Neurochemistry International 128 (2019) 14–20

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Increased REDD1 facilitates neuronal damage after subarachnoid hemorrhage

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A B S T R A C T

Regulated in development and DNA damage responses 1 (REDD1) is a highly conserved stress-response protein and can be induced by hypoxia/ischemia and DNA damage. However, it is not known whether REDD1 involves in neuronal damage caused by subarachnoid hemorrhage (SAH) that is known as one of the most important causes of disability and death worldwide. Here, we first found that SAH markedly induced the increase of REDD1 (35.467 ng/ml) in cerebrospinal fluid (CSF) of patients at acute stage (within 24 h from bleeding) compared to that of control (0.644 ng/ml). And, REDD1 level was positively correlated with severity of brain injuries (Hunt-Hess grade of SAH), but it showed an obvious decline at recovery stage 6.201 ng/ml (before discharge from hospital) because of good recovery. Moreover, it was found that the expression of REDD1 was significantly induced by hemolysate in a dose-dependent way in neurons. Knockdown of REDD1 by lentivirus encoded REDD1-shRNA could inhibit the neuronal apoptosis and LDH leakage caused by hemolysate. Importantly, the level of REDD1 in peripheral blood of SAH patients was significantly higher (4.364 ng/ml) than that of healthy persons (1.317 ng/ml) and also was positively correlated with that in CSF. Taken together, our findings provide the novel and direct evidence that REDD1 could play a critical role of process of neuronal damage caused by SAH, suggesting a new molecular target to protect brain function from SAH injury.

1. Introduction

Subarachnoid hemorrhage (SAH) is a life-threatening type of stroke caused by a rupture of aneurysm, arteriovenous malformation or traumatic brain injury, has high rates of morbidity and mortality in which one-third patients will survive with good recovery, one-third will survive with a disability and one-third will die (Connolly et al., 2012; Foreman, 2016). A common and serious complication of SAH is cerebral vasospasm which has been proposed to be the primary treatment target for SAH. Prolonged or pronounced vasoconstriction of major cerebral blood vessels can lead to delayed cerebral ischemia (Francoeur and Mayer, 2016; McBride et al., 2017). In the past decades, many studies focused on finding the effective strategies to facilitate the neurological recovery after SAH injury (Cahill et al., 2006; Long et al., 2017; Nishikawa and Suzuki, 2018; Ostrowski et al., 2006; Shah et al., 2018), however, they are far from satisfactory for SAH treatment. Therefore, it is very urgent and important to improve the survival rate and reduce the mortality rate of SAH. Exploring the underlying mechanisms involved in SAH injury is feasible and valid strategy, it will help to seek the novel and effective molecular target for SAH treatment in the future.

REDD1 (Regulated in development and DNA damage responses 1), also known as RTP801 or DNA-damage-inducible transcript 4 (DDIT4), was identified by two independent groups based on its marked response to hypoxia/ischemia and DNA damage in 2002 (Ellisen et al., 2002; Shoshani et al., 2002). The increased REDD1 may promote differential PC12 cell and neuron death in several conditions of stimulation, on the contrary, the cellular survival rate increased when the expression of REDD1 was interfered (Ellisen et al., 2002; Liu et al., 2014; Noseda et al., 2013; Regazzetti et al., 2010; Schwarzer et al., 2005; Shoshani et al., 2002; Yoshida et al., 2010). In addition, REDD1 also can induce the cellular apoptosis in models of Parkinson's disease and other neurodegenerative disorders through suppressing mammalian target of rapamycin (mTOR) signaling (Canal et al., 2014; Malagelada et al., 2006, 2008; Martin-Flores et al., 2016; Ota et al., 2014). The opposite report is that REDD1 is essential for optimal T cell proliferation and survival depended on cell context (Reuschel et al., 2015). SAH is sudden bleeding into the subarachnoid space because of rupture of aneurysm, which is commonly followed by secondary focal brain ischemia (Francoeur and Mayer, 2016; McBride et al., 2017). Our previous studies demonstrated that the expression of REDD1 increased progressively with prolongation of ischemic duration and the levels of REDD1 mRNA and protein were positively correlated with the degrees of neuronal injury. Neuronal damage could be significantly blocked by

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https://doi.org/10.1016/j.neuint.2019.03.019
Received 28 November 2018; Received in revised form 12 February 2019; Accepted 26 March 2019
Available online 29 March 2019
0197-0186/

inhibiting REDD1 expression in ischemia (Wu et al., 2011, 2015). Based on above opinions, we speculate that there may exist a relationship between REDD1 and SAH injury, but so far, it is unknown whether REDD1 participates in process of SAH injury. Here, we observe for the first time the level of REDD1 in cerebrospinal fluid (CSF) of SAH patients at acute and recovery stage, reveal the role of REDD1 mediating neuronal apoptosis in hemolysate treatment, determining whether REDD1 is one important molecule involved in SAH injury.

