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An Exploratory Study Comparing Blood Metal Concentrations between Stroke and Nonstroke Patients in Koreans

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Abstract: Various heavy metals have been known for causing ischemic stroke. In order to describe the causative relationship between the blood levels of various heavy metals and stroke patients, 116 patients with stroke and 111 patients without stroke were selected from one Oriental medical hospital in Wonju, Korea. Total of 9 kinds of metals such as As, Cd, Co, Cu, Hg, Mn, Ni, Pb, and Zn were analyzed in blood from patients with and without stroke. There were no significant differences in the means of metal concentrations between the stroke and non-stroke patients except for the mean of Co concentration. In the case of Co, the means for stroke and non-stroke patients were 0.44 ug/*l* and 0.40 ug/*l* showing a significant difference at the level of p-value = 0.05. The odds ratios for each metal ranged from 0.96 to 2.86. Most odds ratios were not significant but the odds ratio for Co, 2.86 ± 1.49 was significant, indicating that Co increases the risk of stroke by 2.86 times. In order to identify the specific risk level of stroke increased by a multiple interaction of metals, regression coefficients and odds ratio for a pair or multiple pair of metals were reanalyzed. However, all of regression coefficients and odds ratios were not significant. In conclusion, Co showed the significant level in blood from patients with stroke. In addition, the odds ratio of stroke was significantly different from other metals. Thus, it is considered that Co among various metals analyzed in this study is the important metal for increasing the risk of stroke.

Keywords: heavy metals, blood concentration, stroke

Introduction

A stroke is a disruption in the blood supply to the brain. It is frequently referred to as a cerebrovascular accident. A stroke occurs when the blood supply to part of the brain is suddenly interrupted due to the presence of a blood clot (ischemic stroke) or when a blood vessel in the brain bursts, spilling blood into the spaces surrounding brain cells (hemorrhagic stroke). Brain cells die when they no longer receive oxygen and nutrients from the blood or when they are damaged by sudden bleeding into or around the brain. This results in temporary or permanent neurologic impairment. Ischemic stroke, also known as cerebral infarction, accounts for 80 to 85 percent of all strokes, while hemorrhagic stroke accounts for the other 15 to 20 percent.[1,2] There are risk factors which are specific to stroke but generally the attributable risk fractions have not been accurately estimated. However, many of the risk factors identified for stroke have been associated with the blood supply to the brain caused by vascular injury or blockage.[3]

Various heavy metals have been suggested as one of important factors for vascular injury or blockage.[4-6] It has been known that much of the vascular damage produced by toxic metals stems from the proliferation of oxidative free radicals they cause.[7,8] The brain is particularly sensitive to oxidative stress due to the high generation of reactive oxygen species(ROS) with free radical damage arising due to the brain's high lipid content, an increased oxygen consumption rate, and chemical reactions.[9,10] In addition, experimental researches indicate that the generation of free radicals leading to oxidative stress plays an important role especially in the pathogenesis of ischemic brain injury.[11-14] Thus, the overload of heavy metals in blood could be considered as one of important factors for ischemic stroke.

However, few studies have been done to explain a causative correlation between blood concentrations

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of heavy metals and stroke patients. In addition, the identification of metal with the highest level and the potential risk by co-exposure would be also considered important for explaining the relationship of heavy metals and stroke patients. In this study, the blood levels and odds ratios for various metal related with stroke were identified. Patients with ischemic stroke were selected in this study.

Materials and Methods

Case Selection

For the present study, 116 patients with stroke and 111 patients without stroke were selected from

Table 1. Comparison of general characteristics between stroke and nonstroke patients

