletal system [27]. R Moreno-Reyes reported that low nutritional status for both Se and I was common in KBD patients, Se was seriously deficient in KBD endemic areas, and urine I level was low in 66% of residents, which suggested that low I was another risk factor for KBD [28]. X-ray imaging of Keshan disease was somewhat similar to KBD, meanwhile, and delayed bone age and growth retardation were manifested in KBD. For example, the average bone age

of children in the KBD endemic area was delayed by 1-2 years, and several evaluation parameters of growth and development lagged behind those of children of the same age in non-endemic areas [29]. These common manifestations of Keshan disease and KBD might be associated with I deficiency. In our study, the level of I in the Control External -Chang'an was extremely higher compared to the four groups. Notably, hair I levels in the KBD + Se-Yongshou and in the Control + Se -Yongshou were significantly lower than in the KBD -Bin in this study, which indicates that Se might affect the absorption of I in KBD patients and normal controls.

In this study, there are significant differences in the elements between KBD + Se-Yongshou and Control + Se -Yongshou, Such as Bi, Pb, Pt, Sb, Sn, Th, Ti, Ca, Co, Cu, Cr, Fe, K, Li, Mo, Na, P, Sr, V, Rb and Zr. Moreover, almost same Se contents were found between KBD + Se-Yongshou and Control + Se -Yongshou. Therefore, the absorption of elements mentioned above might be not influenced by Se intake. The geochemical characteristics, socioeconomic factors and dietary patterns in these endemic areas could be associated with the significant differences in the elements between KBD + Se-Yongshou and Control + Se -Yongshou.

The main pathological changes in KBD are necrosis in the deep zone of the growth plate and in the articular cartilage, and impaired cartilage and bone development and secondary osteoarthropathy. The necrotic chondrocytes lose their function of synthesizing collagen and proteoglycan [30]. B is an essential trace element in human cartilage. It has been observed that B deficiency can reduce the synthesis of proteoglycan in cartilage and collagen in chick embryo cartilage [31]. In vitamin D3 deficient humans, B deficiency can affect the growth of chondrocytes [32]. The B content in the joint fluid of patients with osteoarthritis was lower than that of healthy individuals, and joint symptoms can be reduced markedly after supplementation with boron [33]. The present study shows that the B content in the hair of the KBD -Bin and KBD + Se-Yongshou are lower than in Control External -Chang'an. There was a significant difference in the B content between the Control -Bin and KBD -Bin, but there was a statistically significant difference in the B content between the Control -Bin and KBD + Se-Yongshou. However, it still remains to be demonstrated whether the B content in blood and cartilage also affected the synthesis of proteoglycan and collagen in cartilage.

Ge compounds have been shown to have antiviral and anti-inflammatory properties that ease pain, enhance the immune response and inhibit the formation of OH and O₂ radicals. Thus, although not considered to be nutritionally essential, Ge could delay cell ageing by inhibiting lipids and preventing oxygen radical damage [34]. In animal experiments, a germanium compound, 132Ge, was shown to increase the activity of osteoblasts [35]. Because lipid peroxide levels are enhanced in KBD children [36], it may thus be hypothesized that a low tissue Ge content may further aggravate the damage caused by free oxygen radicals. The B and Ge contents in the hair of children in the KBD area did not change in response to Se supplementation, indicating that the observed low levels were not a secondary effect of Se deficiency. Because B and Ge act synergistically with Se, supplementation with a mixture of Se, B, and Ge could thus be beneficial and preferable over administering Se alone.

Ren et al. proposed that Mo deficiency in the environment was

associated with KBD based on the analysis of the relationship between KBD and the distribution of Se and Mo in soil humic acid in Jilin Province [37]. Wu et al. reported that the hair Mo level of residents in the KBD area of Tandian village in Shaanxi Province was significantly higher than that in non-endemic areas [38]. Similarly, the hair levels in the KBD + Se-Yongshou and KBD -Bin were significantly higher than those in Control + Se -Yongshou and Control -Bin. However, the level of Mo in the Control External -Chang'an was higher than in the other groups, and the levels of Mo in the Control + Se -Yongshou and KBD + Se-Yongshou were decreased after Se supplementation. Therefore, the relationship between Mo and KBD was unstable and needs to be further explored in research on pathogenesis with larger sample sizes and more diverse types of samples. K and Na are macro elements that mainly contribute to maintaining homeostasis. Thus far, the relationship between these elements and KBD has only been reported. In this study, the levels of Na and K were higher in the Control + Se -Yongshou than in the other groups, and they all decreased in the KBD + Se-Yongshou compared with the KBD -Bin. This finding indicates that homeostasis in patients may change with the development of the disease. Phosphorus is important components, which are indispensable nutrients for calcification of bone and teeth. Phosphorus is one of the basic components of genetic material nucleic acid, which are important substances to regulate cell metabolism. Phosphorus involved in synthesis of ATP and ATP was decreased in blood form KBD patients that is caused by synthesis disorder, this might be the reason for the level of Phosphorus was higher in KBD -Bin than other groups in this study.

