The Dynamic Changes and Clinical Significance of Serum Neuroglobin Levels in Patients With Acute Ischemic Stroke

CHENG Min[1)], HAN Ya-Ting[1)], LI Na[2)], GUO Huai-Lian[1,3)*]

([1)]*Department of Neurology, People's Hospital, Peking University, Beijing* 100044, *China;*

[2)]*Central Laboratory, People's Hospital, Peking University, Beijing* 100044, *China;*

[3)]*Key Laboratory for Neuroscience, Ministry of Education, National Health and Family Planning Commission, Peking University, Beijing* 100191, *China)*

Abstract **Objective** Neuroglobin (Ngb) has been described as a neuroprotective agent in cerebral ischemia, which has a high affinity for oxygen and helps to prevent hypoxic-ischemic brain damage, and to affect the outcomes after acute ischemic stroke (AIS). In this study, the changes of serum Ngb level after AIS were investigated and the relationship of Ngb and stroke severity and prognosis were evaluated. **Methods** We prospectively measured the serum levels of Ngb in AIS patients at different time points (within 72 h and on day 14 (D14) after onset of cerebral infarction) and in control subjects. Serum Ngb levels were compared between the AIS patients and controls. The serum Ngb levels in the AIS patients with and without diabetes mellitus (DM) were also compared. Correlations between Ngb level and infarct size and that between Ngb level and National Institutes of Health Stroke Scale (NIHSS) score of the patients were analyzed. Receiver operating characteristic (ROC) curve was used to appraise their value in predicting the outcome at day 90 after AIS, which was evaluated using the modified Rankin Scale (mRS). **Results** Serum Ngb levels in patients were 105.7 (88.3, 123.1) μg/L within 72 h and 72.8 (58.7, 86.9) μg/L on D14 after cerebral infarction, respectively. Serum Ngb level in control group was 58.2 (35.0, 81.6) μg/L. Serum Ngb level within 72 h after AIS increased significantly compared with that in the control group ($P<0.05$). Serum Ngb levels of AIS patients with or without DM showed no significant difference within 72 h and on D14 after AIS ($P>0.05$). Serum Ngb level within 72 h was significantly correlated with NIHSS score (Spearman correlation coefficient=0.232, $P=0.038$). Serum Ngb levels had no significant difference in patients with large infarction than in those with small or moderate infarction at each time point ($P>0.05$). ROC curve analysis suggested that the serum Ngb level had a significantly good predictive power for outcomes. **Conclusion** The results indicated that serum Ngb level increased within 72 h after AIS, which is independent of comorbidity with diabetes. Serum Ngb level within 72 h was significantly correlated with NIHSS score, and Ngb might have the potential to be a predictor of stroke severity and prognosis.

Acute ischemic stroke (AIS) is a sudden neurologic deficit that results from impairment of blood supply to the brain, and is a major cause of death and disability in modern life. In recent years, the measurement of blood biomarkers has drawn more interest, but a precise early prognostic biomarker of AIS is still lacking. Therefore, more reliable biomarkers are needed to guide the clinical diagnosis of AIS. Neuroglobin (Ngb) is a member of the tissue globin family with a high affinity for oxygen, and is highly and specifically expressed in brain neurons[1].

Ngb is an oxygen-binding heme protein found in the brains of vertebrates, including humans. The involvement of Ngb in protection against ischemia has been studied *in vitro* and *in vivo*. Knockdown of Ngb expression exacerbated hypoxia and ischemic neuronal damage in rodents[1-2]. Some evidences have

clearly demonstrated that Ngb has neuroprotective effects on the cultured neurons and animal brains against hypoxic and ischemic injuries[2-5]. In Parkinson's disease, Ngb upregulation protected neurons from H_2O_2-induced neurotoxicity[4]. Ngb has been described as a neuroprotective agent in cerebral infarction, and it has a high affinity for oxygen and helps prevent hypoxic-ischemic brain damage, therefore it has the potential to affect outcomes after AIS. Human studies have suggested a relationship between Ngb polymorphisms and risk for cerebrovascular disease. Ischemic stroke increases expression of the neuroprotective protein neuroglobin, suggesting Ngb may represent a novel target for stroke therapy in experiment[6-7]. But the relationship between stroke infarct volume and Ngb level is still not fully understood. In addition, Ngb level also increased in patients with diabetic proliferative retinopathy[8-9], and a lot of AIS patients has vascular risk factors such as diabetes. However, there was no research exploring the difference of Ngb levels between AIS patients with and without diabetes. To investigate these questions, we examined the dynamic changes of serum Ngb after AIS and analyzed the relationship between Ngb levels and clinical outcomes of AIS.