	Stroke patients (N = 116)		Nonstroke patients (N = 111)		
Variable	No	Mean (SD) or %	No	Mean (SD) or %	p-value
Age	116	64.92 (11.72)	111	55.86 (19.22)	0.04
Sex					0.33
Male	48	41.38%	39	35.14%	
Female	68	58.62%	72	64.86%	
Job					0.002
Blue collar	21	18.10%	24	21.62%	
White collar	5	4.31%	7	6.31%	
Non-job	79	68.10%	47	42.34%	
Househole	11	9.48%	21	18.92%	
Students	0	0.00%	6	5.41%	
No answer			6	5.41%	
Smoke					0.99
Never	89	76.72%	80	72.07%	
Post smoke	5	4.31%	5	4.50%	
Current smoke	22	18.97%	20	18.02%	
No answer			6	5.41%	
Alcohol					0.10
Never	88	76.72%	78	70.27%	
Post Alcohol	5	4.31%	12	10.81%	
Current Alcohol	23	19.83%	15	13.51%	
No answer			6	5.41%	
Residence					0.02
Urban	3	2.59%	0	0.00%	
Suburban	30	25.86%	43	39.64%	
Rural	83	71.55%	62	55.86%	
No answer			6	5.41%	
Education					0.02
Non	91	78.45%	60	54.05%	
Elementary	6	5.17%	12	10.81%	
Middle-high	16	13.79%	26	23.42%	
College and over	3	2.59%	7	6.31%	
No answer			6	5.41%	
Disease					0.00
Stroke	116	100%	0	0.00%	
non stroke	0	0.00%	111	100%	
Blood pressure					
Systolic	116	139.57 (18.48)	111	131.06 (20.09)	0.001
Diastolic	116	88.88 (14.19)	111	83.94 (13.32)	0.009

one Oriental medical hospital in Wonju, Korea. The medical treatments were rendered from September, 2000 to May, 2001. Patients with ischemic stroke within 14 days of the first onset of symptom including an intracerebral hemorrhage participated in this study. However, patients with high potential of heavy metals exposure were excluded in this study. Health history including blood pressure measurement and lifestyle (such as dietary habit, smoking and alcohol) were all recorded in Table 1.

Blood Collection

Approximately 10 m*l* blood was drawn from each subject, 4 m*l* was poured into a tube which contained K-EDTA as an anticoagulant and mixed for 10 minutes. Blood samples for elemental analysis were stored at –20°C until analysis. Samples were diluted 5 times for serum with high purity de-ionized water.[15]

Sample Pre-treatment and Metal Analysis

Sample pre-treatment and analytical methods followed US EPA procedures, in part, Goulle's and Mortada's study.[16-18]

1. Cd, Cu, Pb, Co, Mn, Ni, Zn, As

Samples were treated using 65 m*l* of cleaning solution (186 m*l* of ethyl alcohol, 93 m*l* of acetone and 371 m*l* of n-hexane to 1 *l* volumetric flask) for 12 hours. Each aliquot (0.1~0.2 g) from well homogenized samples was moved to a digestion vessel. The aliquots were mixed with 10 m*l* of 1:1 HNO_3, and then covered with a watch glass. Treated samples were heated to 95 ± 5°C and refluxed for 10 to 15 minutes without boiling. After adding 5 m*l* of concentrated HNO_3, the samples were allowed to be cool and were refluxed for 30 minutes. This step was repeated until no brown fume was given off by the sample, showing the complete reaction with HNO_3. Using a ribbed watch glass, the solution was evaporated to approximately 5 m*l* at 95 ± 5°C for two hours. The samples were cooled again and 3 m*l* of 30% H_2O_2 was added. In order for the peroxide reaction the vessels were covered with a watch glass and placed on the heat source for warming until effervescence subsides. Then peroxide solution was added in 1 m*l* aliquots. The aliquots were warmed until the effervescence was minimal. The aliquots were covered with a ribbed watch glass. The acid-peroxide digestate of the aliquot was heated until the volume was reduced to approximately 5 m*l*. The solution was covered over the bottom of the vessel at all times. After cooling, it was diluted to 50 m*l* with water. The particulates in digestates were then removed by filtration. The filtered samples were analyzed by ICP-MS. The ICP-MS analysis for eight elements was carried out using ICP-MS spectrometer Varian Ultramass 700 (USA, 1998) with cross flow nebulizer. The operating conditions are given in Table 2.