5. Conclusions

Elements must be synergistic and complementary to each other, otherwise, disorders such as the alteration of cell structure and dysfunction of organs can occur when elements become imbalanced until they disrupt the autoregulation of homeostasis. This study demonstrated that correlations between various elements were remarkably different among groups. This finding indicates that the interactions between elements are not just a simple reciprocal relationship in the occurrence of KBD. In fact, KBD was associated with an imbalance in multiple elements that may play a dynamic and interactive role in the development of the disease.

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Conflict of interests

The authors have none conflict of interests to declare.

Appendix A

Figs. A9 and A10

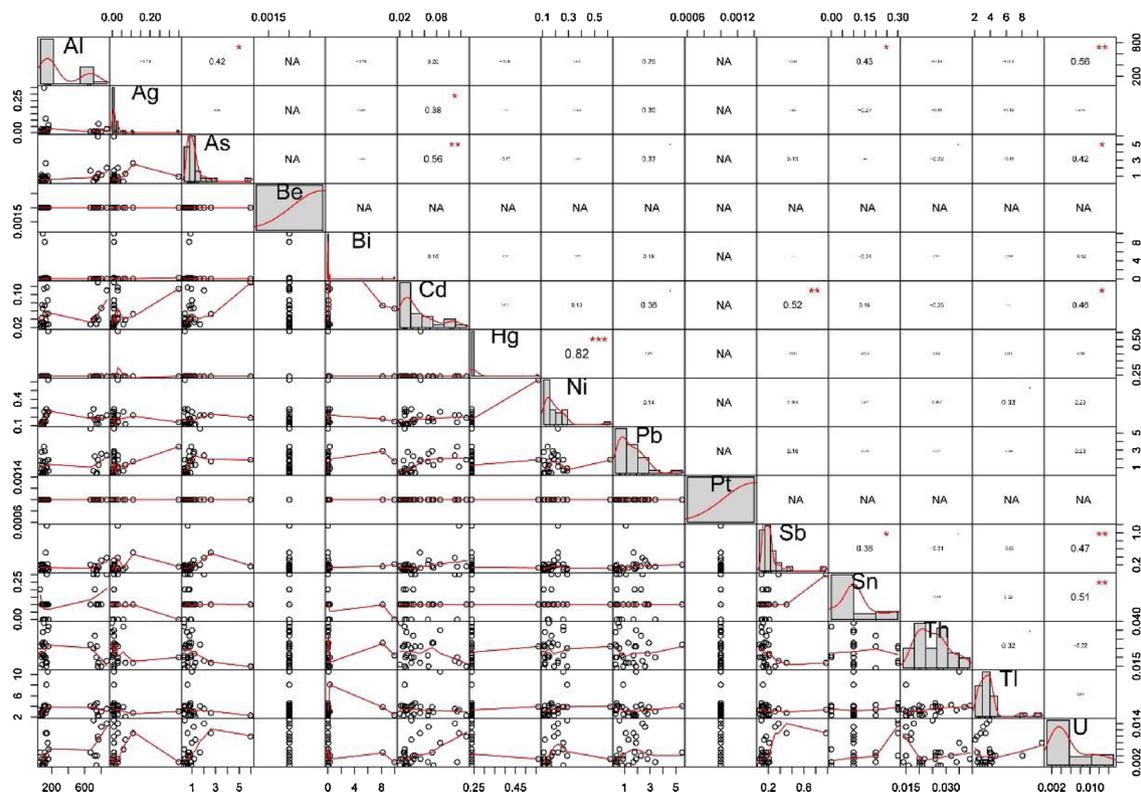


Fig. A1. Correlation analysis of the content ($\mu\text{g/g}$) of 15 toxic elements in KBD -Bin. The distribution graph is displayed on the diagonal line. The bottom left shows a bivariate scatter plot with fitted lines. The upper right shows the correlation coefficient and significance level. * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$

