Study of the Correlation of Ca, Mg, K, and Na Concentrations with the Blackfoot Disease by the Comparison of the Hair from Inhabitants with Blackfoot Disease and Inhabitants without Blackfoot Disease in Hyper Endemic Villages in Taiwan

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Blackfoot disease (BFD) is a local endemic disease that prevails in the southwest coastal areas of Taiwan. From a pathological point of view, BFD is a chronic peripheral vascular atherosclerosis obliterans disease affecting the lower limbs. This study compared the calcium, magnesium, potassium, and sodium concentrations found in the hair of hyper endemic village BFD patients (n = 123), and in the hair of inhabitants without BFD (n = 39) by using an atomic absorption spectrophotometer. Analysis revealed, when compared to the hyper endemic village inhabitants without BFD, BFD patients have a higher mean calcium concentration ($p < 0.05$), but a lower mean magnesium concentration ($p < 0.05$) and a lower mean potassium concentration ($p < 0.01$) in hair. In addition, we found BFD patients have a higher ratio of [Ca/Mg] ($p < 0.05$), but have a lower ratio of [K/Na] ($p < 0.01$) in hair. From this point of view, we hypothesize the lower mean concentrations of magnesium and potassium and the higher mean calcium concentration may have certain correlation with the BFD. Furthermore, this assumption may be used to explain why some of the hyper endemic village inhabitants can avoid the BFD. However, it needs further investigation.

Key words —— atomic absorption spectroscopy, blackfoot disease, hair, minerals

INTRODUCTION

Blackfoot disease (BFD) is a local endemic disease that prevails in the southwest coastal areas in Taiwan. The hyper endemic villages include Hsuehchia and Peimen of Tainan county as well as Putai and Yichu of Chiayi county. This disease has crippled more than one thousand six hundred residents since 1911.[1] From a pathological point of view, BFD is a chronic peripheral vascular disease affecting lower limbs. It causes[2] chronic arterial calcification of the lower limbs and ischemia vascular disease of the lower limbs with initial symptoms of: numbness, coldness, and intermittent claudicating. This is followed by sharp pain, ulceration, and even dry gangrene. Eventually, most of the BFD patients have to amputate their lower limbs by artificial operation. So far, not only the BFD, but also the BFD adverse effects such as hypertension, various peripheral vascular disease, and lower limb ischemia disease were common symptoms found in the hyper endemic village BFD patients, including the inhabitants without BFD.[3–8]

Epidemiological surveys in these areas have attributed the occurrence of BFD to the long-term use of artesian well water. In addition, the prevalence rate of BFD was found to be proportional to the arsenic concentration in the artesian well water.[6–8] Our previous studies found that hyper endemic village BFD patients have higher arsenic (with significant difference; $p < 0.01$) concentration levels in the blood, hair, urine, and plantar digital arterial tissues, when compared to health controls.[9–12] Yet, kisters *et al.*[13] found the increased [Ca/Mg] ratio in hypertensive cells is a pathogenetic factor for the development of arteriosclerosis and hypertension. The magnesium ion is an effective calcium channel blocker. Patients with severe hypermagnesiumia develop profound cardiovascular and neuromuscular dysfunction as a result. Rayssiguier[14] concluded that mag-

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nesium deficiency results in vascular calcification. Also, evidence exists that magnesium deficiency may affect several different stages involved in arteriosclerosis and, additionally, potassium deficiency may exacerbate the arteriosclerosis. In most humans, the [K/Na] ratio and [Ca/Mg] ratio are necessary at both cellular and whole body concentrations which, in turn, require adequate, long term intakes of nutritional magnesium.[15)]