1 Subjects and methods

1.1 Subjects

Between October 2017 and October 2020, a prospective cohort of patients with AIS was collected in the Department of Neurology at Peking University People's Hospital. Patients who suffered from acute cerebral infarction within 72 h for the first time were included. All patients were confirmed AIS through serial neurological examinations and by evaluating with cranial computed tomographic (CT) or magnetic resonance imaging (MRI) scan. To avoid interfering factors, patients who suffered lacunar infarction, cerebral hemorrhagic infarction, epilepsy, history of neurological diseases, myocardial infarction, renal and hepatic abnormalities, hematological system disorders, autoimmune disease, tumor, severe systemic infection, surgery or trauma within 3 months, or taking estrogen drugs were excluded.

Accordingly, demographic data of subjects, including age, gender, and clinical data, such as features and duration of symptoms, and location of infarcts were documented. Control group consisted of people who came to our hospital for regular physical checkups during the same period. All controls finished medical examinations and had negative imaging studies and no neurological disease, such as recent infection, stroke, tumors, vascular anomalies, abnormalities of blood system, transient ischemic attack, head trauma, or serious illness, which were matched with patient group by sex, age, hypertension, diabetes mellitus (DM), hyperlipidemia, and cardiac disease. This study had the approval from the hospital ethics committee and all subjects gave informed consent for participating in the study.

1.2 Measurement of serum Ngb

Blood samples from patients with AIS were obtained from the antecubital vein within 72 h and on day 14 (D14) after stroke, after an overnight fast. Blood samples were also obtained in the controls. Samples were centrifuged (2 000 r/min, 10 min) to segregate serum, and was frozen at −80° C until the later analysis. Ngb in serum was measured by enzyme-linked immunosorbent assay (ELISA) with commercially available kits (Novus Biologicals, USA), according to the manufacturer's instructions.

1.3 Clinical assessments

All patients were evaluated with head CT or MRI and the National Institutes of Health Stroke Scale (NIHSS) score as indicator of neurological deficit. All the imaging studies were reviewed by 2 staffs of the radiology department, who were blinded to clinical diagnosis. Based on the head CT and/or MRI, the cerebral infarct volume was measured and calculated using the formula $(A×B×C)/2$ (where A=maximal longitudinal diameter, B=maximal transverse diameter perpendicular to A and C=number of 10-mm slices containing infarct) [10]. Patients were classified as having a large infarction (LI, volume>10.0 cm^3), a small infarction (SI, volume<5.0 cm^3) or a moderate infarction (MI, volume between 5.0 and 10.0 cm^3). Outcome at day 90 after AIS was evaluated using the modified Rankin Scale (mRS): 0=no symptoms; 1=no significant disability; 2=slight disability; 3=moderate disability; 4=moderately severe disability; 5=severe disability; 6=dead[11]. For statistical comparison, favorable outcome was defined as a mRS score of 0−2, and unfavorable outcome was defined as a mRS score of 3−6. Surviving patients participated in follow-up interviews by telephone.

1.4 Statistical analysis

The statistical package SPSS 27.0 was used for all analyses. All values were expressed as mean± standard or medium (P25, P75). K-S test was used for Ngb levels comparisons between AIS patients at 2 selected time points and controls, and the same statistical analysis was performed to identify differences in Ngb levels and differences in infarction size. Differences in Ngb levels between the SI or MI and LI subgroups were assessed with K-S test. Relations between variables were assessed by Spearman correlation coefficient. All analyses were two tailed. Values were considered significant at $P <$ 0.05. A receiver operator characteristic (ROC) curve and the area under the ROC curve (AUC) were used to assess the accuracy of predictions of outcome from the serum Ngb levels.

