Cerebral hemorrhage therapy by targeting VEGF and HGF in a preclinical trial in rats

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Abstract. Cerebral hemorrhage is the most common type of human cerebrovascular disease and frequently causes paralysis, vegetative state and mortality. The modulatory actions of vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF) are vital in the human nervous system. The present study investigated the association between cerebral hemorrhage and the expression of VEGF and HGF in a rat model of cerebral hemorrhage. The therapeutic potential of cerebral hemorrhage was also evaluated using targeted drugs for VEGF and HGF in the cerebral hemorrhage rat model. Behavioral and preclinical changes and the survival rates of rats were assessed after treatment with VEGF receptor (VEGFR) and HGF receptor (HGFR). The results of Tarlov scores demonstrated that movement of limbs and coordination when walking were significantly improved in moderate and severe hemorrhage lesions in the VEGFR plus HGFR-treated group and mainly alleviated in primary hemorrhage lesions compared with rats in the single VEGFR or HGFR-treated groups and the control group ($^{**}P<0.01$). Decreasing expression levels of VEGF and HGF were observed in the neural tissue of animals treated with VEGFR plus HGFR compared with the control group ($^{**}P<0.01$). These preclinical observations indicated that VEGF and HGF serve a function in the pathological injury and repair of cerebral tissue in rats with cerebral hemorrhages. The therapeutic benefits of VEGFR plus HGFR suggested that VEGFR plus HGFR may be candidate drugs for cerebral hemorrhage, and thus offer a promising treatment for clinicians and doctors.

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Introduction

Cerebral hemorrhage is the most common human type of cerebrovascular disease (accounting for 20-30% of cases) and the least treatable subtype of hemorrhagic stroke, with a mortality rate ~30-40% (1). The most common clinical manifestations are cerebral arteriosclerosis, hypertension and intracranial vascular malformations (2). Cerebral hemorrhage is often provoked by exertion and emotion and the majority of patients present sudden onset during activity. Cerebral hemorrhage usually causes severe dysfunction of the cerebral nervous system and the loss of mobility and independence, often resulting in burden to family and carers (3,4). Subarachnoid hemorrhage is one of the most serious types of cerebral hemorrhage and usually results in mortality as blood bleeds into the subarachnoid space (5,6). Although orally administered anticoagulants or surgical resection are used as the main clinical treatments of cerebral hemorrhage, there is no effective therapeutic schedule to improve functional outcomes in patients with cerebral hemorrhage, especially subarachnoid hemorrhage (7-9). Therefore, there is an urgent requirement for potential therapeutic agents targeting cerebral hemorrhage and an improved understanding of the molecular mechanisms of human cerebrovascular disease.

The majority of cerebral hemorrhages are non-traumatic and caused by rupture of vessels in brain parenchyma (10). An increasing number of detection methods are used for measuring the disease progression of intracranial hemorrhage, including intracranial pressure, positron emission tomography, computed tomography, and magnetic resonance imaging (11). In addition, numerous molecules have been proposed that may aid the diagnosis of, and therapy for, cerebral hemorrhage by targeting cell-associated hemostasis (12,13). Previous studies (14,15) have identified epidermal growth factor receptor (EGFR) and hepatocyte growth factor (HGF) as important risk factors for bleeding in the development, rehabilitation and recurrence of cerebral hemorrhage. In addition, literature and clinical studies indicate that EGFR and HGF are prospective candidate molecules for targeted molecular therapy in cerebrovascular disease (16).

Vascular endothelial growth factor (VEGF) serves as a crucial promoter for angiogenesis in physiological and pathological conditions, and is identified as a survival factor and specific mitogen in endothelial cells (17). Previous studies reported that VEGF was a target for drug therapy including bevacizumab, sorafenib and sunitinib, and as a predictive marker

for hypertension (18-20). Hypertension is the primary risk factor for cerebrovascular disease, and may be a consequence of an inductor in the microvascular network of the brain, resulting in abnormal regulation of functions. The VEGF-mediated angiogenesis target of rapamycin-mediated regulation of cell growth, cell proliferation, cellular metabolism and angiogenesis have been identified as key factors in the development of cerebrovascular disease (21). Therefore, targeting VEGF by inhibiting the VEGF pathway by binding with VEGF receptor (VEGFR) has demonstrated preclinical benefits in cerebrovascular disease (22).

