

Systemically Administered Brain-Targeted Nanoparticles Transport Peptides Across The Blood–Brain Barrier And Provide Neuroprotection

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ABSTRACT

Although growth factors and anti-apoptotic peptides have been shown to be neuroprotective in stroke models translation of these experimental findings to clinic is hampered by limited penetration of peptides to the brain. Here, we show that a large peptide like the basic fibroblast growth factor (bFGF) and a small peptide inhibitor of caspase-3 (z-DEVD-FMK) can effectively be transported to the brain after systemic administration by incorporating these peptides to brain-targeted nanoparticles. Chitosan NPs were loaded with peptides and then functionalized by conjugating with antibodies directed against the transferrin receptor-1 on brain endothelia to induce receptor-mediated transcytosis across the blood-brain barrier. Pre-ischemic systemic administration of bFGF- or z-DEVD-FMK-loaded nanoparticles significantly decreased the infarct volume after 2-h middle cerebral artery occlusion and 22-h reperfusion in mice. Co-administration of bFGF- or z-DEVD-FMK-loaded nanoparticles reduced the infarct volume further and provided a 3-h therapeutic window. bFGF-loaded NPs were histologically detected in the brain parenchyma and also restored ischemia-induced Akt dephosphorylation. The neuroprotection was not observed when receptor-mediated transcytosis was inhibited with imatinib or when bFGF-loaded nanoparticles were not conjugated with the targeting antibody. Nanoparticles targeted to brain are promising drug carriers to transport large as well as small BBB-impermeable therapeutics for neuroprotection against stroke.

Keywords: Cerebrovascular disease/stroke, Growth factors/cytokines, Nanoparticles, Neuroprotection,

INTRODUCTION

Recent research has disclosed that cell death in acute as well as chronic neurodegenerative diseases involves common mechanisms such as caspase activation¹⁻⁵. Therefore, inhibitors of caspases (e.g. Benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethylketone; z-DEVD-FMK) appear to be attractive therapeutic targets for a wide range neurological diseases including stroke⁶⁻⁸. Unfortunately, most of the inhibitor agents are small peptides that cannot cross the BBB. On the other hand, growth factors (e.g. basic fibroblast growth factor; bFGF) suppress cell death by acting at several points on death pathways and, additionally, promote regeneration⁹⁻¹¹. These features make them promising agents for treatment of stroke and other neurodegenerative diseases. Unfortunately, these large peptides too cannot penetrate the brain tissue when systemically administrated. Although it has been proposed that opening of the BBB after ischemia could allow large molecules such as bFGF could enter the brain, recent studies examining the BBB leakage topographically have shown that early leakage is only seen in the deeply ischemic core areas but not in the potentially salvageable penumbra.^{12, 13, 14}

Promoting cell survival while inhibiting death effectors is a potentially promising neuroprotective approach that may provide additive synergy between these two groups of drugs. Indeed, Ma et al. demonstrated that both z-DEVD-FMK and bFGF could reduce infarct size when given intracerebroventricularly and that their combined administration had a synergistic effect; not only the infarct reduction was greater but also the therapeutic time window was prolonged compared to their individual use¹⁵. Therefore, the development of an effective brain delivery system for peptides is needed to translate these promising experimental achievements to clinic.

Brain-targeted nanoparticles (NPs) can transport large amounts of BBB-impermeable agents across the BBB on systemic administration^{16, 17}. We previously demonstrated that chitosan NPs loaded with z-DEVD-FMK and conjugated with anti-transferrin receptor-1 monoclonal antibody (TfRMAb) rapidly crossed the BBB upon systemic administration to mice by way of transferrin receptor-mediated transcytosis and, provided neuroprotection against focal cerebral ischemia¹⁸. However, loading large peptides such as bFGF to NPs and their transport to the brain in an active form and sufficient concentrations remained a challenge. In the present study, we have first developed the conditions to load bFGF to chitosan NPs and then showed that they reduced ischemic injury on systemic administration. Next, we investigated if we could obtain an additive protective activity by combined administration of z-DEVD-FMK and bFGF-loaded NPs and, whether or not this approach could provide a prolonged therapeutic window. We have also demonstrated activation of the downstream pathways in bFGF signaling to confirm that bFGF was transported in sufficient concentrations similar to the inhibition of caspase-3 activity with z-DEVD-FMK-loaded NPs as we previously showed¹⁸. Finally, we verified that neuroprotection was mediated by transcytosis of peptide-loaded NPs across the endothelium by inhibiting transcytosis with imatinib¹⁹. Importantly, the targeted brain delivery of bFGF-loaded NPs provided not only neuroprotection but also about 300 times reduction in the amount of the peptide administered to the mouse compared to the systemic administration of plain bFGF, offering a great advantage to reduce systemic adverse effects²⁰.