Table 2. ICP-MS operating conditions

Nebulizer	Babington
Spray chamber	Scott, 2°C
Radio Frequency (RF) power	1300 W
Sampling depth	6.4 mm
Plasma gas flow rate	15.0 *l* min^{-1}
Auxiliary gas flow rate	1.0 *l* min^{-1}
Nebulizer gas flow rate	1.05 *l* min^{-1}
Sampler	0.5 mm, Ni
Skimmer	0.5 mm, Ni
Integration time	0.3 s/channel (three channels per mass)
Repetitions	5

2. Hg

Samples were treated by using 65 m*l* of cleaning solution (186 m*l* of ethyl alcohol, 93 m*l* of acetone and 371 m*l* of n-hexane to 1 *l* volumetric flask) for 12 hours. Each aliquot (0.1, 0.2 g) from well homogenized samples was placed in the bottom of a BOD bottle. The aliquots (0, 1, 3, 5 m*l*) of mercury working standard containing 0~5 ug/*l* of mercury were transfer to a series of BOD bottles. The reagent water (5 m*l*) and concentrated sulfuric acid (5 m*l*) were added to the aliquots of standard and sample. Also 2.5 m*l* of concentrated nitric acid was added to them and then they were heated for two minutes at 95 ± 3°C. After cooling, samples and standard were added by 15 m*l* 5% potassium permanganate solution and mixed. They were heated for 30 minutes at 95 ± 3°C. After cooling, they were mixed with sodium chloride-hydroxylamine hydrochloride to reduce the excess permanganate. The standard and sample were diluted to 100 m*l*

with de-ionized water. The particulates in digestates were removed by filtration. The filtered samples and standards were analyzed by mercury analyzer (USA, Cetac, M-6000A).

Reagents

All reagents were of analytical reagent grade. High purity de-ionized water (Milli-Q system, Millipore, USA) was used throughout. Analytical reagent nitric acid (Merck, 70%) was used after additional purification by sub-boiling distillation in quartz still. Plastic bottles and glassware were cleaned by soaking in 20% (v/v) HNO_3 for 24 hours. This material was then rinsed three times with de-ionized water.

Statistics

We performed t-test to compare the geometric means between the case group and the control group. We also took percents exceeding upper limit of the reference value ranges of them. Potential confounders such as age, sex, smoking, drinking, job, and residence were adjusted. Multiple regression values (β(SE)) were calculated after adjusting confounding factors. Dependent variables calculated natural logarithm of each metal level plus one, for example, log (Al+1). Odds ratios were taken for exceeding reference values in blood, adjusted for potential confounders. Odds ratios of the control and experiment groups were compared after adjusting confounding factors of out-of-range subjects. For statistical analysis, Stata (2001)[19] statistical package was used for mean (SD) %, geometric mean, t-test, multiple regression, and odds ratios.

Results

Table 3 shows geometric means of blood concentrations for various heavy metals in all patients. WHO reference values (trace elements in Human Nutrition and Health) for each heavy metal are listed and compared to those of the samples. The blood concentrations of all metals exceeded the upper limit of WHO reference value. Especially,

Table 4. Adjusted heavy metal concentration in blood of stroke and non-stroke patients* (N = 227)

unit : ug/l

Metals	Stroke (n = 116)	Non-stroke (n = 111)	p-value
As	1.26 (1.52)	1.13 (1.39)	0.39
Cd	0.44 (0.40)	0.42 (0.49)	0.66
Co	0.44 (0.39)	0.40 (0.43)	0.05
Cu	156.02 (0.38)	151.41 (0.42)	0.41
Hg	3.82 (0.46)	3.71 (0.76)	0.91
Mn	1.52 (0.62)	1.34 (0.83)	0.62
Ni	0.78 (0.46)	0.84 (1.04)	0.73
Pb	1.04 (1.20)	1.04 (1.21)	0.84
Zn	1510.20 (0.38)	1394.09 (0.41)	0.81

*Note : Adjusted geometric mean (SE) for sex, age, smoking, alcohol, job, and residence.