2. Materials and methods

2.1. Cerebrospinal fluid of patients and ethics statement

The samples of CSF were collected from SAH patients who were diagnosed by a neurologist, consisted of 33 SAH patients including 15 men (45.5%) and 18 women (54.5%), within 24 h from the bleeding or before discharge from hospital because of their recovery, during January 2017–September 2017. Their age ranged from 27 to 81 years, with a median of 58.1 years. Control CSF samples were obtained from other conditions (except hemorrhage, ischemia and neurodegenerative disease) with normal biochemical properties in levels of glucose, chloride and protein before patients left from hospital because of their recovery. 27 normal CSF samples were analyzed in this study, including 13 men (48.1%) and 14 women (51.9%), during January 2017–September 2017. Their age ranged from 33 to 74 years, with a median of 51.9 years. The patient characteristics identified as SAH and control have been shown in Table 1. The supernatant of CSF was collected by centrifuging and stored at −80 °C until use. All procedures performed in studies involving human participants conforms to the World Medical Association Declaration of Helsinki. Informed consents were obtained from all SAH and control patients before sample collection. The study protocol (2017-Y030) was approved by the Clinical Research Ethics Committee, Affiliated Hospital of Nantong University.

Table 1
Clinic characteristics and REDD1 levels in CSF of Control and SAH patients.

Variables	Control		SAH	
	Number	REDD1	Number	REDD1
Number of patients	27		33	
Age				
Median [range]	51.9 (33–74)		58.1 (32–81)	
Sex				
Male	13		15	
Female	14		18	
Average value		0.644 ± 0.122		35.467 ± 2.717
Hunt-Hess grade			33	
I			7	22.241 ± 6.870
II			13	35.181 ± 8.007
III			6	51.093 ± 6.567
IV			3	61.751 ± 8.329
Unknown			4	16.394 ± 7.575
Modified Rankin Scale			20	
0 no symptoms			12	5.218 ± 3.024
1 no significant disability			2	5.406 ± 0.477
2 slight disability			0	
3 moderate disability			1	12.039
4 moderate to severe disability			0	
5 severe disability			2	11.385 ± 4.062
Unknown			3	5.269 ± 7.459

2.2. Enzyme-linked immunosorbent assay (ELISA)

REDD1 in CSF of SAH and control patients was measured by ELISA. The content of REDD1 in CSF was analyzed using commercial Quantikine Human Ddit4 immunoassay kits (CUSABIO, China) and calculated as instruction offered by manufacturer. Results were expressed in nanogram per milliliter.

2.3. Primary cortical neurons culture

The primary cortical neurons was prepared from C57BL/6 newborn mice as a method described previously (Wu et al., 2009, 2011). In brief, the cortex was obtained aseptically and incubated in 0.125% trypsin at 37 °C for 15 min. After triturating, the dissociated cortical neurons were suspended in complete DMEM (Dulbecco's Modified Eagle's Medium; Invitrogen-Life Technologies) containing 4.5 g/L glucose and 10% fetal bovine serum (FBS, Invitrogen-Life Technologies) and plated in poly-D-lysine-coated 96, 24-well plates or 3-cm dishes (Corning). Cultures were kept in an incubator with 5% CO_2 + 95% air at 37 °C (NAPCO 5400). The medium was replaced by Neurobasal medium with supplemental B27 (Invitrogen-Life Technologies) 1 h after cells were seeded. All cultures were fed with fresh Neurobasal medium with supplemental B27 every 2 or 3 days. After 6 days in culture, the purity of the neurons was assessed by staining with neuron-specific antibody against microtubule associated protein 2, over 98% of cells were positively stained. The cultures were therefore used for experiments after 7 days *in vitro*. The C57BL/6 mice used in this study were provided by the Animal House of Nantong University (Nantong, JS, China). The Animal Ethics Committee of Nantong University approved the use of animals for this study.

2.4. Preparation of hemolysate

Treating primary cortical neurons with hemolysate is a commonly accepted model to simulate the clinical SAH condition. Hemolysate was prepared from three adult C57BL/6 mice arterial blood as reported previously and recently with minor modification (Li et al., 2017; Matz et al., 2000). Briefly, mice were anesthetized with an intraperitoneal injection of 10% chloral hydrate (300 mg/kg) prior to blood collection. The blood was withdrawn from left ventricle of mice and lysed by three cycles of freezing in dry ice for 10 min and then rapidly thawing in a water bath at 37 °C. Hemolysate was stored at −80 °C until use.

2.5. Assessment of cell viability

Cell viability was assessed using a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay or lactate dehydrogenase leakage as described previously (Malagelada et al., 2006; Wu et al., 2009, 2011). In MTT assay, the yellow monotetrazolium bromide (Sigma Chemical Co., St. Louis, MO, USA) was reduced to a purple formazan by mitochondrial succinate dehydrogenase in live cells. In briefly, the cells were incubated with 100 μl 1% MTT for 4 h at 37 °C, the reaction was stopped by adding 100 μl cell lysis buffer (20% sodium dodecyl sulfate in 50% N′N-dimethylformamide) to each well, and the plate was further incubated at 37 °C incubator for 20 h. Finally, the absorbance was measured at the 570 nm wavelength using a microplate assay reader (Elx800, Bio-tek, USA). The results were expressed as a percentage of absorbance measured in control cells after substracting blank value from all wells. LDH is an intracellular enzyme that leaks into the culture medium when cell membranes are damaged. To eliminate the effect of hemolysate on LDH measure, neurons were treated with hemolysate for 12 h and then recovered with fresh normal medium for 12 h. LDH activity in later medium was determined by a commercial LDH kit according to manufacturer's instructions (Nanjiang Jiancheng Bioengineering Institute). The LDH activity in medium was calculated and converted to the percentage of control level.