HGF is known for its important role in the regulation of cell proliferation, morphogenesis wound healing, motility and angiogenesis (23). A previous study (16) demonstrated that HGF and HGF receptor (HGFR) served a vital function in the formation and progression of human cerebrovascular disease by regulating cell proliferation. HGF expression was closely associated with the state of patients with cerebral hemorrhage. Chu *et al* (24) reported that mRNA stabilization of HGF mediated by hypoxia and HGF may be a risk factor in cerebral hemorrhage.

Materials and methods

Animals. Healthy specific-pathogen-free male Wistar rats (n=50; 40 weeks old; weight, 350±20 g) were purchased from the Animal Center of Hebei Medical University [Shijiazhuang, China; Certification No. SCXK (Hei) 20100026] and used to establish a cerebral hemorrhage model as described in a previous study (25). Following the confirmation of brain hemorrhage, the rats were divided into primary, moderate and severe (n=10 in each group) according to the Tarlov scale. The maximum tolerated doses of VEGFR and HGFR were determined by anesthetization with 50 mg/kg pentobarbital followed by slow injection of various doses of VEGFR and HGR into the right ventricle wall of rats. The rats were treated intravenously with VEGFR or/and HGFR once daily with PBS as a control. The rats were sacrificed by cervical dislocation following 30 days of treatment and the excised brains were studied by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The expression of HGF and VEGF mRNA was examined and analyzed. The remaining rats were observed until 180 days following the commencement of treatment, and every 10 days the state according to the Tarlov scale and the survival rate was recorded. All surgery procedures and euthanasia were performed to cause minimal suffering. All experimental protocols were approved by the Ethics Committee of Second Hospital of Hebei Medical University and were performed in accordance with the guidelines by the National Institutes of Health Guide for the Care and Use of Laboratory Animals (26).

Relative mRNA levels by RT-qPCR. Total cellular RNA was extracted from the hippocampus, midbrain, cerebral cortex and white matter and was subjected to synthesis of cDNA (2 μg) by RT-qPCR. RT was performed using the SuperScript® First-Strand Synthesis System for RT-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. The cycling conditions for RT were as follows: Initial cycling for 5 min at 95˚C, followed by 40 cycles for 15 sec at 95˚C, 30 sec at 60˚C and 30 sec at 72˚C.

Samples were subjected to qPCR using SYBR® Premix Taq (Applied Biosystems; Thermo Fisher Scientific, Inc.) on ABI PRISM 7900 thermocycler (Applied Biosystems; Thermo Fisher Scientific, Inc.) to analyze expression changes of VEGF and HGF. The following primers were used: VEGF, 5'-CTCATC GCAGATGCCTGGAA-3' (forward) and 5'-TTCAGGTAATAG GCACCCTTGAAGA-3' (reverse); HGF, 5'-CTCAGCCAGATG CAATCAAT-3' (forward) and 5'-GCTTCTTTGGGACACTTG CT-3' (reverse); and housekeeping gene GAPDH, 5'-GCACCG TCAAGGCTGAGAAC-3' (forward) and 5'-TGGTGAAGA CGCCAGTGGA-3' (reverse). The cycling conditions for qPCR were as follows: An initial denaturation step at 95˚C for 4 min, followed by 35 cycles at 94˚C for 20 sec, 55˚C for 30 sec, 72˚C for 20 sec, 72˚C for 2 min and a final elongation step at 72˚C for 10 min. The mean value in the control group was identified as the calibrator and the results are expressed as the n-fold difference relative to control (n=3; relative expression levels).

Behavioral assessments. The behavior of the rats was assessed on days 7, 14, 21 and 30 following surgery. The assessment parameters, including left limb movement and co-ordination of movement were evaluated using modified Tarlov scores as follows: Severe level, possible limb movement and partial limb paralysis (1-4 points); moderate level, failure to jump and stand normally (4-7 points); primary level, failure to stand and with joint movement (7-9 points). Healthy rats were scored 9-10 points.

