Title: The Association Between Inflammatory Biomarkers and Vitamin D Level with Evolution and Severity of Stroke

Authors: Anahid Safari[1], Nima Fadakar[2], Afshin Borhani-Haghighi[2,*]

1. *Stem Cells Technology Research Center, Shiraz University of Medical Sciences Shiraz, Iran.*
2. *Clinical Neurology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.*

***Corresponding Author**: E-mail: neuro.ab@gmail.com

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Abstract

Background and purpose: Vitamin D deficiency has been shown to be linked to evolution of ischemic stroke, but the data regarding the association between stroke severity and vitamin D level is scarce.

Materials and methods: Patients with first-ever acute ischemic stroke in the middle cerebral artery (MCA) territory within 7 days after the stroke were recruited. The control group included age and gender matched individuals. We compared 25-OH vitamin D (vitamin D), high sensitive C-reactive protein (hsCRP), serum amyloid A (SAA), and osteopontin levels between stroke patients and the control group. We also investigated the association between stroke severity according to National Institutes of Health Stroke Scale (NIHSS) and Alberta stroke program early CT score (ASPECTS) and vitamin D level as well as inflammatory biomarkers.

Results: Current study showed the association between hypertension (P= 0.035), diabetes mellitus (P= 0.043), smoking (P= 0.016), history of ischemic heart disease (P= 0.002), higher SAA (P< 0.001), higher hsCRP (P< 0.001), and lower vitamin D levels (P= 0.002) with stroke evolution in a case-controlled study. Meanwhile, in stroke patients, its severity was associated with higher SAA (P= 0.04), higher hsCRP (P= 0.001), and lower vitamin D levels (P value= 0.043) according to clinical scale (higher admission NIHSS). According to ASPECT score, higher SAA (P= 0.017), higher hsCRP (P= 0.007), but not lower vitamin D level were associated with more infarct areas (P= 0.149).

Conclusion: Vitamin D might have a role in both evolution and severity of stroke.

Keywords: Ischemic stroke; Cerebrovascular accident; Vitamin D; High sensitive C-reactive protein; Serum Amyloid A; Osteopontin

Introduction

Stroke is a leading cause of mortality and morbidity globally (Borhani-Haghighi, Safari et al. 2013); however, our armamentarium for its treatment is limited (Shahtaheri, Borhani Haghighi et al. 2012). Numerous evidences have revealed the critical role of inflammation in the development of acute ischemic stroke. Hence, inflammatory biomarkers might predict the functional outcome of the stroke (Safari, Safari et al. 2016).

C- reactive protein (CRP), Pentraxin1(Ptx1),is produced by liver cells in response to interleukin-1 (IL-1), interleukin-6 (IL-6) and tumor necrotic factor alpha (TNF-α). High sensitivity CRP (hsCRP) picks up even low CRP levels (Mohebbi, Ghabaee et al. 2012).

Serum amyloid A (SAA) is also an acute phase reactant that increases within hours during inflammatory reaction. Some studies suggest the superiority of SAA over erythrocyte sedimentation rate (ESR) and CRP in quantifying inflammatory reactions (Cunnane, Grehan et al. 2000).

Osteopontin (OPN) is a secreted extracellular phosphoprotein, which plays a pivotal role in a normal mineralization of bone as well as upregulating calcified plaques of the vessels. In addition, OPN has immunomodulatory activities via chemotaxis, cell attachment, cytokine production, and anti-apoptosis mechanism (Wang and Denhardt 2008).

Low 25-hydroxyvitamin D (25-OH vitamin D) concentration has been reported to be associated with obesity, diabetes mellitus, hyperlipidemia and hypertension, which are the major risk factors in ischemic stroke (Muscogiuri, Annweiler et al. 2017).

Even though there are increasing evidences that shows vitamin D deficiency is linked to an increased risk of stroke “evolution” (Zhou, Wang et al. 2018), the association between vitamin D level and severity of stroke and its inflammatory aspects are still unclear. Severity of stroke is assessed by both clinical measures, such as National Institutes of Health Stroke Scale (NIHSS)

and radiologic measures like Alberta Stroke Program Early CT Score (ASPECTS), the two available criteria for assessing stroke severity.

There are a few studies that have investigated the association of NIHSS as a stroke severity measure and vitamin D level at the time of admission (Daubail, Jacquin et al. 2013, Tu, Zhao et al. 2014, Wang, Ji et al. 2014, Park, Chung et al. 2015, Wei and Kuang 2018, Zhang, Wang et al. 2018). To the best of our knowledge, there is no study that has investigated the association between radiological severity scales, such as ASPECTS and vitamin D level. In addition, in none of the aforementioned studies, the acute phase reactants were investigated.