Gibson[16)] suggests the use of hair as a good biopsy material for assessment of trace elements status. However, the assessment must follow a strict exercise in sampling and only proximal, recently-grown hair should be collected. Appropriate standard washing procedures must also be employed and sensitive analytical techniques used for the determination of hair trace element concentrations. Despite the successful use of hair in forensic medicine, screening populations for heavy metal poisoning, and monitoring environmental pollutants, its use for the diagnosis of an individual for disease or nutritional status has been challenged.[17)] In our previous study,[18)] it was concluded that hair could reflect the burden of calcium, magnesium, potassium, and sodium concentrations in the human body. Chen *et al.*,[19)] and He *et al.*[20)] also demonstrated either hair or serum both can reflect the trace elements in the body and show the same trend. In this large scale experiment of the hyper endemic village BFD patients and the hyper endemic village inhabitants' without BFD, hair was used. Hair was used for the stricter and better understanding of the relationship between calcium, magnesium, potassium, and sodium concentrations, including a comparison of the variation of [Ca/Mg] and [Ca/Mg] in the hair with the hyper endemic village BFD patients and the hyper endemic village inhabitants without BFD.

There were no documented reports coupling hair sample analysis with the same age to determine calcium, magnesium, potassium, and sodium concentrations by the hyper endemic village BFD patients and the hyper endemic village inhabitants without BFD. Meanwhile, we tried to provide a factor concerning the BFD. A number of reports have emphasized the influence of arsenic,[5–8)] but the role of calcium, magnesium, potassium, sodium, and substances other than arsenic has not been studied in Taiwan or elsewhere. Particularly, no study has been documented with a focus on the hyper endemic village inhabitants who are without BFD.

MATERIALS AND METHODS

Reagents and Apparatus —— Reagents used for digestion procedures and deionized distilled water (18 MΩ) were purchased from E. Merck. Standard solutions (1000 ppm in H_2O) of calcium, magnesium, potassium, and sodium were obtained from the same company. Standard human hairs material NIES No. 5, Japan (National Institute for Environmental Studies Certified Reference Material) were purchased and used as the base for the determination of precision and accuracy. The containers were made of inert materials such as quartz, Teflon, and polypropylene. All containers were first immersed in 8 N HNO_3 for 24 hr, rinsed under deionized distilled water, and dried at ambient temperature before use.

Hair Sampling —— Dr. Lin, D T. from the Chiayi hospital was responsible for confirming the diagnosis of BFD in patients. In this study of 123 BFD patients, 77 were male and 46 were female. Average age was 65.4 ± 10.5 (mean ± S.D.) years. Of the 39 hyper endemic inhabitants without BFD, 24 were male and 15 were female with an average age of 60.4 ± 12.3 years. It is necessary to consider the difference of samples. Calcium, magnesium, potassium, and sodium concentrations were affected by various factors including: sex, gender, nutritional condition, genetic factors, and living conditions.[18)] All of the subjects provided their informed consent as approved by the college medical ethic committee.

The stringent screening and quality control are required in collecting samples so as not to affect the accuracy of the result of the analysis. Hair samples were cut near the scalp area with thin-blade stainless steel scissors. The average length of hair samples ranged from 1.0 to 3.0 cm. Hair samples were accurately weighed to 1.000 ± 0.200 g. All hair samples were then placed inside polyethylene bags. The samples were stored at a controlled temperature (25°C) and humidity (65%RH).

Hair Cleaning —— Samples were immersed in a 65 ml mixture of normal-hexane, ethyl alcohol, and acetone (4 : 2 : 1; v/v) two times. Each immersion lasted 1.5 hr. Then the samples were rinsed under deionized distilled water four times and immersed in acetone 65 ml for 15 min. The samples were given a final rinse under deionized distilled water three times, filtered with paper dried at ambient temperature, and prepared for the digestion procedure.[18)]

Hair Digestion —— A hair sample was weighed (0.200 ± 0.100 g), and then placed inside a 250 ml

Table 1. Recoveries and Coefficient Variations of Calcium, Magnesium, Potassium, and Sodium in the Standard Material of NIES No. 5. Human Hair