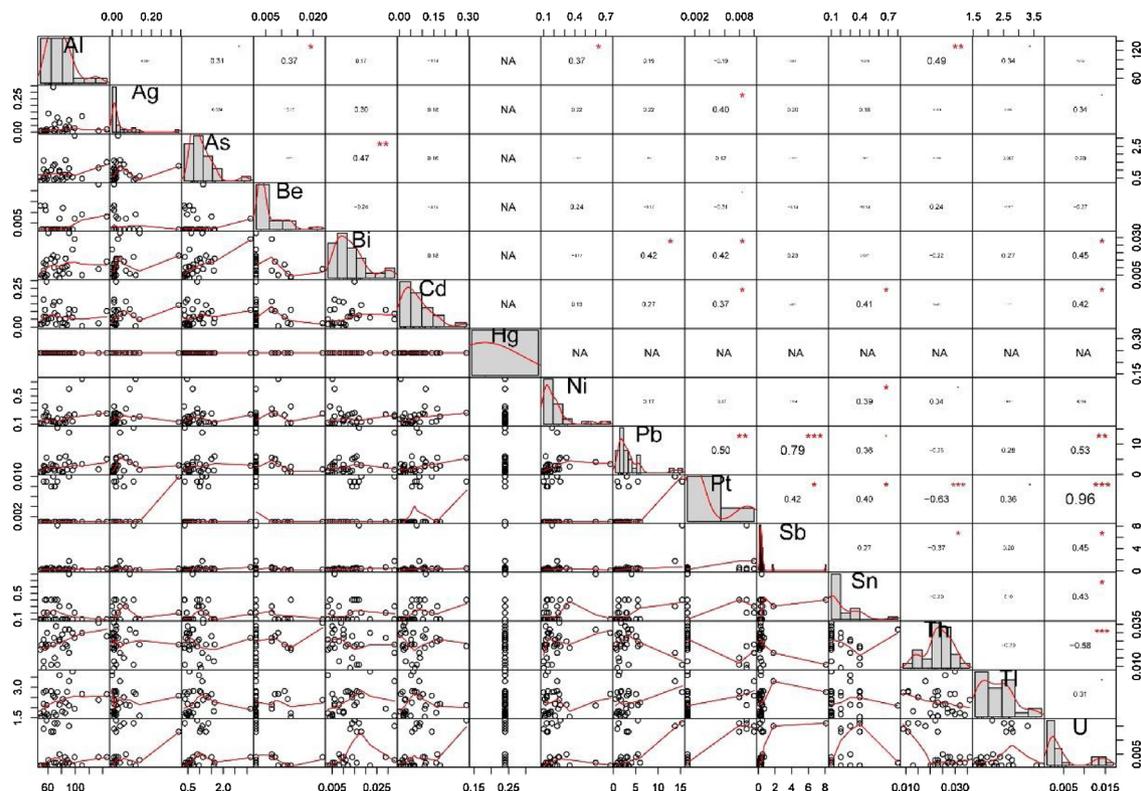


Fig. A2. Correlation analysis of the content ($\mu\text{g/g}$) of 15 toxic elements in Control + Se-Yongshou. The distribution graph is displayed on the diagonal line. The bottom left shows a bivariate scatter plot with fitted lines. The upper right shows the correlation coefficient and significance level. * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$