2 Results

2.1 Characteristics of AIS patients and controls

Of the 40 patients (22 men and 18 women, 21 of them with DM) included in this study, their age ranged from 40 to 84 years (average age 69.7 years). Locations of infarct, as evidenced by radiologic and neurologic symptoms or signs, included the Internal carotid artery infarction (35 cases) and vertebrobasilar infarction (5 cases). 14 patients had LI, and 26 patients had SI or MI. The control group contained 10 men and 12 women. Their age averaged 59.3 years and ranged from 49 to 79 years. Clinical characteristics (sex ratio, age, smoking, complications with hypertension or hyperlipidemia) of the two groups are summarized in Table 1. Comparison of clinical characteristics between the two groups showed no significant difference ($P >$0.05).

Table 1 Characteristics of patients with AIS and controls

Characteristics	AIS (n=40)	Control (n=22)	P
Age, years	69.7±6.4	59.3±9.4	>0.05
Gender ratio, M/F	22/18	10/12	>0.05
With hypertension	23	8	>0.05
Smoking	17	8	>0.05
ALT/(U·L^{-1})	21.9±6.6	21.8±5.3	>0.05
LDL-C/(mmol·L^{-1})	2.9±0.8	2.7±0.6	>0.05
CRP/(mg·L^{-1})	5.8±2.6	5.4±1.4	>0.05

AIS, acute ischemic stroke; ALT, alanine transaminase; LDL-C, low density lipoprotein cholesterol; CRP, C-reactive protein.

2.2 Serial changes in serum Ngb levels in AIS patients

The mean serum Ngb level of the control group was 58.2 (35.0, 81.6) μg/L. Ngb levels in serum tended to increase after cerebral infarction. Ngb level increased significantly within 72 h [105.7 (88.3, 123.1) μg/L)] compared with control (P=0.004), and it decreased significantly on D14 [72.8 (58.7, 86.9) μg/L] compared with that within 72 h (P=0.029). The Ngb levels had no significant difference between control and D14 group (P=0.149) (Table 2). No significant differences were found in the subgroups with or without DM (Table 3).

Table 2 Serum Ngb levels in AIS patients at different time points and in control group

μg/L

Controls (n=22)	Patients	
	Within 72 h (n=40)	On D14 (n=40)
58.2 (35.0, 81.6)	105.7 (88.3, 123.1)*	72.8 (58.7, 86.9)#

*P=0.004 (within 72 h vs control); #P=0.029 (on D14 vs within 72 h); P=0.149 (on D14 vs control). Data are expressed as medium (P25, P75).

Table 3 Serum Ngb levels in AIS patients with or without DM

μg/L

Time	AIS with DM (n=21)	AIS without DM (n=19)	P
Within 72 h	116.4 (93.1, 140.2)	81.8 (50.7, 112.9)	0.115
On D14	90.7 (70.6, 110.8)	62.7 (39.1, 86.3)	0.225

P=0.115 (within 72 h, AIS without DM vs AIS with DM); P=0.225 (on D14, AIS without DM vs AIS with DM); Data are expressed as medium (P25, P75).

2.3 Serum Ngb levels and infarct size and NIHSS score

Based upon the calculated infarct volume, the patients were divided into SI or MI (n=26) and LI (n=14) groups. The serial changes of serum Ngb levels in these two groups were shown in the Table 4. The Ngb levels in patients have no significant difference with LI group and SI or MI groups. Significant correlations were found between serum Ngb level within 72 h and NIHSS score on admission (Spearman correlation coefficient=0.232, P=0.038).

2.4 Serum Ngb levels and outcome

Outcome at day 90 after AIS was favorable for

<div align="center">Table 4　Serum Ngb levels in subgroups of patients with LI and SI or MI at different time points</div>

µg/L

	SI or MI (*n*=26)	LI (*n*=14)	P
Serum Ngb level within 72 h	106.1 (83.8, 128.5)	104.8 (73.4, 136.2)	0.92
Serum Ngb level on D14	82.8 (63.1, 102.5)	62.3 (40.4, 94.2)	0.98

P=0.92 (within 72 h, SI+MI *vs* LI); *P*=0.98 (on D14, SI+MI *vs* LI); Data are expressed as medium (P25, P75). LI, large infarction (volume> 10.0 cm^3); SI, small infarction (volume<5.0 cm^3); MI, moderate infarction (volume between 5.0 and 10.0 cm^3).