In the current study, we compared serum 25-OH vitamin D (vitamin D), hsCRP, SAA and osteopontin levels between patients with middle cerebral artery (MCA) infarction and age-gender matched normal individuals. We also investigated the association between stroke severity according to NIHSS and ASPECTS, and vitamin D levels with the above mentioned inflammatory biomarkers. Our hypothesis is vitamin D deficiency might induce larger infarct area according to ASPECTS through modulation of immunologic factors such as hsCRP, SAA and osteopontin. As a perspective Vitamin D administration in early phases of stroke might decrease the stroke severity.

Patients and Methods

This is a prospective cross-sectional study conducted in Namazi hospital affiliated to Shiraz University of Medical Sciences from June 2017 to March 2018. This is a high volume referral center for stroke in southwestern Iran.

Inclusion criteria

Patients with first-ever acute ischemic stroke in the middle cerebral artery (MCA) territory within 7 days before recruitment were enrolled. According to the Recognition of Stroke in the

Emergency Room (ROSIER) scale (Nor, Davis et al. 2005) ischemic stroke was defined as a focal neurological deficit of sudden onset, which continued beyond 24 hours and was documented by a brain CT or an MRI (if necessary), indicating the presence of infarction in the MCA territory. Other inclusion criteria were age higher than 18 years and providing their written informed consent.

The exclusion criteria

Patients with primary intracranial hemorrhage were excluded. Patients with recurrent ischemic stroke, ischemic stroke lasted more than 7 days were also not included.

Patients with lacunar stroke [Causative Classification System for Ischemic Stroke (CSS type III)] (Ay, Furie et al. 2005) were also excluded.

Patients with uncommon causes of stroke (CCS type IV), such as acute arterial dissection, cerebral vasculitis, cerebral venous thrombosis, acute disseminated intravascular coagulation, heparin-induced thrombocytopenia type II, hypoperfusion syndromes, iatrogenic causes, thrombosed intracranial aneurysm, meningitis, primary infection of the arterial wall, thrombotic thrombocytopenic purpura - hemolytic uremic syndrome, Moyamoya disease, and segmental vasoconstriction or vasospasm were also excluded.

Conditions that could affect vitamin D, hsCRP, SAA, and osteopontin levels, such as atrial fibrillation (Cheng, Wang et al. 2012, Thompson, Nitiahpapand et al. 2015), fever or any other sign of infection, any previously proven neoplastic (Garland, Garland et al. 2006, Moshkovskii 2012), rheumatologic or inflammatory diseases (Hwang, Balasubramani et al. 2016), renal failure or severe chronic non–dialysis-dependent kidney disease (Tonelli, Sacks et al. 2005, Dieter, McPherson et al. 2016), acute or chronic liver failure (Maury, Teppo et al. 1983), respiratory failure (Bozinovski, Hutchinson et al. 2008), recent surgery (Habib, Scrocco et al. 2009), thyroid (Reza, Shaukat et al. 2013), and parathyroid diseases (Maser, Lenhard et al. 2018), Alzheimer's disease (Chung, Liang et al. 2000), Parkinson (Knekt, Kilkkinen et al. 2010) and

any history of osteoporosis (Abdu-Allah, El Tarhouny et al. 2015) or its related risk factors were also excluded. Patients with excessive alcohol intake were not recruited as well (Hillborn 1998). Those who were receiving vitamin D or calcium supplements, anticoagulants, glucocorticoids and other anti-inflammatory drugs, calcitonin, parathyroid hormone, and osteoporosis related medications were also excluded.

Control group

The control group included age and gender matched individuals who did not have neither any history of ischemic stroke, transient ischemic attack (TIA) nor any of the above mentioned exclusion criteria.

Sample size

Total of 46 patients and 46 controls were considered to be suitable candidates for the current study according to $\alpha = 0.05$, $\beta = 0.80$, and the effect size of 0.6 calculated by G Power Software.

Body mass index (BMI) was measured in both case and control groups. Major cerebrovascular risk factors including current or previous cigarette smoking habit, hypertension and diabetes mellitus and hyperlipidemia were investigated for all participants.

Hyperlipidemia was defined as current or positive history. fasting total cholesterol level> 200 mg/dL, LDL> 130 mg/dL and/or fasting triglycerides level>180 mg/dL (Nelson 2013). Hypertension was defined as positive history, systolic blood pressure of 140mmHg and/or diastolic pressure> 90 mmHg or electrocardiographic or retinal sign of hypertension (Chobanian, Bakris et al. 2003). Diabetes mellitus was defined as positive history and/ Hb A1C more than 7 (Association 2014).