MATERIALS AND METHODS

Preparation and characterization of the TfRMAb-conjugated or unconjugated chitosan NPs and, evaluation of z-DEVD-FMK and bFGF release from them are given elsewhere¹¹. Briefly, NPs were prepared by loading either peptide to chitosan-polyethyleneglycol-biotin graft polymers in a process aided by reticulation with pentasodium tripolyphosphate. Polyethyleneglycol chains were added to the NP surface to reduce clearance by the reticuloendothelial system, whereas biotin was used to conjugate streptavidinylated anti-transferrin receptor-1 monoclonal antibody by way of streptavidin-biotin binding. These procedures yielded NPs in sizes of 715 ± 32 and 747 ± 42 nm when z-DEVD-FMK or bFGF were loaded, respectively.

The experiments were performed on 75 Swiss albino, male mice (25-30 g). The study protocol was approved by the Hacettepe University Animal Experiments Local Ethics Committee (2008/78-1). Focal cerebral ischemia was induced with the intraluminal filament middle cerebral artery occlusion (MCAo) method as described previously²¹. Briefly, animals were anesthetized with 2% isoflurane and maintained on 1.5% isoflurane and 30% oxygen. After ligating the right common carotid and external carotid arteries, a nylon filament (8-0) inserted into the common carotid artery was advanced to the origin of MCA. The distal 3 mm of filaments was coated with silicone resin/hardener mixture. For reperfusion, the filament was withdrawn. The regional cerebral blood flow (rCBF) in ischemic area was measured by laser-Doppler flowmetry. Rectal temperature was maintained at $37.0\pm 0.5^{\circ}\text{C}$ with a homeothermic blanket. Systolic blood pressure was measured noninvasively. Tissue oxygen saturation was monitored from the paw by a pulse oximeter (V3304 Tabletop Pulse Oximeter; Surgivet).

Seven groups of mice were subjected to 2 h proximal MCAo and 22 h reperfusion. Animals were randomly allocated to study groups in blocks. Fifteen years of experience of our lab shows that this stroke model in Swiss albino mice results in an infarct volume of 50 ± 6 mm³ (mean \pm SD). Based on a previous study¹⁵ demonstrating neuroprotection by intracerebroventricular administration of caspase inhibitors and bFGF against 2 h MCA occlusion in mice, we anticipated a decrease in infarct size by at least 20% in our model. Accordingly, sample size calculation indicated that six animals were required per group for 80% power and a α of 0.05. The control group received an equal mixture of non-functionalized (TfRMAb-free) NPs loaded with z-DEVD-FMK (240 ng) or bFGF (17 ng). Treatment groups received functionalized (TfRMAb-conjugated) NPs loaded with z-DEVD-FMK (240 ng) or bFGF (17 ng) or an equal mixture of both. NPs were administered just before inducing ischemia in these groups. The combined treatment was given to another group 3 h post-ischemia. NPs were administered intraperitoneally in 0.1 ml ultrapure water in the above groups. To compare the efficacy of intravenous and intraperitoneal administrations, another group was treated intravenously with bFGF-loaded NPs in 0.1 ml ultrapure water pre-ischemia. Finally, mice treated with imatinib (150 mg/kg twice a day for 2 days) before the day of the MCAo were administered functionalized bFGF-loaded NPs (i.p.) pre-ischemia. Details of the groups and treatments are given in Table 1.

Motor functions were scored as described previously²². Twenty-two hours after reperfusion, prior to sacrifice, clinical motor functions were assessed and scored in each animal as: grade 0: no observable motor deficit (normal); grade I: failure to extend left forepaw on lifting the whole body by the tail (mild); grade II: circling to the contralateral side (moderate); grade III: leaning to the contralateral side at rest or no spontaneous motor activity (severe). One of the 2 evaluators was blinded with respect to the study groups and, only the values agreed on by both investigators were analyzed.

Mice were then anesthetized with a lethal dose of chloral hydrate (1 g/kg, i.p.), transcardially perfused with 100 ml of heparinized saline (10 IU/ml) followed by 4% paraformaldehyde and then decapitated. Brains were stored in 4% paraformaldehyde for 24 hours, after which 2-mm thick 5 brain slabs were serially cut in coronal plane and embedded in paraffin. Five μm -thick sections were obtained from the posterior surface of each slab and, stained with hematoxylin and eosin. To calculate the infarct volume the boundary of infarct area, characterized by reduced eosin staining on hematoxylin and eosin-stained coronal sections under light microscopy was outlined with image analysis software (ImageJ 1.44o). The size of infarcted area in each section was calculated by subtracting the non-infarcted region in the ischemic hemisphere from the total surface area of the contralateral hemisphere. The infarct volume was then calculated by multiplying each sequential infarct area by the distance between sections (2 mm) and by 5 (the number of sections from each brain).