2.6. Quantitative polymerase chain reaction (qPCR) assay

Total RNA was isolated from neurons using Trizol Reagent (Invitrogen). Reverse transcription of extracted mRNA was performed by the First Strand cDNA synthesis kit (Invitrogen). The following sequence-specific primers were used in real-time RT-PCR. For RTP801: forward, 5′-TGGACAGCAGCAACAGTGGCTTC-3'; reverse, 5′-TCATCCT CGGGGTCACTGAGCAG-3' (Genebank, NM_029083). For β-actin, forward, 5′-CACCCACACTGTGCCCATCTACG-3'; reverse, 5′-GCCACGCTC GGTCAGGATCTTC-3' (Genebank, X03765). The housekeeping gene β-actin was used as an internal standard for RNA preparation and reverse transcriptase reaction. The qPCR reaction was performed in a 20 μl of system contained 1 × Power SYBR Green Master Mix (Applied Biosystems), 250 nM forward and reverse primers and 2 μl cDNA. The cycling conditions were 95 °C for 10 min, followed by 40 cycles at 94 °C (15 s), 56 °C (30 s), and 72 °C (25 s). All reactions were performed in duplicate. The qPCR data were analyzed using $2^{-\Delta\Delta CT}$ methodology.

2.7. Western blot analysis

The cells were lyzed in ice-cold Radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitor cocktail as previous reported (Su et al., 2018). Aliquots of the cell extract containing 20 μg of protein were separated on a 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline with 0.1% Tween 20 buffer (TBST) for 1 h and incubated in primary anti-RTP801 antibody (1:1000, ProteinTech) overnight at 4 °C. After three washes with TBST, the membrane was incubated with goat anti-rabbit IRDye 800 CW IgG (1:10,000) for 1 h at room temperature. The intensity of the specific bands was detected and analyzed by Odyssey infrared imaging system at a resolution of 169 μm (Li-Cor Biosciences, USA).

2.8. Lentivirus infection

The recombinant lentivirus that encoded the core shRNA sequence for down-regulation of REDD1 included 5′-AACCTGATGCAGCTGCTG CAG-3' (REDD1-shRNA) and a scrambled sequence 5′-TTCTCCGAACG TGTCACGT-3′ which had no significant homology to any known mouse genes designed as negative control (Scramble) fused to green fluorescent protein (GFP) were constructed from GenePharma Co., Ltd. (Shanghai, China). Neurons were infected with the recombinant lentivirus according to instruction of manufacturer, chosen the optimal efficiency of infection that was at a multiplicity of infection of 10, almost obtaining 100% of green fluorescent protein-positive cells after 72 h of infection. The efficient knockdown of REDD1 in neurons was confirmed by testing the levels of protein.

2.9. Hoechst 33342 staining

Neuronal apoptosis was tested by Hoechst 33342 staining as described elsewhere (Gabai et al., 2002). Briefly, after treatment with hemolysate, the neurons grown on coverslips were washed by phosphate buffered solution, fixed with 4% paraformaldehyde for 15 min, and stained with Hoechst 33342 (5 μmol/L) for 1 h at 4 °C. By mounting with glycerol, the nuclei of neurons were observed under a fluorescent microscope equipped with a 350 nm excitation laser (Leica DM4000B, Germany). Rounded cells with condensed or fragmented nuclei were considered apoptosis. The number of apoptotic nuclei was counted, and the percentage of these nuclei was calculated in relation to total nuclei viewed in same field. There were at least six data to be collected from different coverslips in three individual experiments.

2.10. Statistical analysis

All data were presented as mean ± SD. Before defining the statistical tests, data were evaluated by the Kolmogorov-Smirnov normality test to address whether or not the data followed a Gaussian distribution. Statistical significance was evaluated using the two-tail unpaired or paired Student's t-test when data was normally distributed. The differences between the means of cell viability and REDD1 expression were determined by One-Way ANOVA followed by a Newman-Keuls post-hoc test for multiple comparisons using GraphPad Prism 6.0 software. Analysis of neuronal apoptosis was performed using two-way ANOVA in appropriate experiments followed by Newman-Keuls post-hoc test. Correlation was performed using linear regression analysis. The results were considered to be statistically significant if $p < 0.05$.

3. Results

3.1. Subarachnoid hemorrhage causes a significant increase of REDD1 in cerebrospinal fluid of patients

First, we investigated whether SAH could cause the increase of REDD1 in CSF of patients by ELISA. The patient characteristics identified as SAH and control have been shown in Table 1. Compared with 27 control persons (0.644 ± 0.122 ng/ml), SAH markedly induced the increase of REDD1 in CSF of all 33 patients (35.467 ± 2.717 ng/ml) at acute stage (within 24 h from the bleeding, $p < 0.001$, Fig. 1A). Associations between REDD1 content and clinical features of SAH were examined. The average of REDD1 values raised progressively with the increase of Hunt-Hess grade which was widely used for categorizing severity of SAH. REDD1 levels positively correlated with Hunt-Hess grade of SAH at acute stage (Table 1). There were significant differences among four groups when performing multiple comparisons except a pair of Hunt-Hess grade III and IV (Fig. 1B). Moreover, 20 cases of above 33 SAH patients were tracked to observe the changes of REDD1 content in their CSF before discharging from hospital (Hunt-Hess grade to 0 or I). Very interestingly, it was found that the level of REDD1 was dramatically decreased from 34.816 ng/ml at acute stage (admission to hospital) to 6.201 ng/ml at recovery stage (discharge from hospital) in CSF of these 20 patients (Fig. 1C). Except for small minority patients, most of patients got good recovery with level 0 of modified Rankin Scale (mRS) that was used to measure disability or dependence in activities of daily living in victims and a prognosis indicator of SAH at discharge from hospital. The level of REDD1 at level 0 of mRS was only 5.218 ng/ml in CSF as shown in Table 1. To exclude the possibility that hemolytic product itself caused a false positive, the hemolysate of peripheral erythrocytes from SAH patients was used as the separate sample to be tested by ELISA simultaneously, in which the data were similar to that of negative control and hard to be detected out. Taken together, our results demonstrate that SAH can remarkably induce the rise of REDD1 in human CSF and REDD1 level is positively correlated with degree of brain injury, implying that REDD1 is closely related to cerebral damage caused by SAH.