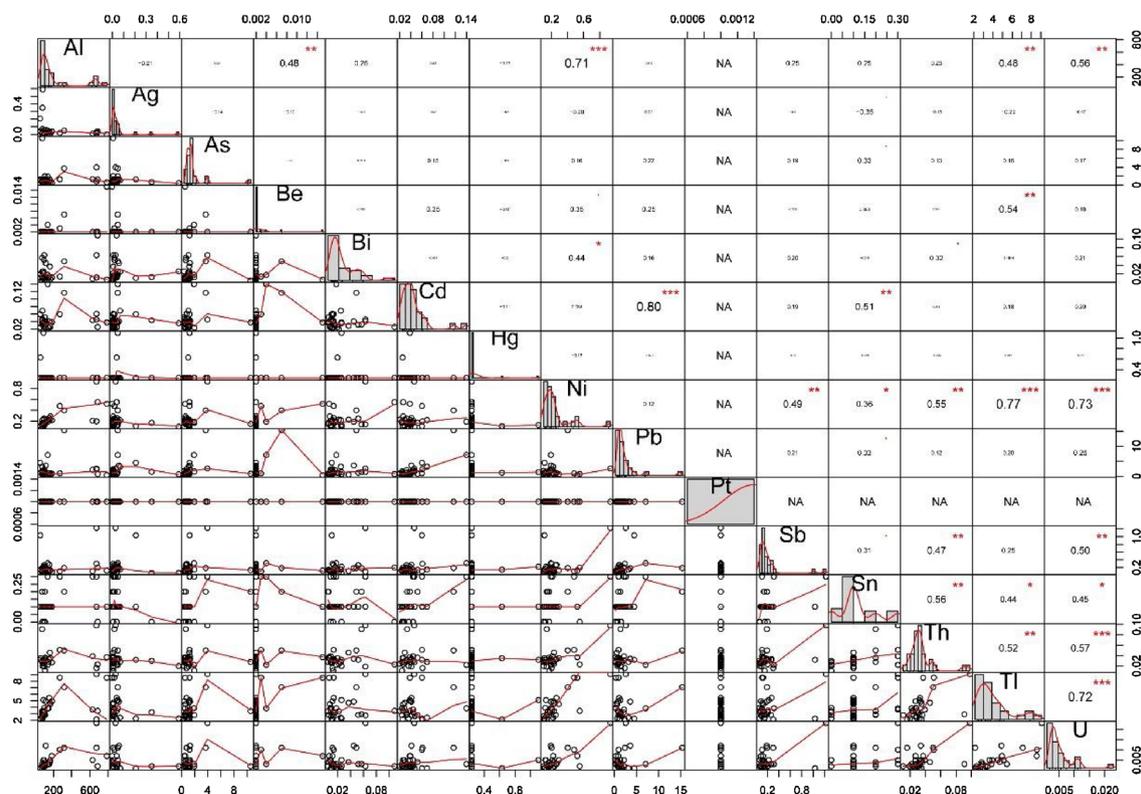


Fig. A3. Correlation analysis of the content ($\mu\text{g/g}$) of 15 toxic elements in KBD + Se-Yongshou. The distribution graph is displayed on the diagonal line. The bottom left shows a bivariate scatter plot with fitted lines. The upper right shows the correlation coefficient and significance level. * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$

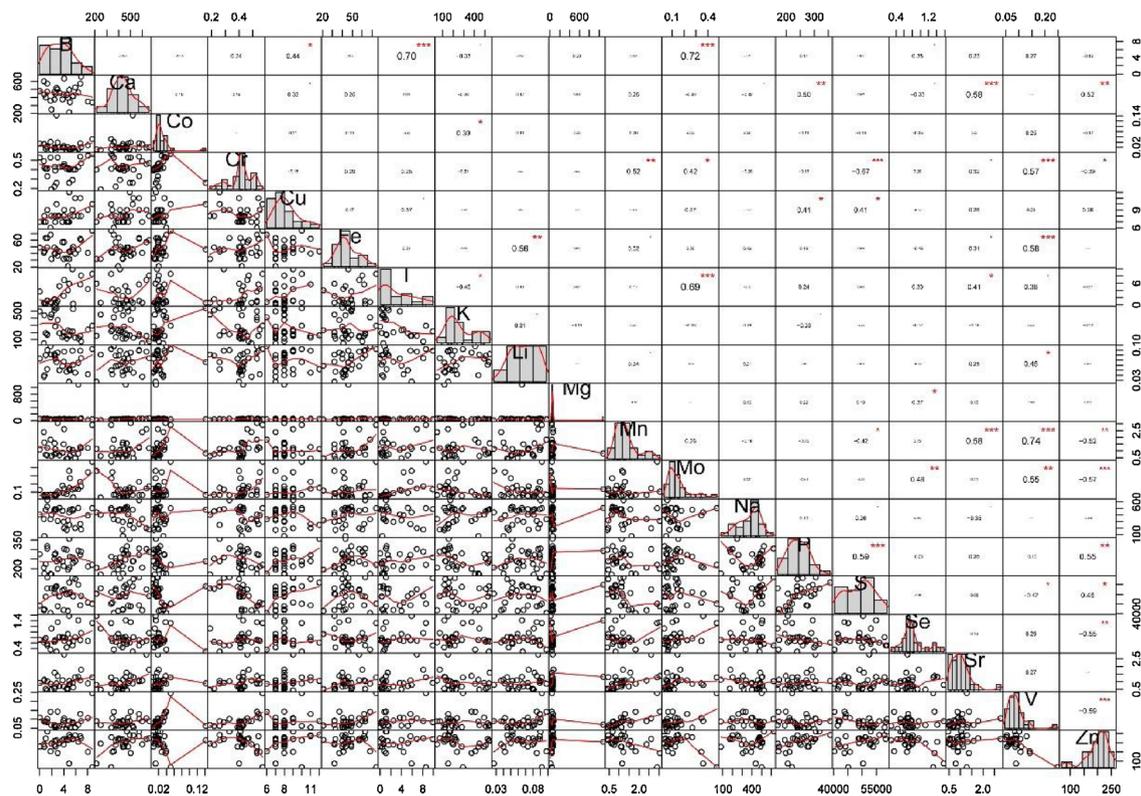


Fig. A4. Correlation analysis of the content ($\mu\text{g/g}$) of 19 nutrient elements in Control External -Chang'an. The distribution graph is displayed on the diagonal line. The bottom left shows a bivariate scatter plot with fitted lines. The upper right shows the correlation coefficient and significance level. * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$