In another set of animals, brains were examined to illustrate the NPs penetrated into parenchyma. Ten naïve mice were given functionalized bFGF-loaded NPs (pre-ischemia, i.p. or i.v.) 30 min (n=2), 1 h (n=2) and 3 h (n=6) before sacrifice. Mice administered non-functionalized bFGF-loaded NPs served as controls (n=3). NPs were also searched in brain sections of 4 mice subjected to 2 h ischemia and 1 h reperfusion and, received functionalized (n=2) or non-functionalized (n=2) bFGF-loaded NPs. Two mice given the vehicle of NP suspensions (0.1 ml ultrapure water, i.p.) served as sham controls. NPs in sections were detected by either labeling free biotin molecules on the NP surface with streptavidin–horseradish peroxidase or immunolabeling against the human bFGF loaded to NPs.

For conjugating streptavidin to biotin molecules decorating the NP surface, 4-5 μm -thick coronal sections were prepared from the paraffin-embedded blocks. Sections were deparaffinized at 60°C overnight and then in xylene for one hour. Sections were hydrated in graded alcohol solutions, washed in PBS for 10 min and then treated with streptavidin-

horseradish peroxidase complex (Reagent C of Acu-Stain Mouse+Rabbit HRP Kit, Genemed Biotechnologies, CA, USA) for 1 h at room temperature. After three 10-min washes with PBS, sections were treated with filtered diaminobenzidine (DAB) (Genemed Biotechnologies, CA, USA) for 1 h at room temperature. Sections were cover-slipped with Hoechst 33258 following three 10-min washes with PBS

For immunolabeling, paraffin-embedded 4-5 μm -thick sections were deparaffinized and hydrated in xylol and graded alcohol solutions. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase solution. Antigen retrieval was performed with 10 mmol/L citrate buffer (pH=6) in a pressure cooker for 20 minutes. The sections were then rinsed with PBS (pH 7.4) at room temperature and nonspecific binding was blocked by using 3% bovine serum albumin (Sigma, USA) in PBS for 20 minutes. Next, the sections were incubated with anti-human-FGF-2 / basic FGF polyclonal antibody (Millipore, 1:100 in PBS) at room temperature for 1 hour. Immunodetection of bFGF was performed using the conventional streptavidin–horseradish peroxidase technique (Dako cytometry kit, CA, USA). Filtered DAB (Sigma, St Louis, Missouri, USA) was used as chromogen. Negative controls were carried by omitting the primary antibody.

In another set of animals, brain samples of the MCA area from mice subjected to 2 h ischemia and 22 h reperfusion and received (i.v.) either functionalized NPs (n=6) or non-functionalized NPs loaded with bFGF (n=4) as well as from sham-operated naïve mice (n=4) were used for the blotting procedure. Samples obtained by wedge resection of the MCA area were placed on ice and lysed in RIPA buffer (25 mM Tris–HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, and 0.1% SDS) with protease (Sigma) and phosphatase inhibitors (Sigma) by ultrasonic homogeniser (Bandelin Sonopuls). The lysates were centrifuged at 14000 RPM for 15 minutes at 4°C. The supernatants were collected and protein concentrations of the samples were determined by Qubit Protein Assay Kit

(Invitrogen). Equal amounts of protein were loaded to 4-12% SDS-PAGE (Invitrogen), and subsequently transferred to PVDF membranes (Invitrogen) by iBlot dry blotting system (Invitrogen). Nonspecific protein binding was blocked by incubating the PVDF membranes in 3% BSA (Sigma) in TBS-T for 1 hour at 4°C, and then the membranes were incubated with anti-rabbit phospho-Akt (Ser473) antibody (1:1000, Cell Signaling Technology) and anti-rabbit Akt antibody (1:1000, Cell Signaling Technology) overnight at 4°C. Next day, after washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody (1:3000, Cell Signaling) for 1 hour at room temperature. The protein bands were visualized by chemiluminescence (Super Signal West-Femto; Pierce). Membranes were stripped and re-probed for anti-rabbit beta-tubulin (1:7500, Sigma) in order to normalize p-Akt to Akt band intensities on different gels. Images were captured by Kodak 4000MM image station. ImageJ was used for data analysis.