3.2. Hemolysate decreases the viability of cultured neurons

In this study, we used an *in vitro* model to mimic the clinical scenario caused by SAH as previously reported (Li et al., 2017; Matz et al., 2000) with minor modification. Primary cultured cortical neurons were treated with hemolysate at a dilution of 1:100 or 1:50 (the ratio of hemolysate in medium) for 12 h. After treatment, the cell viability was detected by MTT assay. As shown in Fig. 2, the cell viability was slightly reduced to 89% of control group at the 1:100 dilution of hemolysate and significantly declined to 67% at the 1:50 dilution, implying that hemolysate could obviously decrease the viability of primary cultured neurons. This observation was consistent with the results obtained from LDH assay, in which the LDH leakage increased to 1.5 and 3.4 fold of control at 1:100 or 1:50 of dilution respectively. These results suggest that in our experimental system the hemolysate mimicked SAH could cause neuronal damage.

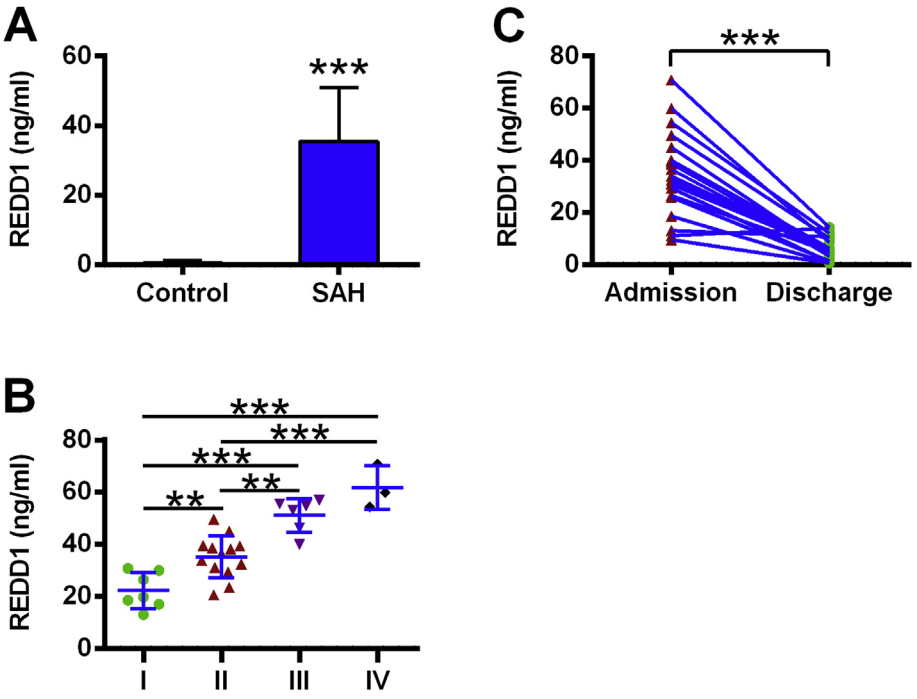

Fig. 1. Subarachnoid hemorrhage causes a significant increase of REDD1 in cerebrospinal fluid. REDD1 in CSF of SAH patients and control persons was detected by ELISA. Data were calculated to nanogram REDD1 per milliliter CSF. (A) The level of REDD1 in CSF of patients after SAH. Statistical significance was assessed by two-tail unpaired Student's t-test, $t(58) = 11.57$, ***$p < 0.001$ versus Control ($n = 27$ in Control, $n = 33$ in SAH). (B) The REDD1 level at different Hunt-Hess grade. Statistical significance was assessed by One-Way ANOVA followed by a Newman-Keuls post-hoc test for multiple comparisons, $F(3, 25) = 27.02$, **$p < 0.01$, ***$p < 0.001$. (C) Compare the REDD1 levels in CSF of SAH patients between acute stage (Admission) and recovery stage (Discharge). Statistical significance was assessed by two-tail paired Student's t-test, $t(19) = 8.657$, ***$p < 0.001$ versus Admission ($n = 20$).

3.3. REDD1 expression is significantly increased in cultured neurons with hemolysate treatment

To study whether REDD1 is involved in SAH-induced neuronal damage, we observed the expression of REDD1 mRNA by RT-PCR and the level of REDD1 protein by western blot as well as ELISA in neurons after hemolysate treatment. It was found that hemolysate significantly enhanced the expression of REDD1 mRNA which amounted to 1.7-fold (1:100 dilution) and 2.7-fold (1:50 dilution) of the control in neurons (Fig. 3A). Likewise, the expression of REDD1 protein also was obviously increased after hemolysate treatment compared to that of control by western blot (Fig. 3B and C). In order to keep in line with above experiments, we also used ELISA method to measure the level of REDD1 in neurons besides western blot. ELISA results showed that REDD1 markedly rose to 41.2 ng/mg protein (1:100 dilution) and 61.4 ng/mg protein (1:50 dilution) after hemolysate treatment, which were significantly higher than that of control (22.7 ng/mg protein, Fig. 3D). These results demonstrated that hemolysate could promote the high-level expression of REDD1 in primary cortical neurons, revealing that REDD1 may be involved in neuronal damage caused by SAH.