Efficacy safety assessments. Efficacy assessments included the maximum toxicity dose in cerebral hemorrhage and the dose-limiting toxicity in the presence and absence of VEGFR (0.18 mg) and/or HGFR (0.24 mg). A significant decrease of median percent change in cerebral hemorrhage was observed following 30-day treatment in the VEGFR plus HGFR-treated group. Safety assessments included the incidence rates (>10%) of the most frequent treatment-emergent adverse events in the 30-day treatment period in the experimental and control groups. The efficacy and safety data included all rats with cerebral hemorrhage receiving the therapeutic drugs and control.

Statistical analysis. Statistical analysis was performed using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). All data were reported as the means and standard error. Statistical significance of differences between mean values was assessed by Student's t-test for unpaired data. Comparisons of data between multiple groups were performed with analysis of variance. $^{*}P<0.05$ was considered to indicate a statistically significant difference.

Results

Characteristic of rats with cerebral hemorrhage. The rats were induced to develop cerebral hemorrhage through autologous blood injection and were designated into three categories of cerebral hemorrhage according to the severity of the illness. The rats were divided randomly into four groups (n=40) in each category subsequent to the confirmation of cerebral hemorrhage. The characteristics of the rats with cerebral hemorrhage are presented in Table I. All rats received therapy with at least one agent and the control group received normal saline.

Duration of treatment, maximum tolerated dose, and dose-limiting toxicity. Median overall duration of treatment was

Table I. Characteristics of the male rats (age, 40 weeks; weight, 350±20 g; treatment cycle, 30 days).

Characteristic	Primary	Moderate	Severe
Total number	80	80	80
Received drugs	VEGFR or/plus HGFR	VEGFR or/plus HGFR	VEGFR or/plus HGFR
Score	8.4±0.3	5.6±0.4	3.4±0.2
Pressure (cm H_2O)	3.23±0.52	7.24±0.63	13.97±0.64

VEGFR, vascular endothelial growth factor receptor; HGFR, hepatocyte growth factor receptor.

Table II. Treatment-associated hypertension and proteinuria by common toxicity criteria grade.

		0.18 mg VEGFR (n=24)			0.24 mg HGFR (n=24)			18 mg VEGFR + 0.24 mg HGFR (n=4)		
Adverse event	Total (n=72)	Primary	Moderate	Severe	Primary	Moderate	Severe	Primary	Moderate	Severe
Hypertension	38	1	4	6	2	5	6	3	5	6
Grade 1	11	0	1	1	0	1	2	2	2	2
Grade 2	11	0	1	2	1	2	1	1	2	1
Grade 3	16	1	2	3	1	2	3	0	1	3
Proteinuria	28	0	2	4	1	4	6	1	5	5
Grade 1	4	0	0	1	0	1	1	0	0	1
Grade 2	10	0	1	1	0	1	2	1	2	2
Grade 3	14	0	1	2	1	2	3	0	3	2

VEGF, vascular endothelial growth factor; HGFR, hepatocyte growth factor receptor.

14 days in all dosing cohorts. These were 0.06, 0.12, 0.18, 0.24 and 0.30 mg VEGFR, and 0.08, 0.16, 0.24, 0.30 and 0.36 mg HGFR for the respective cohorts. The maximum tolerated dose was 0.18 mg of VEGFR and 0.24 mg of HGFR once daily, identified by slow injection into the right ventricle wall in the preclinical study. The lowest-dose cohort of VEGFR and HGFR had the fewest number of VEGFR and HGFR dose reductions. Rats with primary, moderate or severe cerebral hemorrhages received a minimum of dose of therapy with a post baseline safety evaluation included in the safety population in the present study. The most common treatment-associated adverse events were hypertension and proteinuria during the treatment with VEGFR and HGFR in rats with moderate or severe of cerebral hemorrhage (Table II).