Statistical analysis

Data are expressed as mean±SEM. Blood pressure, rCBF, tissue oxygen saturation, motor function scores; infarct volumes and p-Akt/Akt ratios of groups were compared with Kruskal–Wallis test. Statistically significant data were further analyzed with Mann–Whitney U test. The infarct volumes (parametric values) were correlated with the neurological deficit scores (non-parametric values) by using Spearman's test. A p value of < 0.05 was regarded as statistically significant. P values were corrected for multiple comparisons.

RESULTS

Systemically administered peptide-loaded NPs provide neuroprotection

For neuroprotection study, mice were randomly allocated to 7 groups of 6 animals to avoid any selection bias. The mean (\pm SEM) infarct volume of the control group receiving non-functionalized NPs loaded with an equal mixture of bFGF or z-DEVD-FMK, which can release bFGF and z-DEVD-FMK to the plasma but are unable to cross BBB, was 51 ± 1 mm³ after 2h MCAo and 22h reperfusion (Figure-1A). In contrast, functionalized bFGF-loaded NPs significantly decreased the infarct volume when administered either i.v. (37 ± 3 mm³) or i.p. (33 ± 1 mm³) (Figure-1A, C). z-DEVD-FMK-loaded, functionalized NPs (i.p.) also reduced the infarct size (37 ± 3 mm³) similarly to when they were administered i.v., as previously reported¹⁸. Administration of an equal mixture of bFGF- or z-DEVD-FMK-loaded, functionalized NPs further reduced the infarct volume to 24 ± 2 mm³ (n=5, one animal from this group was excluded because we detected ischemic changes also in the contralateral hemisphere). To test if the neuroprotection observed with the pre-ischemic treatments above might have clinical significance, an equal mixture of bFGF- and z-DEVD-FMK-loaded NPs was administered 3 hours after stroke (1 h after reperfusion), which also significantly reduced the infarct volume to 34 ± 2 mm³. Treatments with NPs improved motor function deficit scores parallel to the reductions in infarct size although the decreases in deficit scores were less striking possibly because some sensorimotor fibers ascending to or descending from the recovered cortex could not escape damage in the ischemic subcortical area (Figure-1B). However, we found a moderate but statistically significant correlation ($r=0.59$, $p=0.001$, $n=29$) between the infarct volumes and the neurological deficit scores.

To confirm that the neuroprotection provided by NPs is mediated by peptides transported into brain parenchyma, we treated another group of mice with imatinib, which inhibits the receptor-mediated endothelial transcytosis, two days prior to administering NPs¹⁹. Imatinib treatment suppressed the neuroprotective effect of bFGF-loaded functionalized NPs; the infarct volume of this group (49 ± 2 mm³, n=5, one mouse allocated to this group died

during surgery) was not significantly different than the control group receiving non-functionalized NPs (Figure-1D). These mice slightly lost weight (3 ± 0 g in 2 days); however, we did not detect any histological abnormalities in brain endothelia or other vascular structures or in parenchyma in non-ischemic hemispheres by light microscopy as reported²¹.

Since no significant differences in rCBF changes, arterial blood pressure and tissue oxygen saturation were observed between groups during ischemia (Table 2), we attributed the reduction in infarct volume and motor function to neuroprotective action of the brain-delivered peptides but not to any potential hemodynamic or systemic changes induced by circulating NPs. Moreover, to verify that effective concentrations of bFGF in the parenchyma were achieved, we measured the phosphorylation level of Akt (a downstream step in bFGF signaling) in wedge-shaped ischemic MCA territory specimens in 14 mice. p-Akt/Akt ratio significantly dropped to $39\pm 4\%$ in mice subjected to 2 h ischemia and 22 h reperfusion and treated with non-functionalized NPs ($n=4$) compared to $66\pm 3\%$ in non-ischemic, sham-operated mice brains ($n=4$, $p<0.05$). Treatment with functionalized NPs restored the p-Akt/Akt ratio in ischemic brain specimens near to sham-operated brain levels ($60\pm 9\%$, $n=6$, $p=0.61$)(Figure-2). Of note, effective inhibition of caspase-3 activity in ischemic mouse brain with z-DEVD-FMK-loaded NPs given at the same dose used in the present study had previously been shown¹⁸.