3.4. Knockdown of REDD1 inhibits neuronal damage induced by hemolysate

To further explore whether REDD1 involved in SAH-induced damage on neurons, primary cultured neurons were infected by lentivirus encoded shRNA targeting to REDD1 (REDD1-shRNA) or non-targeting shRNA (Scramble) for 72 h before being treated with hemolysate. Knockdown of REDD1 induced by REDD1-shRNA lentivirus was verified by protein analysis (Fig. 4A). By Hoechst 33342 staining, it was found that hemolysate markedly induced the apoptotic occurrence as reflected by more bright and concentrated nuclei to 30.9% in neurons pre-infected Scramble lentivirus (Fig. 4B). However, the percentage of apoptotic cells had only 6.8% in the neurons pre-infected with REDD1-shRNA lentivirus in hemolysate treatment (Fig. 4B and C). These results demonstrated that knockdown of REDD1 could significantly inhibit the neuronal apoptosis caused by hemolysate. This observation was also consistent with the result obtained from the LDH assay that LDH leakage from neurons pre-infected with REDD1-shRNA lentivirus was 1.4 fold of the control, which was significantly less than that of neurons pre-infected with Scramble lentivirus (that reached to 3.3 fold of the control). These findings suggest that REDD1 may play a critical role in

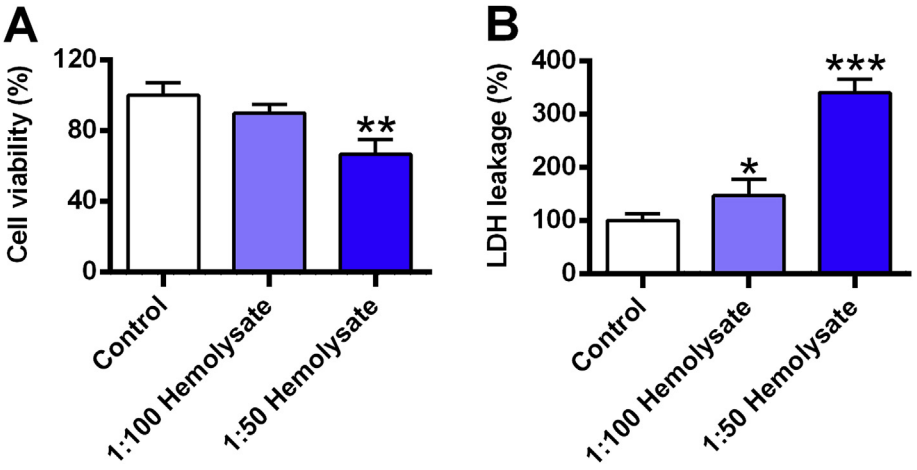

Fig. 2. Effect of hemolysate on cell viability of primary cortical neurons. Primary cultured neurons were treated with hemolysate at a dilution of 1:100 or 1:50 (the ratio of hemolysate in medium) for 12 h. Neuronal viability was analyzed by MTT assay after hemolysate treatment and by LDH assay after hemolysate treatment followed by recovery for 12 h. Statistical significance was assessed by One-Way ANOVA followed by a Newman-Keuls post-hoc test. Data were presented as percentage of the control and shown as Mean ± SD ($n = 6$). (A) The viability of neurons by MTT assay. $F(2, 15) = 36.84$, **$p < 0.01$ versus Control. (B) LDH leakage of neurons. $F(2, 15) = 167.6$, *$p < 0.05$, ***$p < 0.001$ versus Control.