The mRNA expression of VEGF and HGF in rats with cerebral hemorrhage. RT-qPCR was performed to detect the expression levels of HGF and VEGF mRNA and protein in the cerebral tissue of adult rats with cerebral hemorrhage after treatment with VEGFR or/and HGFR. The results (Fig. 1) demonstrate that mRNA expression levels of HGF and VEGF were significantly decreased in the hippocampus, midbrain, cerebral cortex and white matter in the rat brains of the HGFR plus VEGFR-treated groups. However, HGF and VEGF mRNA expression levels were significantly increased after two cycles treatment in control group. Furthermore, although rats with primary cerebral hemorrhage also received two cycles of treatment, mRNA and protein expression levels of HGF and VEGF were significantly decreased compared with the moderate and severe cerebral hemorrhage groups.

VEGFR and HGFR enhance healing and prolong survival rates of rats. In order to explore whether the combination therapy of *VEGFR and HGFR* is effective for rats with cerebral hemorrhage *in vivo*, the recurrent activity of VEGFR and HGFR in the cerebral hemorrhage rat model was studied. The results (Fig. 2) demonstrated that movement capacities of limbs and coordination in walking were notably improved in cases of moderate and severe hemorrhage lesions in the VEGFR plus HGFR-treated group and mainly alleviated in cases of primary hemorrhage lesion compared with rats in the single VEGFR or HGFR-treated groups and the control group ($^{**}P<0.01$). There were no significant changes in arterial blood pressure, body weight or body temperature, and injected arterial blood gas data were not detected across all the experimental groups. The results of the present study also demonstrated that there was no significant difference between the VEGFR-treated and HGFR-treated groups as evaluated by Tarlov scores. Tarlov scores of the rats with cerebral hemorrhage indicated that the therapeutic effects were significant in the primary, moderate and severe hemorrhage categories in the VEGFR plus HGFR group ($^{*}P<0.05$, $^{**}P<0.01$) compared with the control group (Fig. 3). Furthermore, there was a long-term survival rate observation in the severe cases of cerebral hemorrhage in the 180-day period after treatment with VEGFR plus

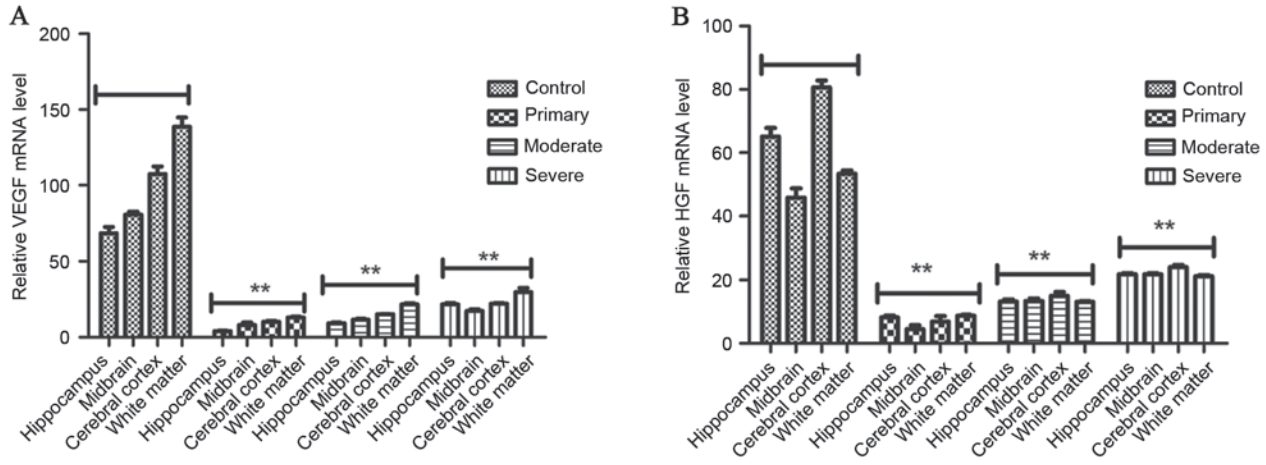

Figure 1. Effects of VEGFR plus HGFR on the expression levels in the brain of VEGF and HGF in rats with cerebral hemorrhage. (A) The mRNA expression of VEGF. (B) The mRNA expression of HGF. All data are presented as the mean ± standard error of triplicate samples. **P<0.01 was considered to indicate a statistically significant difference between the model and control groups. VEGFR, vascular endothelial growth factor receptor; HGFR, hepatocyte growth factor receptor, VEGF, vascular endothelial growth factor; HGF, hepatocyte growth factor.