Detection of NPs in brain parenchyma

Investigators blinded to the treatment evaluated the brain sections to detect NPs in parenchyma in 19 mice (Figure-3). NPs were scattered throughout the brain parenchyma and as the mice were thoroughly perfused before sacrifice, none was found inside vessel lumens except a few adhered to the endothelium. Numerous parenchymal NPs were detected 30 min and 1h after administration, decreasing by 3 h. This observation conforms to the time profile

of NP penetrance to the brain parenchyma by way of transferrin receptor-mediated transcytosis as detected with in vivo brain imaging in intact mice under anesthesia¹⁸. Since NPs could unambiguously be detected only at 1500X magnification, quantification of their distribution across 5 μm -thick sections was not feasible and, hence, was limited by sampling error; nevertheless, NPs appeared to be similarly distributed in ischemic as well as non-ischemic hemispheres with visual screening. In contrast, we did not observe any NPs in brain parenchyma (including the ischemic hemisphere) of the mice given non-functionalized NPs, excluding the possibility that NPs could penetrate the brain through the ischemia-damaged BBB in accordance with the neuroprotection and biochemistry data. NPs had a clearly distinguishable spherical shape (less than 1 μm) when visualized with the streptavidin-horseradish peroxidase technique exploiting the presence of free biotin molecules on the NP surface (Figure-3A). The specificity of this labeling was further confirmed by immunostaining of bFGF peptides loaded to the NPs with anti-bFGF antibodies (Figure-3C). There was no evidence of inflammation or astrocyte end-feet swelling in the non-ischemic brain parenchyma around the NPs, conforming with the idea that chitosan is a biocompatible polymer and does not induce tissue toxicity at microscopic level²³.

DISCUSSION

In this study, we demonstrated that (I) a relatively large peptide like bFGF (17.2 kDa) can be successfully encapsulated into chitosan NPs and targeted to the brain as well as z-DEVD-FMK, a small peptide consisting of four amino acids; (II) systemically administered NPs can transport bFGF to the brain rapidly, efficiently and, importantly, in an active form, which provides significant neuroprotection against stroke induced by 2 hours of MCAo; (III) this effect can totally be suppressed by inhibition of transcytosis across the BBB; (IV)

combined treatment with z-DEVD-FMK- and bFGF-loaded NPs provides additive neuroprotective effect and, is effective even when administered 3 hours post-ischemia.

The active substances encapsulated to NPs were composed of amino acids. Therefore, during preparation of NPs, we chose mild chemical conditions to preserve their three dimensional structures and functional groups²⁴. Indeed, both peptides were found to be biologically active in the brain after systemic administration (see also¹⁸). Systemically administered bFGF-loaded NPs showed neuroprotection only when they were conjugated with antibodies against the TfR-1 located on the brain vascular endothelium. This excludes the possibilities that bFGF released from the NPs to circulation or direct access of bFGF-loaded NPs to the brain through the ischemia-damaged BBB may have provided neuroprotection and, strongly suggests that transcytosis of NPs mediated by the TfR-1 is the main mechanism transporting bFGF into the brain parenchyma. This idea was further supported by complete suppression of the neuroprotection after inhibiting endothelial transcytosis with imatinib^{19, 25}. Despite unambiguous microscopic and pharmacological evidence (i.e. neuroprotection) provided by previous studies^{18, 26}, we did not exclude the possibility that the infarct sparing effect might have been secondary to an endothelial action of the peptides (e.g. as proposed for systemically administrated bFGF²⁷). The present study with imatinib, which allows penetration into the endothelium but disrupts transcytosis by inhibiting the exocytosis from the endothelium to parenchyma, convincingly shows that, for neuroprotection, NPs must be exocytosed from the endothelium to the brain parenchyma and that an endothelial action alone is not sufficient¹⁹. We should emphasize that the total amount of bFGF that we administered to a mouse with NPs is too small (680 ng/kg) compared to the dose (200 µg/kg i.v.) used in studies reporting neuroprotection with systemically administered bFGF to the mouse²⁰, supporting the idea that rather than bFGF spilled to plasma from NPs, bFGF transported to the brain provides neuroprotection, possibly by

achieving parenchymal concentrations comparable to that obtained with i.c.v. injection of 30 ng of bFGF in the study by Ma and his colleagues¹⁵. Rich and selective expression of Tfr1 on brain vasculature unlike the Tfr2 subtype expressed in other tissues may have facilitated preferential targeting of Tfr1MAb-conjugated NPs to the brain. Therefore, targeted delivery of bFGF to the brain not only provides neuroprotection but also reduces the possibility of systemic side effects by reducing the amount of bFGF administered about 300 times. Finally, we should note that the administration of nanoparticles either before inducing ischemia when circulation was still intact or 1 h after reperfusion when the ischemic tissue was at least partly reperfused must have allowed distribution and transport of nanoparticles to all MCA territory. The collateral blood flow to the penumbra must have further sustained the delivery during 2 hours of MCA occlusion.