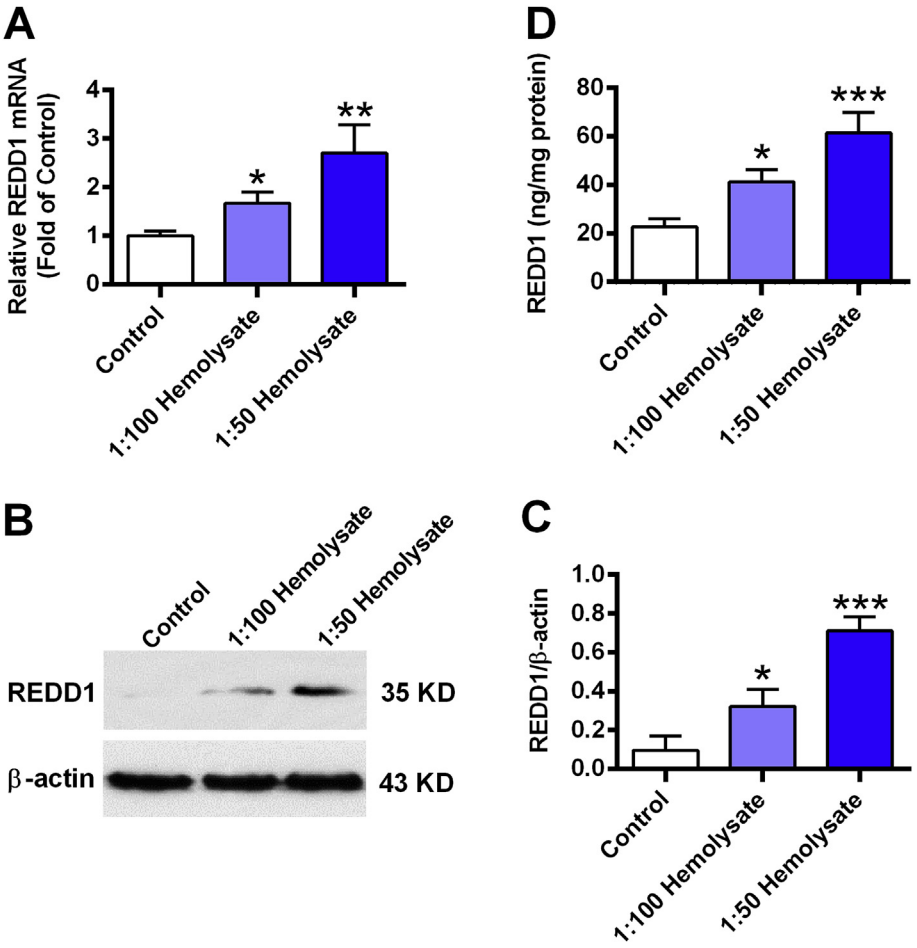

Fig. 3. Effect of hemolysate on REDD1 expression in primary cortical neurons. Primary cortical neurons were treated with hemolysate at a dilution of 1:100 or 1:50 (the ratio of hemolysate in medium) for 12 h. The expression of REDD1 mRNA was measured by RT-PCR and the level of protein was tested by western blot as well as ELISA. Statistical significance was assessed by One-Way ANOVA followed by a Newman-Keuls post-hoc test. (A) Expression of REDD1 mRNA by RT-PCR (n = 3). Data were normalized to the control. $F(2, 6) = 16.78$, $^{*}p < 0.05$, $^{**}p < 0.01$ versus Control. (B) Representative western blot of REDD1 in neurons. (C) Quantitative analysis of western blot for REDD1 (n = 3). $F(2, 6) = 47.2$, $^{*}p < 0.05$, $^{***}p < 0.001$ versus Control. (D) The level of REDD1 protein by ELISA. Data were calculated to nanogram REDD1 per milligram total protein and shown as Mean ± SD (n = 3). $F(2, 6) = 31.89$, $^{*}p < 0.05$, $^{***}p < 0.001$ versus Control.

the process of neuronal damage caused by hemolysate.

3.5. Subarachnoid hemorrhage correspondingly leads to the rise of REDD1 in peripheral blood of patients

Finally, the level of REDD1 in peripheral blood of SAH patients also was measured by ELISA. We found that REDD1 in peripheral serum of SAH patient was about 4.364 ng/ml, however, it was only 1.317 ng/ml in serum of healthy person (Fig. 5A), which demonstrated that SAH not only induced a significant increase of REDD1 in CSF but also in peripheral blood. The correlation between the level of REDD1 in CSF and the level of REDD1 in serum was observed by plotting the values for these two indicators against one another from 33 patients. Compared the level of REDD1 in CSF with that in serum, $r2 = 0.740$ and $p < 0.0001$ (Fig. 5B), which suggested that there was a positive correlation in level of REDD1 between CSF and peripheral blood. We plotted a receiver operating characteristics (ROC) curves by applying the value of REDD1 in serum from all subjects including 32 healthy persons and 33 SAH patients. Serum REDD1 effectively distinguished SAH from normal cases, which was reflected by the area under ROC curve (AUC) of 0.898 (95% confidence interval: 0.814–0.983 and $p < 0.001$). Using a threshold of 3.267 ng/ml for REDD1, Youden index was 0.726, the sensitivity and specificity were, respectively, 0.758 and 0.969 in SAH group. Based on above data, it is conceivable that REDD1 could be a potential biomarker for diagnosis of SAH because its high level in CSF could be reflected into peripheral blood.

4. Discussion

Our study presents for the first time that SAH can markedly induce the increase of REDD1 in CSF of patients, REDD1 level is positively correlated with degree of brain injury at acute stage, but it shows an obvious decline at recovery stage. The expression of REDD1 can be significantly induced in neurons by hemolysate in a dose-dependent way. Knockdown of REDD1 by lentivirus encoded REDD1-shRNA could inhibit the neuronal apoptosis and LDH leakage caused by hemolysate. These findings support the hypothesis that SAH-induced neuronal damage is mainly mediated by increasing REDD1 expression.

One important novel finding of the present study is that REDD1 can be markedly induced by hemolytic product both *in vitro* and *in vivo* conditions. REDD1, is a highly conserved stress-related protein, its expression can be induced by several environmental stresses such as hypoxia/ischemia and DNA damage (Ellisen et al., 2002; Noseda et al., 2013; Regazzetti et al., 2010; Schwarzer et al., 2005; Shoshani et al., 2002; Yoshida et al., 2010). REDD1 transcript is the most abundantly expressed in all 1200 significantly regulated transcripts detected after PC12 cells were treated with neurotoxin 6-hydroxydopamine (Malagelada et al., 2006). REDD1 can attenuate cardiac hypertrophy via enhancing autophagy (Liu et al., 2014). It also obviously increases in lungs of individuals with advanced emphysema and in serious adenocarcinoma of ovary that is positively correlated with late-stage disease (Jia et al., 2014; Yoshida et al., 2010). Our previous study reported that the expression of REDD1 gradually increases with prolongation of ischemic duration in primary cultured neurons (Wu et al., 2011, 2015). However, very little is known about whether REDD1 involves in SAH-induced brain injury. Our data clearly demonstrated that the level of REDD1 markedly increases in CSF after SAH, REDD1 levels were positively correlated with Hunt-Hess grades, whereas hardly no existence in normal CSF. Similar results were obtained from the *in vitro* experiments that the expression of REDD1 mRNA and protein was significantly