Table 3. Geometric mean (SD), range (Min, Max) and % exceeding upper of WHO limit reference range in heavy metals concentration in blood of all patients (N = 227)

unit : ug/l

Metals	Mean (SD)	Min	Max	Exceeding upper of limit reference (%)	Reference values*
As	1.99 (1.54)	0	82.27	20.70	2-20
Cd	0.68 (0.68)	0	18.92	6.61	0.3-4
Co	0.49 (0.51)	0	14.90	2.64	5-10
Cu	242.26 (0.50)	71.52	3295.70	3.08	800-1400
Hg	1.82 (1.77)	0	1408.10	22.91	2-20
Mn	2.16 (2.10)	0	561.16	36.12	8-12
Ni	1.19 (1.67)	0	64.72	33.19	1-5(?)
Pb	3.97 (2.48)	0	157.59	5.73	50-150
Zn	1339.43 (0.47)	639.06	9743.60	3.02	6000-7000

*WHO (1996), trace elements in Human nutrition and Health. World Health Organization, Geneva. 258-9.
(?) is uncertain as reference value.

concentrations for As, Hg, Mn, and Ni were higher than WHO reference values by over 20%. Other metals showed about 2-6% of all concentrations exceeding WHO reference values.

The comparison of geometric means of metal concentrations in stroke and non-stroke patients is presented in Table 4 after adjusting confounding factors such as sex, smocking, job and residence. There were no significant differences in the means of metal concentrations between the stroke and non-stroke patients except for the mean of Co concentration. In the case for Co, the means for stroke and non-stroke patients were 0.44 and 0.40 showing a significant difference at the level of p-value = 0.05.

Table 5 presents regression coefficients and odds ratios for the metal concentrations of stroke and non-stroke patients. The regression coefficients indicate the level of metals increased or decreased by the factor, stroke. The regression coefficients for each metal ranged from 0.96 to 2.64, indicating the levels of all metals increased by stroke. However, all of regression coefficients were not significant. The odds ratios for each metal ranged from 0.96 to 2.86. Most odds ratios were not significant but the odds ratio of Co (2.86 ± 1.49) was significant, indicating that Co increases the risk of stroke by 2.86 times.

In order to identify the specific risk level of stroke increased by a multiple interaction of metals, regression coefficients and odds ratio for pair or multiple pair of metals were reanalyzed in Table 6. The regression coefficients for multiple metal pairs ranged from 0.18 to 0.51 which indicate that the amounts of all metal pairs were increased in stroke patients. However, all of regression coefficients were not significant. The odds ratios ranged from 1.20 to 1.66 which is higher than those for each metal. However, all of the odds ratios were not significant.

Table 5. Regression Coefficient (SE) and odds ratios (SE) for stroke and nonstroke patients respectively, from multiple regression of heavy metal in blood (N = 227)

unit : ug/l

Metals	Beta (SE)	P	Odds ratios (SE)	P
As	1.09 (1.12)	0.37	1.10 (0.11)	0.38
Cd	1.09 (1.27)	0.37	1.10 (0.26)	0.69
Co	2.64 (1.67)	0.06	2.66 (1.49)	0.04
Cu	1.23 (1.62)	0.66	1.52 (0.76)	0.40
Hg	1.00 (1.09)	0.96	1.01 (0.09)	0.92
Mn	1.04 (1.08)	0.63	1.04 (0.09)	0.64
Ni	0.96 (1.11)	0.65	0.96 (0.09)	0.68
Pb	1.05 (1.08)	0.52	1.04 (0.08)	0.62
Zn	1.23 (1.82)	0.73	1.18 (0.72)	0.78

Note : Adjusted for sex, age, smoking, alcohol blue color job, big city residence.
Beta (SE), odds ratios (SE), values in Table 5 compared nonstroke patients group (control).

Table 6. Regression Coefficient (SE) and odds ratios (SE) for stroke and nonstroke patients who exposured multiple metal, from multiple regression of heavy metal in blood (N = 227)

Multiple metal exposure	Beta (SE)	P	Odd Ratio	P
Co * Cu	0.51 (0.31)	0.10	1.66 (0.51)	0.10
Co * Pb	0.38 (0.29)	0.19	1.47 (0.43)	0.19
Cu * Pb	0.18 (0.30)	0.55	1.20 (0.36)	0.55
Co * Zn	0.51 (0.31)	0.10	1.66 (0.51)	0.10
Cd * Co	0.29 (0.29)	0.33	1.33 (0.39)	0.33
Co * Cu * Pb	0.38 (0.29)	0.19	1.47 (0.43)	0.19
Co * Cu * Zn	0.51 (0.31)	0.10	1.66 (0.51)	0.10
Cd * Co * Cu * Pb	0.21 (0.32)	0.51	1.23 (0.39)	0.51
Cd * Co * Cu * Zn	0.29 (0.29)	0.33	1.33 (0.39)	0.33
As * Hg * Mn * Pb * Cd	0.30 (0.53)	0.57	1.35 (0.71)	0.57
As * Cd * Co * Cu * Zn	0.20 (0.30)	0.52	1.22 (0.37)	0.52

Note: Adjusted for sex, age, smoking, alcohol, blue color job, big city residence.
Beta (SE), odds ratios (SE) values in Table 6 compared nonstroke patients groups (control).