Earlier work from our group demonstrated that chitosan NPs loaded with small peptides are rapidly transported into the brain when they were functionalized with TfrMAb^{18, 26}. The neuroprotection observed and the activation of the brain bFGF-p-Akt pathway in the present study further illustrate that they can efficiently carry large peptides to the brain as well. This finding is important from a technological viewpoint because it shows that encapsulating large molecules does not unfavorably modify NP transport kinetics and that the preparation procedures used do not lead to loss of the peptide activity, which is extremely vulnerable to minor alterations in the 3D structure or to modifications by small molecules. Importantly, the peptides were also neuroprotective when co-administered 3 h after ischemia onset. This conforms to the 3-hour therapeutic window detected after their intracerebroventricular co-administration¹⁵ and, suggests that a significant amount of the loaded peptide must have been released from NPs in this time window. It is likely that, once inside the brain parenchyma, peptides are rapidly released from the NPs due to degradation of chitosan by enzymatically active forms of human chitinases such as the acidic mammalian

chitinase (AMCase), the di-N-acetylchitobiase and chitotriosidase²³. Supporting this, histologically detectable NPs in parenchyma were decreased 3 h after administration. The upper limit of the therapeutic time window remains to be detected by future studies, as the main aim of the present extensive study was to replicate with systemically administered NPs the neuroprotection and synergy obtained with i.c.v. injection of bFGF and z-DEVD 3 h post-ischemia. The present study also shows that co-administration of NPs loaded with different agents is a viable strategy to obtain additive effects and, technically, is a more practical approach than incorporating multiple agents into the same NP. Therefore, NPs loaded with several other protease inhibitors and survival factors shown to be neuroprotective in stroke models can be combined for more effective suppression of cell death mechanisms without inducing systemic toxicity²⁸.

In conclusion, chitosan NPs targeted to the brain appear to be promising drug carriers to transport large as well as small BBB-impermeable therapeutic agents. They are easy to prepare, rapidly transported to the brain, readily release their content without loss of activity, allow combination of different agents and, do not have any gross toxicity. Therefore, these exciting findings warrant further research to translate the developments to clinical applications.

Conflict of Interest: We do not have any conflict of interest.

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Table 1. Experimental groups.

Groups	Administration time and route	Treatment*
1) TfRMAb-free, control	pre-ischemia, i.p.	Equal mixture of TfRMAb-free NPs loaded with z-DEVD-FMK or bFGF
2) z-DEVD-FMK	pre-ischemia, i.p.	TfRMAb-conjugated NPs loaded with z-DEVD-FMK
3) bFGF	pre-ischemia, i.p.	TfRMAb-conjugated NPs loaded with bFGF
4) bFGF and z-DEVD pre	pre-ischemia, i.p.	Equal mixture of TfRMAb-conjugated NPs loaded with z-DEVD-FMK or bFGF
5) bFGF and z-DEVD post	3 hours post-ischemia, i.p.	Equal mixture of TfRMAb-conjugated NPs loaded with z-DEVD-FMK or bFGF
6) bFGF i.v.	pre-ischemia, i.v.	TfRMAb-conjugated NPs loaded with bFGF
7) bFGF + imatinib	Imatinib; 150 mg/kg i.p. twice per day for two days before the experiment NPs; pre-ischemia, i.p.	TfRMAb-conjugated NPs loaded with bFGF

Groups consist of 6 mice except groups 4 and 7 (n=5 in each). *z-DEVD-FMK (240 ng) or bFGF (17 ng) loaded NPs were administered in 0.1 ml ultrapure water.

Table 2. Physiological parameters of groups

Groups	Blood pressure (mmHg) *	rCBF during ischemia (%)	Tissue oxygen saturation (%)
TfRMAb-free, Control	93±7 (n=4)	29±3	96±1
z-DEVD	95±4 (n=3)	27±2	94±2
bFGF	90±5 (n=5)	28±3	97±1
bFGF and z-DEVD (pre-ischemia)	83±10 (n=4)	30±3	96±2
bFGF and z-DEVD (3 h post-ischemia)	94±5 (n=3)	25±4	95±3
bFGF (i.v.)	98±8 (n=5)	26±3	96±2
bFGF+imatinib	94±3 (n=3)	28±4	96±1

Values are given as mean±SEM. Group means were not significantly different ($p>0.05$).

*Blood pressure could not be measured in some mice for technical reasons. Physiological parameters were recorded during the first 30 min of ischemia before terminating anesthesia.