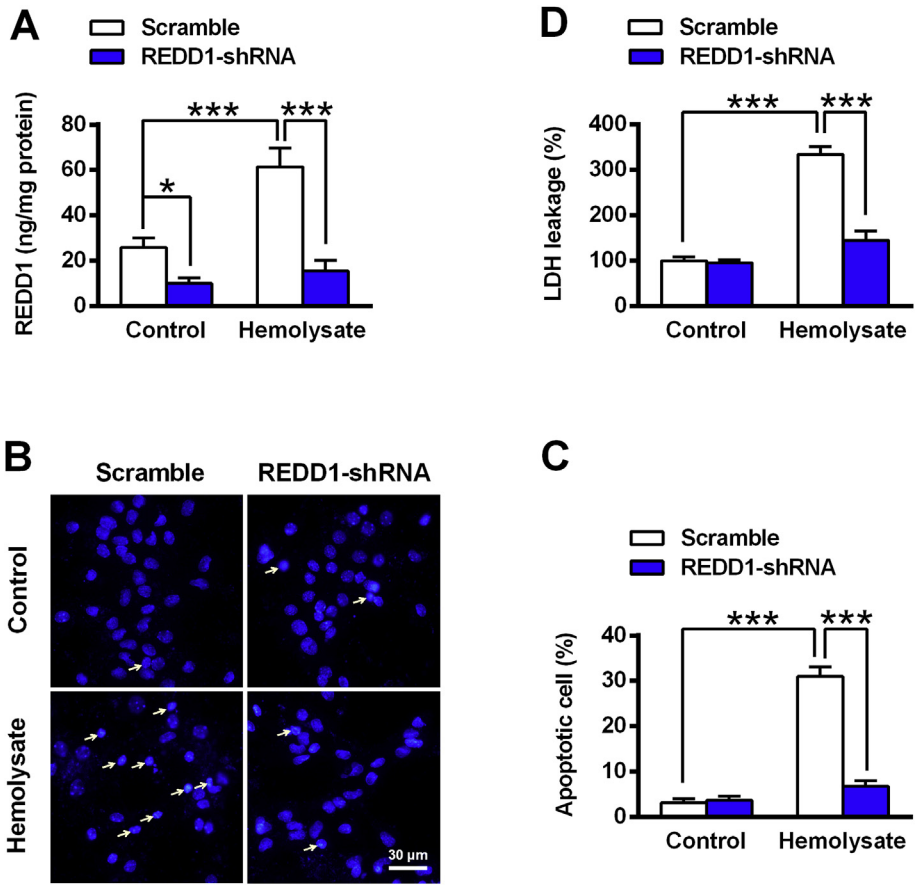

Fig. 4. Effects of REDD1 knockdown on apoptosis and LDH leakage of primary cortical neurons. Primary cortical neurons were infected by lentivirus encoded shRNA targeting to REDD1 (REDD1-shRNA) or non-targeting shRNA (Scramble) for 72 h before being treated with 1:50 dilution of hemolysate for 12 h. Neuronal apoptosis was observed by Hoechst 33342 staining and cell viability was analyzed by LDH assay. Statistical significance was assessed by Two-Way ANOVA followed by a Newman-Keuls post hoc test. (A) Knockdown of REDD1 induced by REDD1-shRNA lentivirus was verified by ELISA. Data were calculated to nanogram REDD1 per milligram total protein. Hemolysate $F(1, 8) = 44.23$, REDD1-shRNA $F(1, 8) = 99.53$, Interaction $F(1, 8) = 23.49$, $^{*}p < 0.05$, $^{***}p < 0.001$. (B) Representative experiments of Hoechst 33342 staining on neurons (The bright concentrated nuclei pointed by arrows). (C) The percentage of apoptotic nuclei in total nuclei. Hemolysate $F(1, 8) = 785.3$, REDD1-shRNA $F(1, 8) = 460.1$, Interaction $F(1, 8) = 504$, $^{***}p < 0.001$. (D) LDH leakage of neurons after hemolysate treatment followed by recovery for 12 h. Data were normalized by the control and presented as Mean ± SD ($n = 6$). Hemolysate $F(1, 8) = 581.9$, REDD1-shRNA $F(1, 8) = 272.2$, Interaction $F(1, 8) = 244.7$, $^{***}p < 0.001$.

increased by hemolysate in neurons. At the same time, the MTT and LDH data showed that neuronal viability was obviously decreased by hemolysate. These results imply that there could be a close relationship between REDD1 and SAH-induced neuronal damage.