25 patients and unfavorable for 15 patients. The serum Ngb level within 72 h was significantly higher in patients with an unfavorable outcome than that in patients with a favorable outcome. Using the ROC, we found that the serum Ngb level within 72 h had a significantly good predictive power for prognosis (Figure 1).
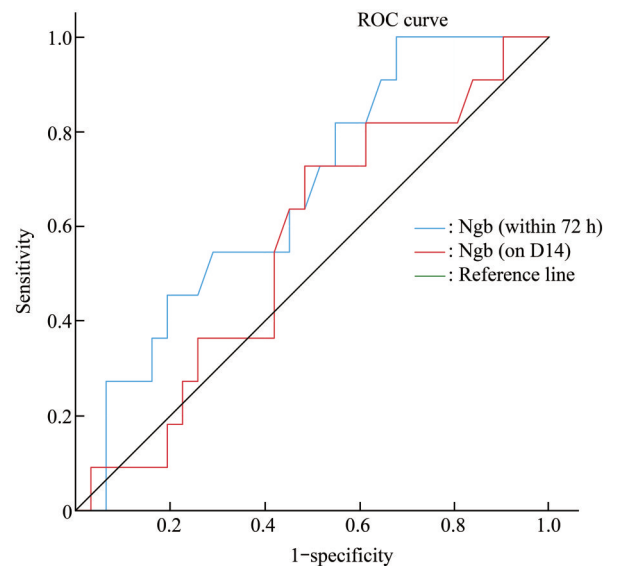

Fig. 1　ROC curve to predict the possibility of outcome at day 90 after acute ischemic stroke by the serum levels of Ngb

3　Discussion

Currently, a number of biomarkers have been used to indicate brain injury, such as S100B, glial fibrillary acidic protein (GFAP), and neuron-specific enolase (NSE) [12]. Although biomarkers in the cerebrospinal fluid (CSF) are more sensitive and specific to indicate brain damage than in blood, the CSF samples are difficult to obtain. In our study, we detected the dynamic changes of the serum levels of Ngb, which can be used as indicators for neuronal damage.

Previous studies have reported that Ngb was upregulated and protective in ischemic stroke *in vivo*[13]. Sun *et al*.[14] found that Ngb expression is increased after hypoxia and ischemia in cultured cortical neurons in rats, and that post-ischemic neuronal survival and animal functional neurological outcome are both improved by Ngb overexpression but worsened by inhibiting Ngb expression. Shang *et al*.[13] reported that the Ngb expression was significantly upregulated in the cerebral cortex after ischemia-reperfusion, and the level of Ngb in serum was significantly increased at 8 h and reached a peak at 48 h following ischemia. Zhao *et al*.[15] has shown that the level of Ngb in serum increased at 1−6 h after the onset of acute cerebral infarction patients, peaked at 24 h, descended to normal at 72 h. Xue *et al*.[12] reported Ngb level increased in serum after AIS and peaked at 72 h, and it was suggested as a predictor of poor prognosis. Jin *et al*.[6] reported that Ngb was detected in several brain areas, and its expression was increased in the cortical peri-infarct region after stroke, suggesting Ngb may represent a novel target for stroke therapy.

Although the level of Ngb in serum after ischemia in rats and in AIS patients has been reported previously, the results of previous studies on the changes of serum Ngb levels in patients with AIS were inconsistent, most of which were changes in the early onset, and did not analyze the results with or without DM. In this study, we analyzed the changes in serum Ngb levels following AIS. Additionally, we analyzed the Ngb levels with or without DM. We present an analysis of the relationship between serum Ngb levels and infarction volume and outcomes at day 90 in patients with AIS. Our results demonstrated that the level of Ngb was increased after AIS in the serum, and upregulation of Ngb significantly correlated to the NIHSS score, and Ngb levels can be used as a predictor for prognosis of AIS. However, our study only collected the serum in 2 time points. Without continuous collecting samples, we haven't

found the peak level of serum Ngb in patients with AIS.