Laboratory investigations

Total of 10cc blood was taken from stroke patients between days 2-7 after stroke and from the controls. Serum were separated from cells by centrifugation at 3000 rpm for 10 min. The sera were stored at -80°C and then thawed. Calcium, phosphor, alkaline phosphatase (ALP), fasting blood sugar (FBS), blood urea nitrogen (BUN), creatinine (Cr) were assessed by routine laboratory methods for both case and control groups.

25-OH vitamin D (KAP1971, DIAsource ImmunoAssays S.A., Louvain-la-Neuve, Belgium), SAA level (KHAoo11, Invitrogen, California, US), osteopontin (E1525Hu, Bioassay Technology, Shanghai, China), hsCRP (4360, Diagnostics Biochem Canada, Ontario, Canada) were measured by enzyme-linked immunosorbent assay (ELISA) method.

Study ethics

This study was approved by institutional review board (IRB) of Shiraz University of Medical Sciences (No#1396-S104), written informed consent was obtained from each patient and healthy controls. If patients were severely ill, consent was obtained from their relatives.

Statistical analysis

Statistical analyses were carried out using SPSS version 20. In normally distributed groups, the result was presented with mean and 95% confidence interval. Chi-square and independent t-test were applied to test the differences in variables. The logistic regression model was used to determine the effects of hypertension, diabetes mellitus, smoking, ischemic heart disease and serum levels of hsCRP, SAA, osteopontin and vitamin D on stroke evolution. Pearson correlation coefficient was applied to determine the association between stroke severity scales (NIHSS and ASPECT) and serum concentration of biomarkers (hsCRP, SAA, osteopentin and vitamin D). The significance level in this study was considered to be 0.05.

Results

Eighty-nine patients with acute ischemic stroke were initially recruited. After applying all the inclusion and exclusion criteria 46 patients with first ever attack of MCA infarction were enrolled. The 46 age and gender matched healthy subjects were recruited as controls. Table 1 shows demographic variables, stroke risk factors, calcium, vitamin D level, hsCRP, SAA, and osteopontin levels.

On evaluating the risk factors and serum biomarkers with stroke evolution, univariate analysis revealed association of diabetes mellitus, hypertension, smoking and ischemic heart disease in addition to higher SAA, higher hsCRP and lower vitamin D levels with evolution of stroke (Table 2).

In stroke patients, there was a significant reverse association between vitamin D and SAA (R= -0.241, P value= 0.025) in addition to vitamin D and hsCRP (R= -0.267, P value= 0.011). But there was no significant association between vitamin D and serum osteopontin levels (R= 0.051, P= 0.642).

In stroke patients, mean and 95% confidence interval (CI) of admission NIHSS score were 12.97 and 10.65-15.35, respectively. Mean and 95% CI of their ASPECT score were 6.24 and 5.57-6.83, respectively. There was a significant reverse linear association between NIHSS score and vitamin D level (R= -0.306, *P* value= 0.043). There were significant linear association between NIHSS score and hsCRP (R= 0.482, *P*= 0.001), as well as SAA levels (R= 0.315, *P*= 0.04). However, the linear reverse association between NIHSS score and osteopontin level was not significant (R= -0.091, *P*= 0.56).

When stroke severity was evaluated according to ASPECT, there were significant reverse linear association between ASPECT score and hsCRP and SAA levels (R= - 0.401, *P*= 0.007 and R= -

0.361, P= 0.017). Meanwhile, there were no significant association between ASPECT score and vitamin D and osteopontin levels (R= 0.221 and - 0.071, p= 0.149 and 0.651).

Discussion

Current study showed the association of hypertension, diabetes mellitus, smoking, history of ischemic heart disease, higher SAA, higher hsCRP, and lower vitamin D levels with stroke evolution in a case-controlled study. Meanwhile, in stroke patients, its severity was associated with higher SAA, higher hsCRP and lower vitamin D according to clinical scale (higher admission NIHSS). According to ASPECT score, higher SAA and hsCRP, but not lower vitamin D levels were associated with more infarct areas. NIHSS and ASPECT scores association with osteopontin level was not significant.

Our results are consistent with previous studies, which showed the association between vitamin D deficiency and stroke evolution (Zhang, Li et al. 2017). Anticalcific protective effect of vitamin D in the process of atherogenesis might prevent the evolution of atherothrombotic ischemic stroke (Mozos and Marginean 2015).