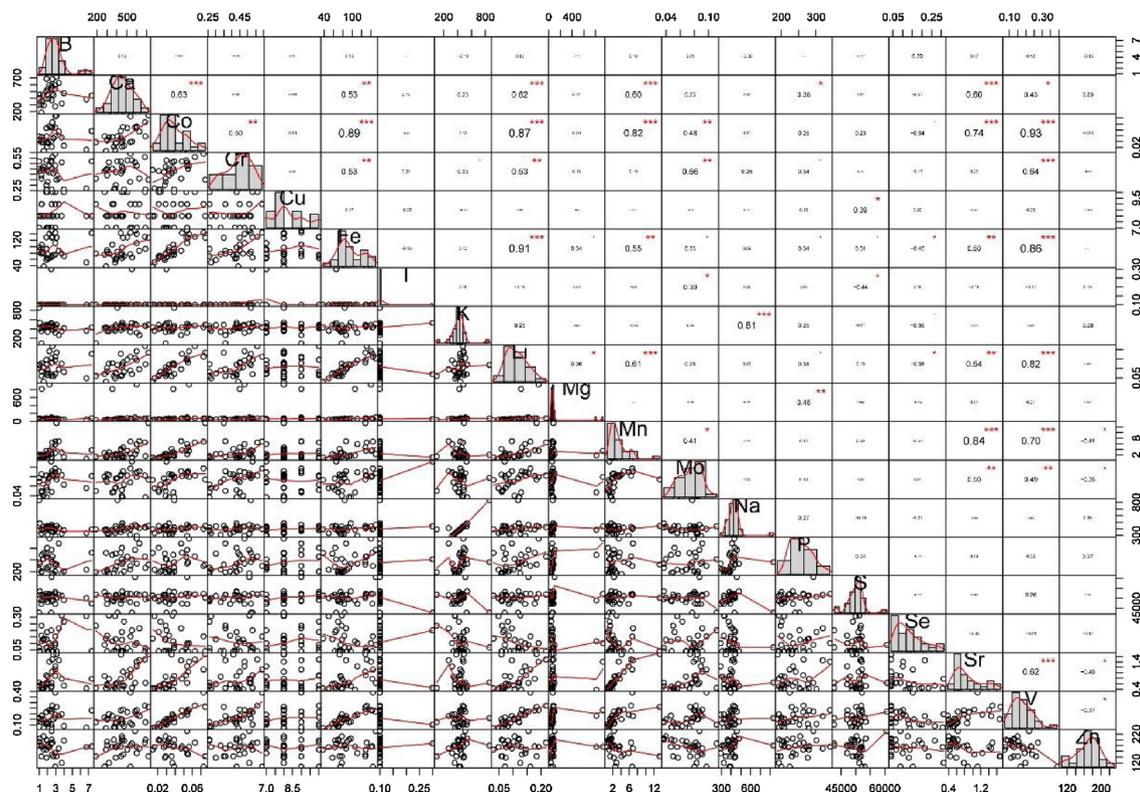


Fig. A5. Correlation analysis of the content (µg/g) of 19 nutrient elements in Control -Bin. The distribution graph is displayed on the diagonal line. The bottom left shows a bivariate scatter plot with fitted lines. The upper right shows the correlation coefficient and significance level. * means P < 0.05, ** means P < 0.01, *** means P < 0.001