Discussion

Free radical generation and consequent oxidative stress in cerebrovascular stroke have a distinctive role in the pathogenesis of brain injury. Much of the vascular damage produced by toxic metals stems from the proliferation of oxidative free radicals they cause.[7,8,20,21] Thus, compounds that reduce free radicals (e.g., free radical trapping agents) protect tissues against the damage caused by free radicals that occurs during stroke.[22] The damages also are produced by inhibited anti-oxidative enzymes by heavy metals.

It has been known that two major anti-oxidative enzymes inhibited by heavy metal such as lead, mercury and copper are superoxide dismutase (SOD), catalase and glutathione peroxidase.[23,24] By acting as a pro-oxidant and inhibiting anti-oxidative processes, these heavy metals could result in excess levels of free radicals, which are particularly disruptive to mitochondrial function and the vascular system. Heavy metals can also increase the acidity of the blood.[25-27] The body draws calcium from the bones to help restore the proper blood pH. Furthermore, toxic metals set up conditions that lead to inflammation in arteries and tissues, causing more calcium to be drawn to the area as a buffer, contributing to hardening of the artery walls with progressive blockage of the arteries. Thus, the overload of heavy metals in blood could be considered as one of important factors for stroke.

In order to compare the metal levels of all patients in this study to those of a general population, WHO reference values were used. The blood levels for analyzed metals exceeding WHO reference values were identified in both stroke and non-stroke patients. As, Hg, Mn, and Ni among analyzed metals showed higher percentages of the levels exceeding WHO reference values than those of the levels in other metals. However, this result did not accord with the comparison of means for each metal in both stroke and non-stroke patients after adjusting compounding factors. Only Co level of all metals in stroke patients showed a significant difference from its level in non-stroke patients. In addition, the odds ratio for Co was 2.86 indicating that Co increase the potential risk for stroke. Thus, Co could be considered as one of the important metals elevating the risk of stroke. In addition, the level of Co in blood increased by 2.64 times in stroke patients than non-stroke patients.

The cobalt is a transition element occurring in four valences (0, +2, +3, and +4), the divalent oxidation state being the most common. There is evidence from both *in vivo* and *in vitro* experiments supporting the view that Co^{+2} induces the production of reactive oxygen species (ROS) with subsequent oxidative stress.[28,29] It was known the Co undergoes redox-cycling reactions inducing depletion of glutathione or bonding to sulfhydryl groups of proteins.[30] In addition, the Co induce heme-oxygenase which increases the oxidative stress, resulting in vascular injury,[31] supporting that Co could be one of the risk factors.

The level of oxidative stress by Co administration is significantly increased by co-exposure with tungsten.[32] This was explained by a physical-chemical mechanism of interaction through their valence. Especially the odds ratios for the various metals analyzed in our study were recalculated with Co whether multiple metal's exposure with Co affects on the odds ratio for stroke. The odds ratios for all metals together analyzed with Co were not significant for stroke risk. However, there is a trend that each odds ratio of coexposured metals with Co is higher than that of each metal itself. Thus, it is considered that the multiple exposure of metals with Co showed the increased odds ratio but was not an important risk factor for stroke.

In this study, especially the levels of As, Hg, Mn, and Ni among the analyzed metals in all the patients exceeded WHO reference values by over 20%. However, only Co among the analyzed metals showed a significant difference in the odds ratio for stroke and a higher level in stroke patients than non-stroke patients. Many studies suggested the possibility that Co would cause vascular damages through oxidative stress. Thus, it is estimated that Co level in the blood can be one of the risk factors for stroke. However, further study should be carried out in terms of vascular damage in brain caused by Co-induced oxidative stress.

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