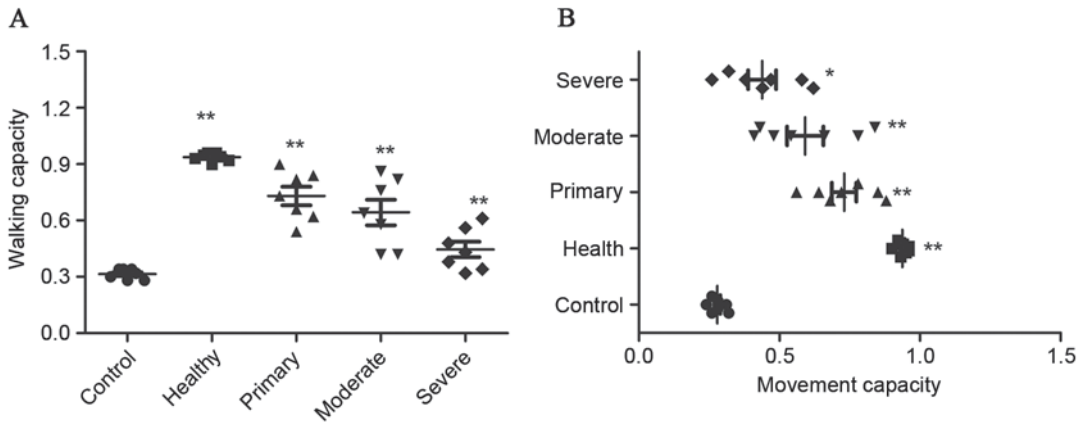

Figure 2. Walking and movement capabilities of rats with cerebral hemorrhage after treatment with VEGFR or/and HGFR. (A) Walking capability in experimental and control groups. (B) Movement capability in experimental and control groups. All data are presented as the mean ± standard error of triplicate samples. *P<0.05 and **P<0.01 were considered to indicate a statistically significant difference between the model and control groups. VEGFR, vascular endothelial growth factor receptor; HGFR, hepatocyte growth factor receptor.

HGFR. The results (Fig. 4) demonstrated that that the survival rates of the rats were extended, and Tarlov scores improved, after treatment with VEGFR plus HGFR compared with the control group (n=10 in each group). The results indicated that the therapeutic agents used for rats with cerebral hemorrhage in the VEGFR plus HGFR group are effective enough to relieve and heal the animals, which translated into long-term survival.

Discussion

Spontaneous, non-dramatic cerebral hemorrhage leads to high morbidity and mortality worldwide. Numerous studies have demonstrated that cerebral hemorrhage causes neuronal damage and further aggravates brain damage to the extent of developing contralateral limb dysfunction (27-29). In cerebral hemorrhage the blood often overflows directly into the brain parenchyma. A previous study (11) indicated that the possible mechanism may be associated with the leakage from small intracerebral arteries caused by a function loss of endothelial growth cells. Furthermore, this transformation may occur within one week of ictus (12). Therefore, dysfunction of endothelial growth cells may be an important pathophysiological factor in cerebral hemorrhage and human cerebrovascular disease. Chu *et al* (16) reported that mRNA and protein expression levels of VEGF were increased in the cerebral tissue of adult rats with chronic hydrocephalus after subarachnoid hemorrhage. These observations suggested that the inhibition of VEGF expression is beneficial in the reduction of the degree of brain injury and the promotion of a functional recovery (30). In the present study, the function of VEGF was analyzed and the therapeutic effects of its receptor were studied in rats with cerebral hemorrhage. The results were consistent with previous studies and VEGFR exhibited a beneficial treatment in the preclinical trial. However, in terms of the final survival rate of the experimental rats, improvements in therapy or combination therapy are required for an improved therapeutic effect.