FIGURE LEGENDS

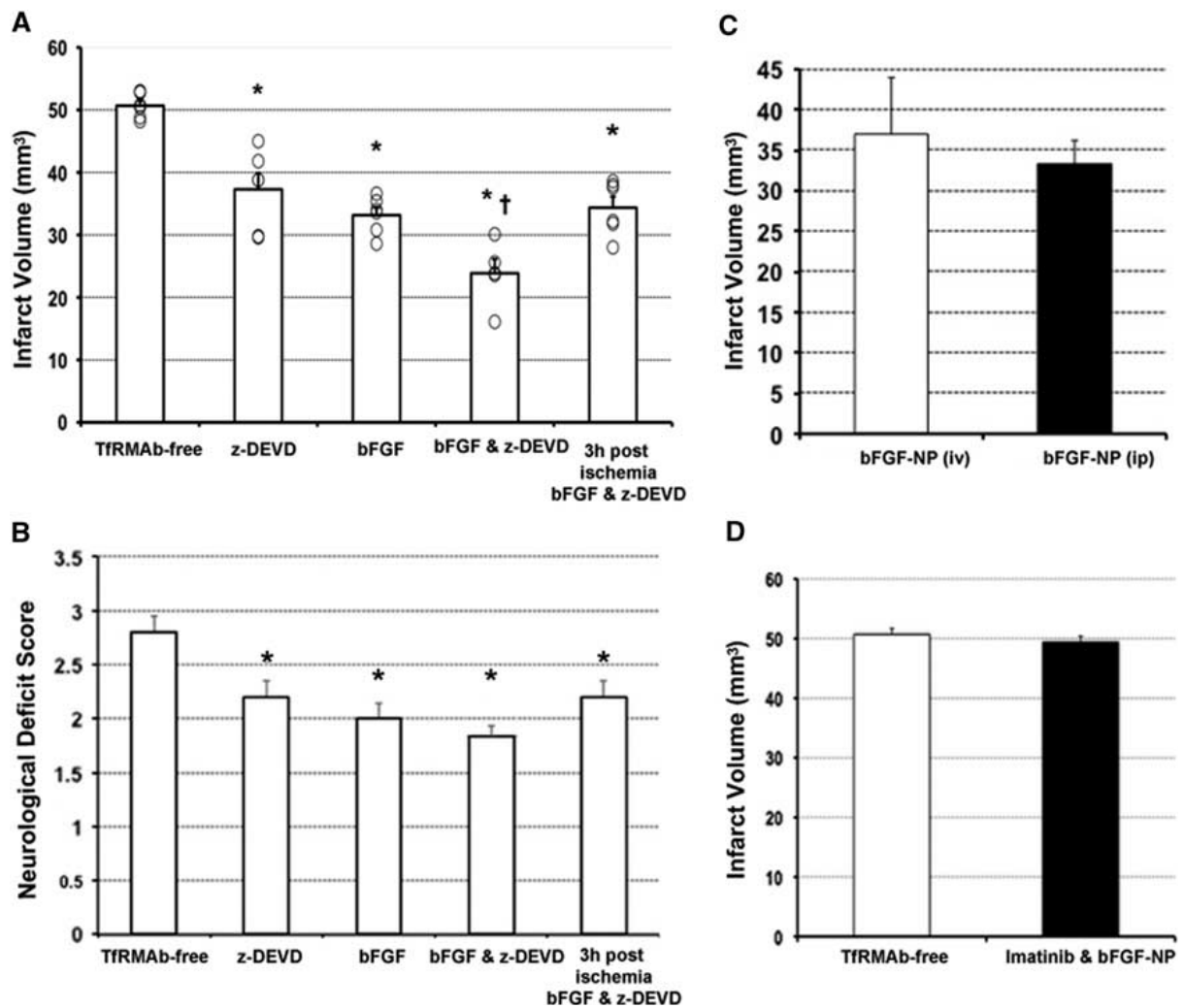


Figure 1: The infarct volumes and motor function deficit scores of the control and treatment groups. Data are presented as mean±SEM. **o**: denotes individual values. **A)** After 2 h ischemia and 22 h reperfusion, infarct volumes of the groups receiving functionalized NPs loaded with z-DEVD-FMK, bFGF or an equal mixture of bFGF and z-DEVD-FMK decreased significantly compared with the group receiving non-functionalized NPs loaded with an equal mixture of bFGF and z-DEVD-FMK (TfRMAb-free, control). Combined treatment was more effective and had a 3-h therapeutic window. **B)** Changes in motor function scores paralleled the infarct volume changes. **C)** i.p. or i.v. administration of functionalized NPs loaded with bFGF was equally effective in decreasing the infarct volume. **D)** When the endothelial transcytosis was inhibited with imatinib treatment, no

neuroprotection was obtained with bFGF-loaded functionalized NPs. * $p < 0.05$ compared to the TfRMAb-free group. # $p < 0.05$ compared to the bFGF or Z-DEVD-FMK-treated groups. The bFGF-NP (i.p.) group in C and TfRMAb-free group in D were reproduced from the data illustrated in A for comparison.