	Certified value (μg/g)	C V%	Analized value (μg/g)	Recovery %	C V %
Calcium	728 ± 30	4.7	727.2 ± 31.9	99.9	4.4
Magnesium	208 ± 10	4.8	201.7 ± 9.07	97.0	4.5
Potassium	34 ± 3	8.8	33.1 ± 1.78	97.6	5.4
Sodium	26 ± 1	3.8	25.6 ± 1.43	98.5	5.6

Each value is the mean ± S.D. of 3 runs. S.D.: Standard deviation. CV%: Coefficient of variation.

microwave digester vessel. 10 ml of nitric acid was added and then heated in a microwave (CEM-MD2000 microwave digester, CEM Corp., Matthews, North Carolina, U.S.A.) using less than 30% power for 5 min. Then, 10 ml of deionized distilled water was added followed by 40% power heating for 25 min and 0% power heating for 10 min. Finally, 2 ml H_2O_2 was added followed by 65% power heating for 5 min. After the heating procedures, the vessels were removed at a normal pressure and temperature. All digested solutions were diluted to specific volumes with deionized distilled water for atomic absorption spectrophotometer (AAS) determination.[18]

Analytical Methods —— With some minor variations in AAS conditions, the instrumentation, and procedures (Hitachi Crop., Tokyo, Japan, Hitachi Z-8200, Atomic Absorption Spectrophotometer coupled with Flame Atomizer) were the same as used in our previous study.[18]

Accuracy and Precision —— An external standard method was used for the quantitative determination of the metal elements in hair. A series of standard solutions containing the following concentrations of calcium, magnesium, potassium, and sodium ions were prepared using deionized distilled water and stock solutions (1000 parts-per-million): 0.00, 0.10, 0.20, 0.40, 1.00, 2.00, and 4.00 μg/ml. To obtain accurate quantitative data, the regression coefficient of the standard calibration curve for each element was made greater than 0.9998. The NIES. No. 5, Japan human hair recovery is used for comparing the accuracy of calcium, magnesium, potassium, and sodium. Our result shows that the overall average recovery for these four elements is greater than 97.0%. Table 1 shows the recovery for these elements. The recovery for calcium, magnesium, potassium, and sodium are 99.9, 97.0, 97.6, and 98.5%, respectively. The coefficient variation (CV %) for the standard materials will be used for precise comparison. According to Table 1, the coefficient variation for these four elements is less than 5.6% (calcium 4.4%, magnesium 4.5%, potassium 5.4%, and sodium 5.6%). Therefore, we conclude that our method is applicable for the analysis of calcium, magnesium, potassium, and sodium concentrations in hair.

Statistical Analysis —— In this study, for the conceptual simplicity and ease of comparison parametric analysis, the changes in the clinical findings of the groups were tested for the significance by two-simple *t*-test analysis. Means between two groups were subjected to two side analysis of variance and compared by Gosset range (student's *t*-test range). The statistical graphic package (Microsoft Statistica, U.S.A.) was used to complete the computation of various statistical data. If the *p*-value for the mean concentration of calcium, magnesium, potassium, sodium, and the ratio of [Ca/Mg] and ratio of [K/Na] between any two groups is less than 0.05 ($p < 0.05$), then the difference will be significant. Values were expressed as means ± S.D.[12,18]

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RESULTS

The Comparison of the Arsenic, Calcium, Magnesium, Potassium, and Sodium Concentrations in the Hair of the Hyper Endemic Village BFD Patients and the Hair of the Hyper Endemic Village Inhabitants without BFD

Table 2 shows that BFD patients have a higher mean concentrations of arsenic (0.329 ± 0.157 μg/g > 0.195 ± 0.105 μg/g), and calcium (901.4 ± 594.2 μg/g > 679.2 ± 305.1 μg/g) than those of the hyper endemic village of inhabitants without BFD and show significant differences with $p < 0.01$ and $p < 0.05$. On the contrary, the hyper endemic BFD patients have a lower mean concentrations of mag-