HGF is a multifunctional cytokine with numerous roles in humans (31). Although the underlying mechanism of HGF in the formation and development of cerebral hemorrhage remains to

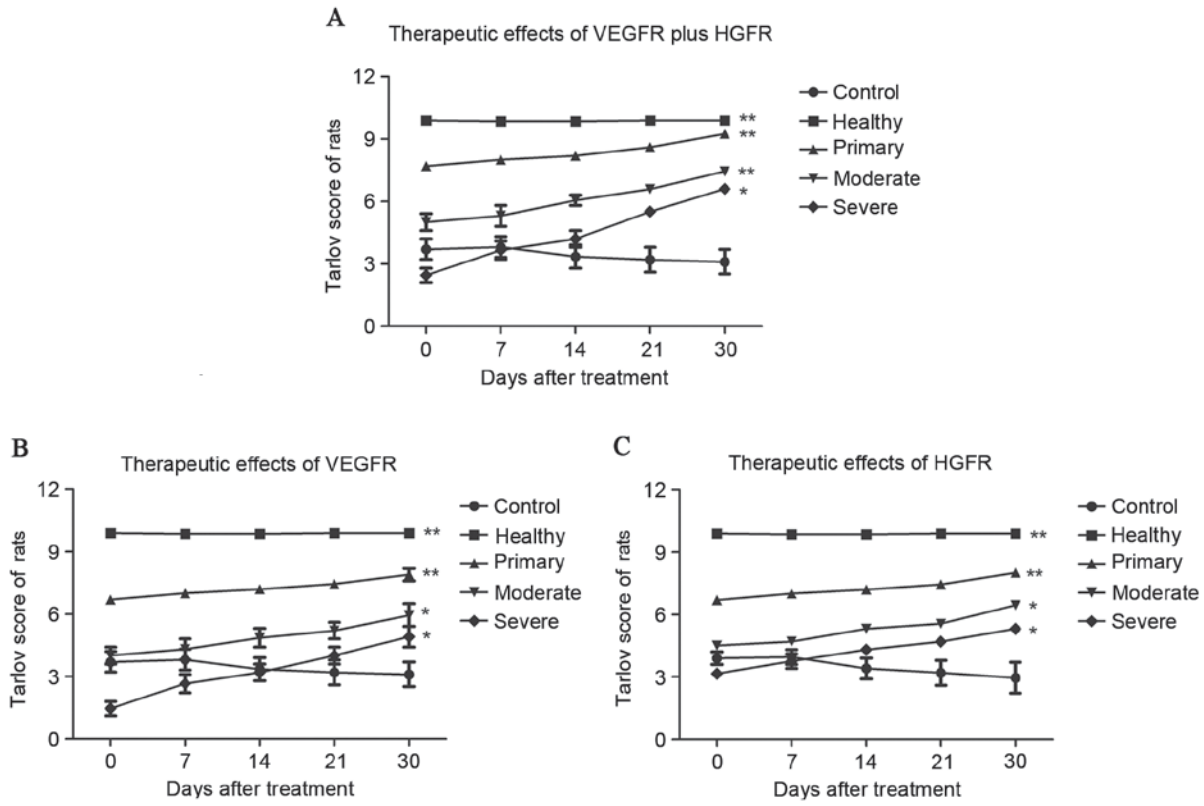

Figure 3. Behavioral assessments using the evaluation of motor function in rats with cerebral hemorrhage. (A) Therapeutic effects of VEGFR plus HGFR in rats with cerebral hemorrhage. (B) Therapeutic effects of VEGFR in rats with cerebral hemorrhage. (C) Therapeutic effects of HGFR in rats with cerebral hemorrhage. Behavioral assessment was based on the modified Tarlov scale. The assessment parameters including left limb movement. All data are presented as the mean ± standard error of triplicate samples. *P<0.05 and **P<0.01 were considered to indicate a statistically significant difference between the model and control groups. VEGFR, vascular endothelial growth factor receptor; HGFR, hepatocyte growth factor receptor.