The biological effect of REDD1 may be either protective or detrimental for cells under different physiological and pathological conditions (Brugarolas et al., 2004; Dennis et al., 2013; Dungan et al., 2014; Hernandez-Saavedra et al., 2017; Noseda et al., 2013; Wu et al., 2011; Yoshida et al., 2010). Exceeded REDD1 appears toxicity for differential neurons, increasing their sensitivity to oxidative stress. On the contrary, REDD1 also can protect non-differential MCF7 and PC12 cells from hypoxia and H_2O_2-triggered apoptosis (Shoshani et al., 2002; Wu et al., 2011, 2015). As a suppressor of mTOR signaling via TSC1/2, REDD1 is an essential mediator of cigarette smoke-induced pulmonary injury and also is involved in mutant Huntington-induced cell death (Martin-Flores et al., 2016; Yoshida et al., 2010). REDD1/TXNIP complex expression is sufficient to induce ROS, suppress ATG4B activity and activate autophagy. In *Redd1*$^{-/-}$ mice, deregulated ATG4B activity and disabled autophagic flux cause accumulation of defective mitochondria leading to impaired oxidative phosphorylation (Reuschel et al., 2015). Thus, the increased REDD1 may either cause or prevent death depending on cell context. Our previous study found that the viability of neurons was increased when the expression of REDD1 was inhibited by neuroprotective agents such as ginkgolide B and ligustilide in ischemia (Wu et al., 2011, 2015). In the present study, our novel finding that knockdown of REDD1 significantly inhibited the neuronal apoptosis caused by hemolysate implies that REDD1 could participate in process of SAH-induced damage. Moreover, REDD1 in CSF of SAH patients remarkably increased at acute stage (admission to hospital) but it significantly decreased at recovery stage (discharge from hospital). In addition, the high level of REDD1 in CSF could be reflected into peripheral blood. SAH not only could induce a significant increase of REDD1 in CSF but also in peripheral blood. It is extremely necessary to consider the facilitative effect of REDD1 on neuronal damage in SAH and REDD1 may be an important therapeutic and diagnostic target for SAH treatment. The potential mechanism is definitely worthy of further study.

In conclusion, the findings in this study are significant in the

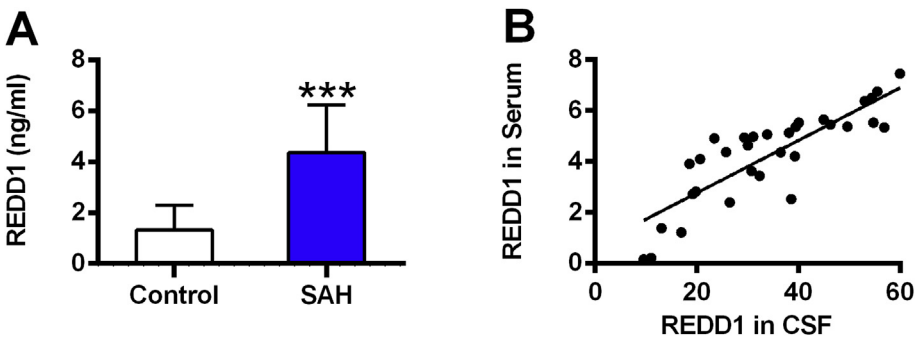

Fig. 5. Correlation of REDD1 level between peripheral serum and cerebrospinal fluid after subarachnoid hemorrhage. REDD1 in peripheral serum of SAH patients was detected by ELISA. Data were calculated to nanogram REDD1 per milliliter serum. (A) The level of REDD1 in peripheral serum of patients after SAH. Statistical significance was assessed by two-tail unpaired Student's t-test, $t(63) = 8.173$, $^{***}p < 0.001$ versus control ($n = 33$). (B) Correlation analysis between the level of REDD1 in CSF and the level of REDD1 in serum. Correlation was performed using linear regression analysis. $R^2 = 0.740$, $p < 0.001$ ($n = 33$).

treatment of SAH in two aspects. First, we highlight the importance of REDD1 involves in SAH because REDD1 significantly increases in CSF of SAH patients and in primary cortical neurons treated with hemolysate. Second, and more importantly, REDD1 knockdown by shRNA obviously inhibits neuronal apoptosis caused by hemolysate. REDD1 may be a potential therapeutic and diagnostic target of SAH. Further work that aims to achieve this objective is worth pursuing.

Abbreviations

CSF, Cerebrospinal fluid; DDIT4, DNA-damage-inducible transcript 4; ELISA, Enzyme-linked immunosorbent assay; LDH, Lactate dehydrogenase; mTOR, mammalian Target of rapamycin; MTT, 3-(4,5-dimethylathiazlo-2-yl)-2,5-diphenyletertrazolium bromide; qPCR, quantitative Polymerase chain reaction; REDD1, Regulated in development and DNA damage responses 1; REDD1-shRNA, short hairpin RNA targeted to REDD1; RT-PCR, Reverse transcription polymerase chain reaction; SAH, Subarachoid hemorrahge; Scramble, Scrambled non-target shRNA; TBST, Tris-buffered saline with 0.1% Tween 20.

Author contributions

J.S. and M.W. performed the experiments; J.S., Y.Y. and S.J. conducted to the analysis and interpretation of data. J.C. and X.W. conceived and designed the project; J.S., J.C. and X.W. wrote and correct the manuscript. can be shared upon request. can be shared upon request. can be shared upon request.

Conflicts of interest

The authors report no conflicts of interest in this work.

Acknowledgements

This study was supported by grants from Science and Technology Innovation Project of Nantong (MS12017008-6, MS12017014-7) and Jiangsu Provincial Program for Medical Innovation Teams and Leading Talents (LJ201133).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.03.019.

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