Several findings showed that Ngb reduced tissue infarction after stroke, tissue protection by overexpressing Ngb could be sustained for up to 2 weeks, and the volume of cerebral infarcts in Ngb overexpressing transgenic mice was reduced by approximately 30% at 1 day and 14 days after cerebral ischemia[16-17]. In this study, we detected correlation between infarct volume and Ngb level, which is inconsistent with the study results in many experimental ischemic stroke models and in AIS patients. But there were also opposite results, Zindy et al. [18] detected that Ngb-deficiency resulted in reduced tissue infarction, suggesting that, at least at endogenous expression levels, neuroglobin in itself is non-protective against ischemic injury. As a whole, apart from few contestable discrepancies, Ngb overexpression is generally considered beneficial against experimental ischemia. The inconsistent maybe related with the Ngb was detected in the cortical periinfarct region after stroke[6]. Accordingly, Ngb might serve a similar neuroprotective role in humans. Although direct evidence is lacking, one study has shown that Ngb polymorphism had protective effects on large-artery atherosclerosis and small-vessel occlusion stroke in the Southern Chinese Han population[7]. In addition, in our study, a positive correlation was detected between NIHSS and Ngb levels in serum of AIS patients, which is consistent with those observed in ischemic stroke patients[12].

There are some limitations in our study. Firstly, it is unclear whether the Ngb level in serum reflects the Ngb level in central nervous system. Secondly, the number of patients is limited. If we increase sample size, accuracy and sensitivity of the study would be better.

4 Conclusion

Our current observations indicated that the serum Ngb level increased within 72 h, which is independent of comorbidity with diabetes. Ngb might play a role in cerebral infarction, and it might be involved as a predictor of stroke severity and prognosis.

References

[1] Simon R. Neuroglobin: neuroprotection and neurogenesis. Neurosci Lett, 2013, **549**: 1-2

[2] Sun Y, Jin K, Mao X O, et al. Neuroglobin is up-regulated by and protects neurons from hypoxic-ischemic injury. Proc Natl Acad Sci USA, 2001, **98**(26): 15306-15311

[3] Ascenzi P, Di Masi A, Leboffe L, et al. Neuroglobin: from structure to function in health and disease. Mol Aspects Med, 2016, **52**: 1-48

[4] Zhang H, Li Y, Xun Y, et al. Polydatin protects neuronal cells from hydrogen peroxide damage by activating CREB/Ngb signaling. Mol Med Rep, 2022, **25**(1): 9

[5] Yu Z Y, Fan X, Eng H L, et al. Neuroprotective roles and mechanisms of neuroglobin. Neurol Res, 2009, **31**(2): 122-127

[6] Jin K, Mao Y, Mao X, et al. Neuroglobin expression in ischemic stroke. Stroke, 2010, **41**(3): 557-559

[7] Lin Y, Fang L, Xue X H, et al. Association between Ngb polymorphisms and ischemic stroke in the Southern Chinese Han population. BMC Med Genet, 2008, **9**: 110

[8] 李康, 高晓虹, 栗改云, 等. 脑红蛋白在增殖性糖尿病视网膜病变患者眼部及血清中表达的研究. 眼科, 2017, **26**(5): 323-327
Li K, Gao X H, Li G Y, et al. Ophthalmology in China, 2017, **26**(5): 323-327

[9] 刘琼, 于健, 陈林江. 脑红蛋白在糖尿病大鼠视网膜病变发展中的表达变化. 中国实用医药, 2019, **14**(13): 192-194
Liu Q, Yu J, Chen L J. China Practical Medicine, 2019, **14**(13): 192-194

[10] Sims J R, Rezai Gharai L, Schaefer P W, et al. ABC/2 for rapid clinical estimate of infarct, perfusion, and mismatch volumes. Neurology, 2009, **72**(24): 2104-2110

[11] Van Swieten J C, Koudstaal P J, Visser M C, et al. Interobserver agreement for the assessment of handicap in stroke patients. Stroke, 1988, **19**(5): 604-607