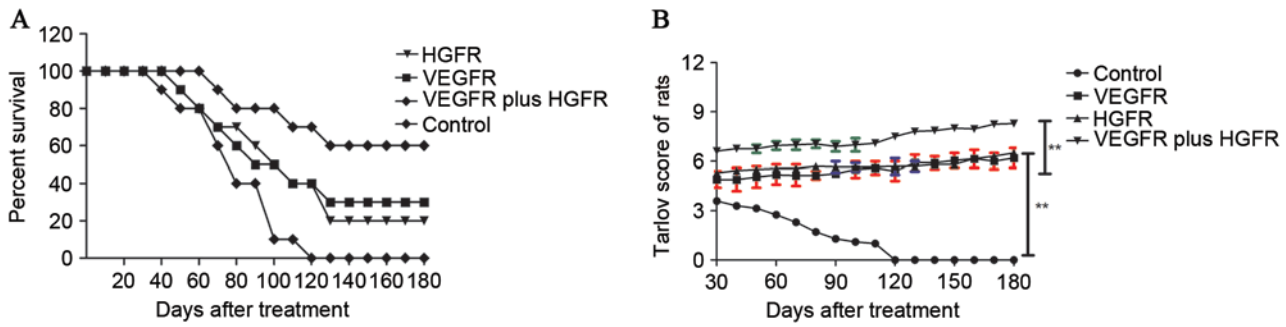

Figure 4. Survival rates of rats with severe cerebral hemorrhage and the Tarlov score of therapy effects in 180-day period. (A) Survival rates of rats with severe cerebral hemorrhage after treatment. (B) Tarlov score of experimental and control groups in 180-day period. All data are presented as the mean ± standard error of triplicate samples. **P<0.01 was considered to indicate a statistically significant difference between the model and control groups.

be fully understood, HGF serves a vital function in human cerebrovascular disease and it is well-known that HGF is frequently unregulated in patients with cerebral hemorrhage (31,32). Previous studies have demonstrated that HGF increased damage by effectively promoting the growth of other cells and the restoration of the nervous system on the injured side (31,33). The present study hypothesized that increasing HGF expression promoted the growth of endothelial growth cells, exacerbating the increase of blood cells that aggravated the state of illness in patients with cerebral hemorrhage. In the present study, the therapeutic effects of the HGFR target for HGF in rats with cerebral hemorrhage were tested. The observations suggest that beneficial effects occurred and that HGFR may be an effective candidate as a treatment for cerebral hemorrhage.

To the best of the authors' knowledge, the current study is the first to investigate the preclinical therapeutic effects of VEGFR and HGFR treatment in rats with cerebral hemorrhage. The purpose of the present study was to evaluate the efficacy and safety of VEGFR and HGFR for treating rats with different levels of cerebral hemorrhage. During the treatment, the maximum tolerated dose, and dose-limiting toxicities were analyzed to ascertain the therapeutic dose. In the present study, VEGFR and HGFR were demonstrated to be efficient and safe for controlling bleeding in rats with cerebral hemorrhage and are

recommended as preferred agents in procedures. Notably, treatment of VEGFR plus HGFR resulted in a statistically significant improvement in walking and limb co-ordination in comparison with the control. In the treatment-associated adverse events, it was also identified that the main side effects of injection with VEGFR and HGFR are lethargy, hypertriglyceridemia, fatigue and proteinuria. However, further trials exploring the VEGFR plus HGFR treatment for cerebral hemorrhage of are required.

In conclusion, the present study focused on the therapeutic effects of VEGFR and HGFR during cerebral hemorrhagic injury, and reached no consistent conclusions on their efficiency. In the current study, a rat model of cerebral hemorrhage was used to investigate the effects the injecting into brain tissue of VEGFR and HGFR. Although the conclusions of the present study were inconsistent, certain results suggest that VEGFR and HGFR mitigated brain injury after cerebral hemorrhage by decreasing VEGF and HGF expression. Further investigation is required in order to establish a preclinical foundation for the effects of VEGFR and HGFR treatment in cerebral hemorrhage.

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