Table 2. Comparison of the Calcium, Magnesium, Potassium, and Sodium Concentrations Found in the Hair of the BFD Hyper Endemic without BFD Inhabitants and the BFD Hyper Endemic of BFD Patients

	BFD hyper endemic without BFD inhabitants ($n = 39$)	BFD hyper endemic of BFD patients ($n = 123$)
Arsenic (μg/g)[a)]	0.195 ± 0.105	0.329 ± 0.157**
Calcium (μg/g)	679.2 ± 305.1	901.4 ± 594.2*
Range (μg/g)	1135.0 – 205.0	2994.0 – 205.0
Magnesium (μg/g)	78.8 ± 32.9	56.9 ± 45.0*
Range (μg/g)	133.8 – 36.0	180.0 – 16.4
Potassium (μg/g)	38.4 ± 22.8	14.0 ± 10.3**
Range (μg/g)	83.0 – 11.0	72.1 – 2.2
Sodium (μg/g)	65.6 ± 16.6	56.5 ± 17.0*
Range (μg/g)	99.3 – 40.4	110.5 – 30.0

Each value is the mean ± S.D. of 3 runs. S.D.: Standard deviation. Significant differences from the BFD hyper endemic without BFD inhabitants are shown by $*p < 0.05$, and $**p < 0.01$. *a*) These values refered from the references 10 and 11.

Table 3. Comparison of the Hair [Ca/Mg], and [K/Na] Ratios with the BFD Hyper Endemic without BFD Inhabitants, and the BFD Hyper Endemic of BFD Patients

	BFD hyper endemic without BFD inhabitants ($n = 39$)	BFD hyper endemic of BFD patients ($n = 123$)
[Ca/Mg]	10.3 ± 4.3	15.7 ± 10.3*
Range	24.88 – 5.69	83.3 – 4.3
[K/Na]	0.31 ± 0.20	0.56 ± 0.38**
Range	1.67 – 0.16	1.08 – 0.32

Each value is the mean ± S.D. of 3 runs. S.D.: Standard deviation. Significant differences from BFD hyper endemic without BFD inhabitants are shown by $*p < 0.05$, and $**p < 0.01$.

nesium (56.9 ± 45.0 μg/g < 78.8 ± 32.9 μg/g), and potassium (14.0 ± 10.3 μg/g < 38.4 ± 22.8 μg/g), than the hyper endemic village inhabitants without BFD and show significant difference with $p < 0.05$, and $p < 0.01$. We also found the hyper endemic inhabitants without BFD have a higher sodium concentration (65.6 ± 16.6 μg/g > 56.5 ± 17.0 μg/g) than the BFD patients and demonstrate significant difference with $p < 0.05$.

The calcium, magnesium, potassium, and sodium concentrations in the hair of the hyper endemic village BFD patients and the hair of the hyper endemic village inhabitants without BFD distribution are shown in Table 2.

Comparison of the Ratio of [Ca/Mg], and [K/ Na] in the Hair of the Hyper Endemic Village BFD Patients and the Hair of Hyper Endemic Village Inhabitants without BFD

Table 3 shows that BFD patients have a higher ratio of [Ca/Mg] than those of hyper endemic village inhabitants without BFD (15.7 ± 10.3 > 10.3 ± 4.3) and with significant differences $p < 0.05$. However, the hyper endemic village BFD patients have a lower ratio of [K/Na] than those of the hyper endemic village of inhabitants without BFD (0.31 ± 0.20 < 0.56 ± 0.38) and have significant differences $p < 0.01$.

The ratios of the [Ca/Mg] and the [K/Na] in the hair distribution both of the BFD patients in hyper endemic villages and the hyper endemic village inhabitants without BFD are shown in Table 3.