[12] Xue L, Chen H, Lu K, et al. Clinical significance of changes in serum neuroglobin and HIF-1alpha concentrations during the early-phase of acute ischemic stroke. J Neurol Sci, 2017, **375**: 52-57

[13] Shang A, Zhou D, Wang L, et al. Increased neuroglobin levels in the cerebral cortex and serum after ischemia-reperfusion insults. Brain Res, 2006, **1078**(1): 219-226

[14] Sun Y, Jin K, Peel A, et al. Neuroglobin protects the brain from experimental stroke in vivo. Proc Natl Acad Sci USA, 2003, **100**(6): 3497-3500

[15] Zhao S C, Chu Z H, Ma L S, et al. Significance of neuroglobin in serum of acute atherosclerotic cerebral infarction patients. Neur Regen Res, 2011, **6**(27): 2140-2145

[16] Wang X, Liu J, Zhu H, et al. Effects of neuroglobin overexpression on acute brain injury and long-term outcomes after focal cerebral ischemia. Stroke, 2008, **39**(6): 1869-1874

[17] Khan A A, Wang Y, Sun Y, et al. Neuroglobin-overexpressing transgenic mice are resistant to cerebral and myocardial ischemia. Proc Natl Acad Sci USA, 2006, **103**(47): 17944-17948

[18] Zindy R, Christian A H, Jesper K, et al. Reduced infarct size in neuroglobin-null mice after experimental stroke in vivo. Exp Transl Stroke Med, 2012, **4**(1): 15

急性缺血性卒中患者血清脑红蛋白的动态变化及临床意义

程　敏[1)]　韩雅婷[1)]　李　娜[2)]　郭淮莲[1,3)*]

（[1)] 北京大学人民医院神经内科，北京 100044；

[2)] 北京大学人民医院中心实验室，北京 100044；[3)] 北京大学教育部及国家卫生健康委员会神经科学重点实验室，北京 100191）

摘要　目的　脑红蛋白（neuroglobin，Ngb）又称神经球蛋白，是一种携氧球蛋白。有研究证明Ngb在脑缺血缺氧疾病中具有神经保护作用，对氧具有高亲和力，有助于预防缺氧缺血性脑损伤，并影响急性缺血性卒中（acute ischemic stroke，AIS）的预后。本研究观察了AIS后血清Ngb水平的变化，并评估了Ngb与卒中严重程度及预后的关系。**方法**　前瞻性地募集AIS患者和健康对照者进行研究。在AIS患者起病后不同时间点（脑梗死发病后72 h内和第14天）及对照组中分别检测血清Ngb水平，并比较AIS患者与对照组之间的血清Ngb水平。对合并与不合并糖尿病的AIS患者的血清Ngb水平进行比较。分析患者血清Ngb水平与梗死体积及国家卫生研究院卒中量表（NIHSS）评分之间的相关性。应用受试者操作特征（ROC）曲线评估Ngb对AIS预后的预测价值。**结果**　AIS患者的血清Ngb水平在脑梗死后72 h内和第14天时分别为105.7（88.3，123.1）µg/L和72.8（58.7，86.9）µg/L。对照组血清Ngb水平为58.2（35.0，81.6）µg/L。AIS发病后72 h内血清Ngb水平较对照组显著升高（$P<0.05$）。合并与不合并糖尿病的AIS患者血清Ngb水平在发病后72 h内及第14天均无显著差异（$P>0.05$）。AIS发病72 h内的血清Ngb水平与NIHSS评分显著相关（Spearman相关系数=0.232，$P=0.038$）。在各时间点，大体积脑梗死组患者血清Ngb水平与中小体积脑梗死组相比无显著性差异（$P>0.05$）。ROC曲线分析表明，血清Ngb水平对AIS患者的预后具有良好的预测能力。**结论**　AIS发病后72 h内血清Ngb水平升高。合并与不合并糖尿病的AIS患者血清Ngb水平无显著差异。发病后72 h内的血清Ngb水平与NIHSS评分显著相关。Ngb可能作为卒中严重程度和预后的预测因子。