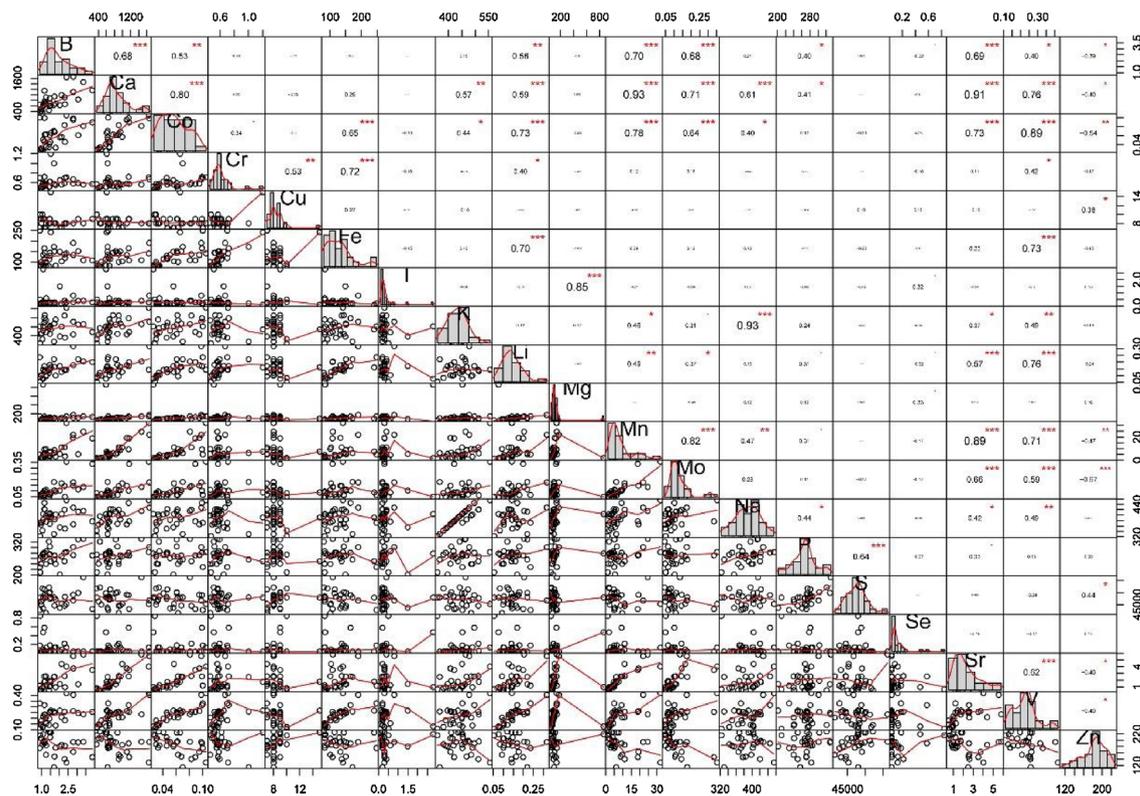


Fig. A6. Correlation analysis of the content (µg/g) of 19 nutrient elements in KBD -Bin. The distribution graph is displayed on the diagonal line. The bottom left shows a bivariate scatter plot with fitted lines. The upper right shows the correlation coefficient and significance level. * means P < 0.05, ** means P < 0.01, *** means P < 0.001

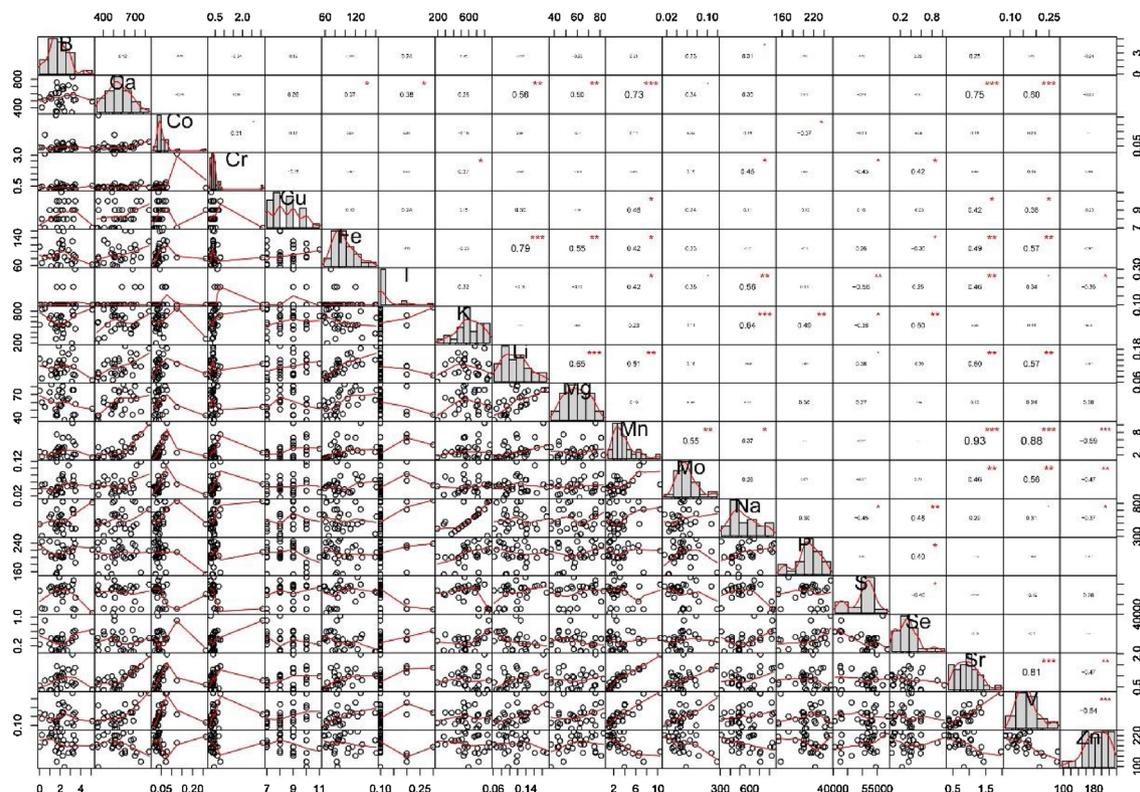


Fig. A7. Correlation analysis of the content ($\mu\text{g/g}$) of 19 nutrient elements in Control + Se-Yongshou. The distribution graph is displayed on the diagonal line. The bottom left shows a bivariate scatter plot with fitted lines. The upper right shows the correlation coefficient and significance level. * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$