DISCUSSION

The Possible Correlation of the Calcium Concentrations, Magnesium Concentrations, and the Ratio of [Ca/Mg] Found in the Hair of the Hyper Endemic Village BFD Inhabitants

In this study, we found hyper endemic village BFD patients have higher mean calcium concentrations, but lower mean magnesium concentrations in hair. The possible cause for the higher mean calcium concentrations, but the lower mean magnesium concentrations found in hyper endemic village BFD patients' hair could be attributed to the calcium released from the bone with aging and calcium suspended in the blood and even tagged in arteries endogenous. The higher calcium concentration in blood will reduce the magnesium concentration in the cellular arteries. If this circumstance is maintained for a period of time, it will proliferate or may influence vascular flexibility. As we all know, magnesium deficiency induces arterial damage.[14] When the development of the initial lesion is in the lower limb arterial wall, the development may be facilitated by the loss of arteries cellular magnesium. Finally, an increase in the calcium content of the lower limb cellular arteries may form calcification. This assumption may be the reason why most of the hyper endemic village BFD patients, when examined, showed heavy calcification in their lower limb arteries.[5,21] However, the higher endemic village inhabitants without BFD were seldom found. In this study, our finding agrees with the Ondrus *et al*.[22] found the magnesium concentration sharply decreased in the chronic leg ischemia patients (BFD patients). Base on this finding, we suspect the higher ratio of [Ca/Mg] in hyper endemic village BFD patient hair may have the same implication with the lower limb arteries calcification. This evidence may suggest the ratio may affect several different stages involved in arteriosclerosis. Finally, it could exacerbate the BFD or its adverse effects. But, it need further study.

A Possible Correlation of the Potassium Concentrations, Sodium Concentrations, and the Ratio of [K/Na] in the Hair of the Hyper Endemic Village BFD Inhabitants

Lower mean potassium concentrations and the lower ratios of [K/Na] were found in the hyper endemic village BFD patients' hair. The possible cause for the lower potassium concentrations and the lower ratios of [K/Na] found in hyper endemic village BFD patients' hair could be attributed to the lower magnesium concentration found in hyper endemic village BFD patients as previously mentioned. As a result, potassium and magnesium deficiency frequently occurs together. In this study, we found the BFD patients have lower mean magnesium concentrations than those of the hyper endemic village of inhabitants without BFD. The potassium deficiency may be an aggravating factor in pathogenesis.[14] The development of the initial lesion in the lower limb arterial wall of BFD patients may be facilitated by the loss of cellular potassium and increased calcium. Meanwhile, the concentrations of calcium increased in the lower limb arteries of BFD patients in the BFD hyper endemic villages. This aforementioned symptom (lower limb arteries calcification) was found in both the Tseng report[5] and Wang report.[21] Therefore, we suggest that the lower mean potassium concentrations and the lower ratios of [K/Na] in BFD patient hair may have a close relationship with the BFD and it adverse effects.

In addition, Chen *et al*.[23–25] found the BFD hyper endemic village inhabitants suffered with a higher risk of hypertension than others without BFD hyper endemic village inhabitants. In this experiment, we found hyper endemic village inhabitants had higher hair sodium content, but a lower arsenic content than the BFD patients. Therefore, we suspect the higher sodium concentration may be one of the cause factors (besides the arsenic) for the higher risk of hypertension in this area. However, it needs further investigation.

In conclusion, with great confidence, we conclude that analysis of the concentrations of calcium, magnesium, potassium, and sodium including the ratio of [Ca/Mg], and [K/Na] change in hyper endemic village BFD patients' hair can serve as a guide to opening a new outlook in the prevention and treatment of BFD from simply the arsenic as a factor. Particularly, measuring the ratio of [Ca/Mg], and [K/Na] in hair not only might be used as a biomarker for screening the arteriosclerosis obliterans (lower limb arteries calcification) to prevent any form of the BFD, but also might be used in the prevention of the BFD adverse affect of hypertension as a prediagnosis method.

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