Meanwhile, vitamin D deficiency was associated with higher NIHSS in all (Daubail, Jacquin et al. 2013, Tu, Zhao et al. 2014, Wang, Ji et al. 2014, Afshari, Amani et al. 2015, Park, Chung et al. 2015), as well as subgroups of acute ischemic stroke patients (Zhang, Wang et al. 2018). Anti-inflammatory effect of vitamin D might play a role in stroke severity. There are both animal and human studies supporting this hypothesis. Balden et al. showed larger infarct volumes and more severe behavioral disturbances in rats fed with vitamin D deficient diet in comparison to controls. They hypothesized the activation of inflammatory markers and suppression of inherent neuroprotective agents, such as Insulin-growth factor-1. Interestingly, vitamin D injection in acute stage did not improve infarct volume or behavioral disturbance (Balden, Selvamani et al.

2012). Wang et al. stated that serum vitamin D levels were adversely associated with serum levels of interleukin-6 and high-sensitivity C-reactive protein (hsCRP) in stroke patients (Wang, Zhu et al. 2018).

As far as we know, there are only a few if any studies that have investigated the association between ASPECT score with vitamin D levels, hsCRP, SAA and osteopontin. Current study failed to show the association between ASPECT score and vitamin D or osteopontin levels in the whole population, but it revealed a significant association between lower ASPECT score (i.e. larger infarct areas) and higher levels of hsCRP and SAA.

We eliminated all the confounding factors that might have affected vitamin D, hsCRP, SAA, and osteopontin levels, such as atrial fibrillation, neoplastic, rheumatologic or inflammatory diseases, renal or hepatic failure, Alzheimer's disease, Parkinson, and osteoporosis. Post-stroke infection as a common complication was also excluded. One of the major limitations of the current study was to find a pure group of MCA infarction with the exclusion of the above-mentioned confounding factors who also had given their informed consent. Even though the defined exclusion criteria might have increased the reliability of the results, the small sample size could be justified.

In conclusion, the current study showed the speculative role of vitamin D in both evolution and severity of stroke. Protective effects of vitamin D might be due to its immune-modulatory activities. Larger studies might pave the way by investigating vitamin D supplementation benefits in preventing and treating stroke patients.

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Conflicts of Interest

The authors have no potential conflicts of interest to disclose.

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Tables

Table 1. Comparison between demographic, risk factors and laboratories in patients who developed acute ischemic stroke in middle cerebral artery territory and age and gender matched healthy subjects (CI= confidence interval).

Variables (Mean and 95% CI)	Case (No#46)	Control (No#46)	P Value
Age	64.22± (59.88-68.30)	66.72±(63.78-69.65)	0.33
Sex%	23 (50%)	23 (50%)	-
BMI	25.55± (24.52-27.11)	26.85± (24.39-29.86	0.34
Hypertension%	25 (54.3%)	15 (32.6%)	0.035
Hyperlipidemia%	13 (28.3%)	11 (24.4%)	0.68
Diabetes Mellitus%	14 (30.4%)	6 (13.0%)	0.043
Smoking%	13 (28.3%)	4 (8.7%)	0.016
Ischemic heart disease%	15 (32.6%)	3 (6.5%)	0.002
Received Tissue-Plasminogen activator (tPA)%	5 (10.9%)	0 (0%)	-

Serum Amyloid A (ng/mL)	808.39± (214-340.36)	233.07± (130.83-398.48)	<0.001
hsCRP (ng/mL)	7054.70± (6461.68-7871.40)	1833.62± (1194.45-4067.38)	<0.001
Osteopontin (ng/mL)	15.34± (10.90-23.76)	16.92± (13.34-45.70)	0.70
25-OH vitamin D (ng/mL)	22.40± (18.83-25.86)	32.07± (20.13-39.12)	0.002

Table-2 Univariate analysis of various risk factors with stroke evolution (OR= Odds Ratio and CI= 95% confidence interval).

Variables	OR	CI	P value
Hypertension	2.46	1.05-5.73	0.037
Diabetes Mellitus	2.91	1.007-8.44	0.048
Smoking	0.24	0.072-0.81	0.021
Ischemic heart disease	6.93	1.84-26.03	0.004
Serum Amyloid A	1.006	1.004-1.009	<0.001
hsCRP	1.001	1.001-1.002	<0.001
Osteopontin	0.99	0.97-1.001	0.70
25-OH vitamin D	0.94	0.91-0.98	0.005