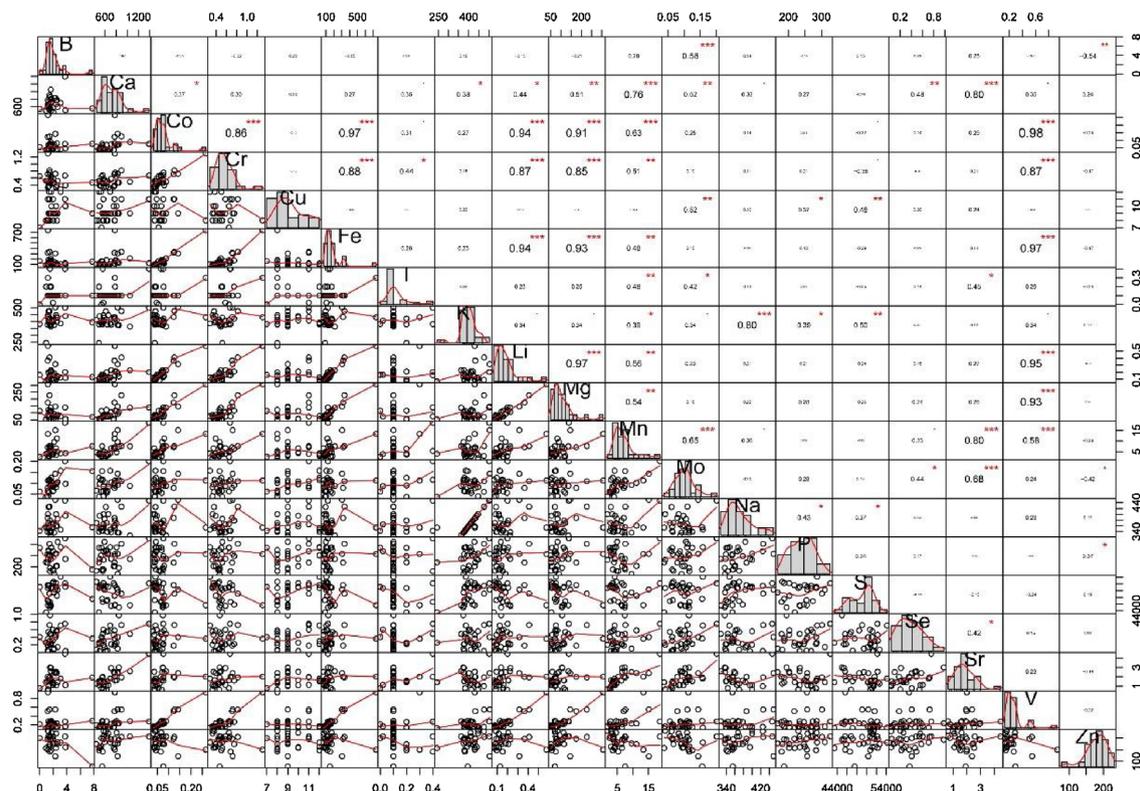


Fig. A8. Correlation analysis of the content ($\mu\text{g/g}$) of 19 nutrient elements in KBD + Se-Yongshou. The distribution graph is displayed on the diagonal line. The bottom left shows a bivariate scatter plot with fitted lines. The upper right shows the correlation coefficient and significance level. * means $P < 0.05$, ** means $P < 0.01$, *** means $P < 0.001$

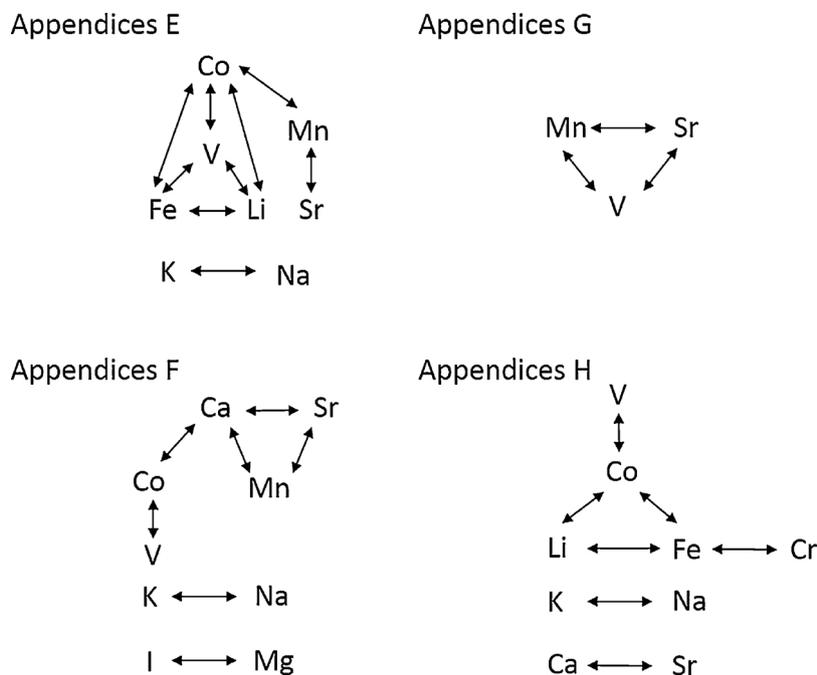


Fig. A9. Correlation relationship among elements in different groups (Appendices E, F, G and H). Bi-directional arrow means there is a correlation between the two elements.

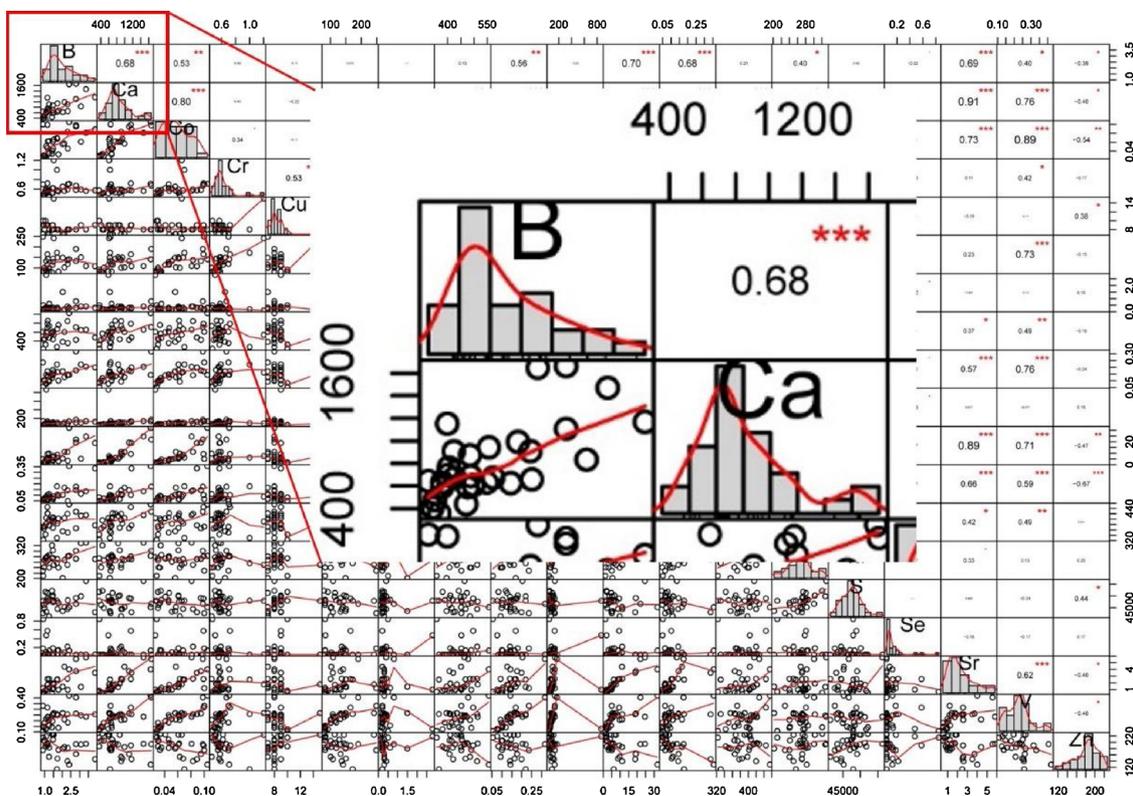


Fig. A10. The white circles in square in lower left quarter were Scatter plot of B and Ca, and the line within the square among the white circles was the fitted lines which present the correlated tendency between two elements, the correlation coefficient between B and Ca is 0.68 in the square in Upper right quarter. *** means $P < 0.001$

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