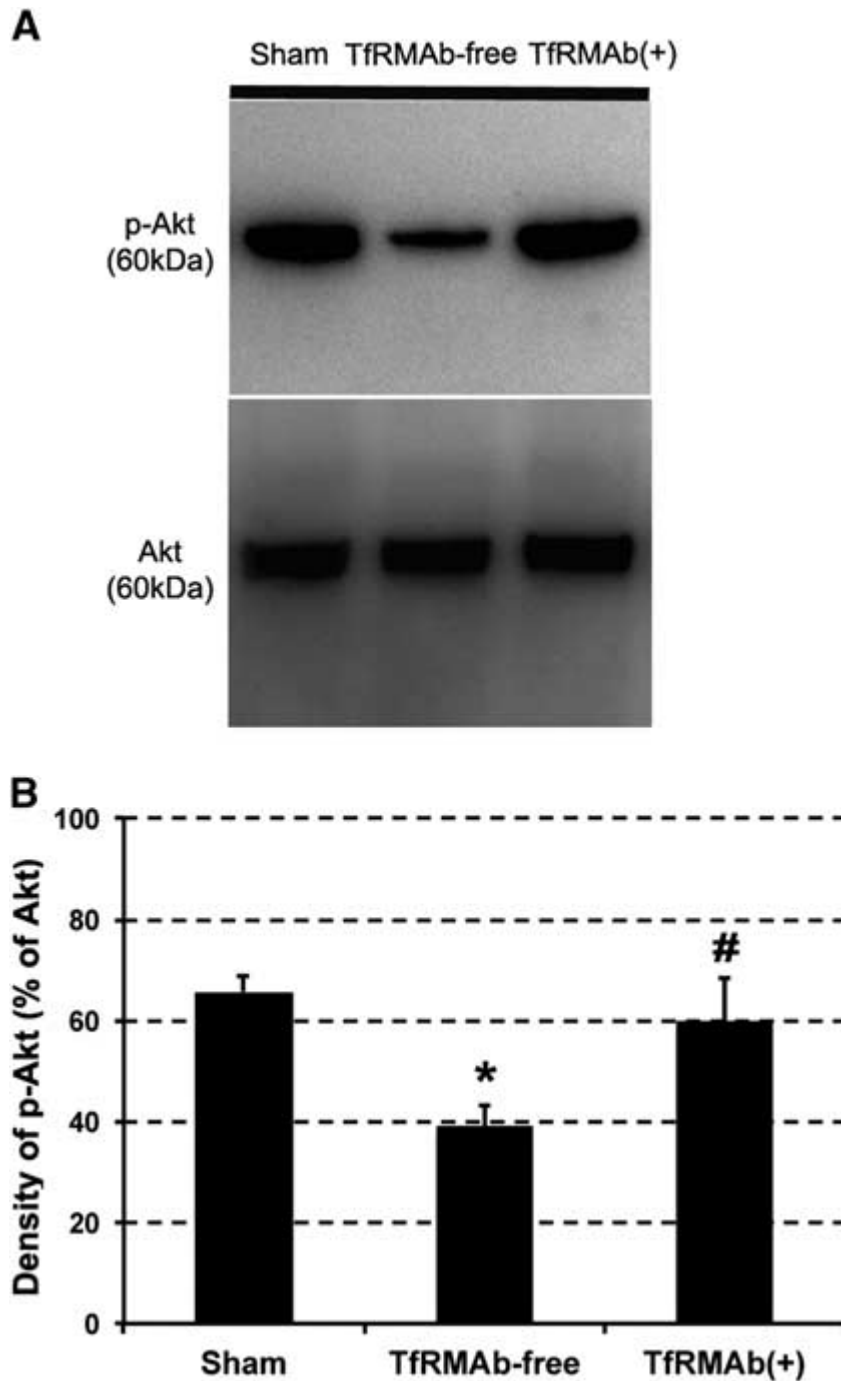


Figure 2: p-Akt/Akt Western blots of the MCA area of mice subjected to 2 h ischemia and 22 h reperfusion. Akt phosphorylation (p-Akt/Akt ratio) decreased in the ischemic MCA area of the group treated with non-functionalized NPs (TfRMAb-free). Treatment with functionalized NPs (TfRMAb+) restored the p-Akt/Akt ratio close to the sham-operated brain levels. *, $p=0.029$ and #, $p=0.61$ compared to the sham-operated group.

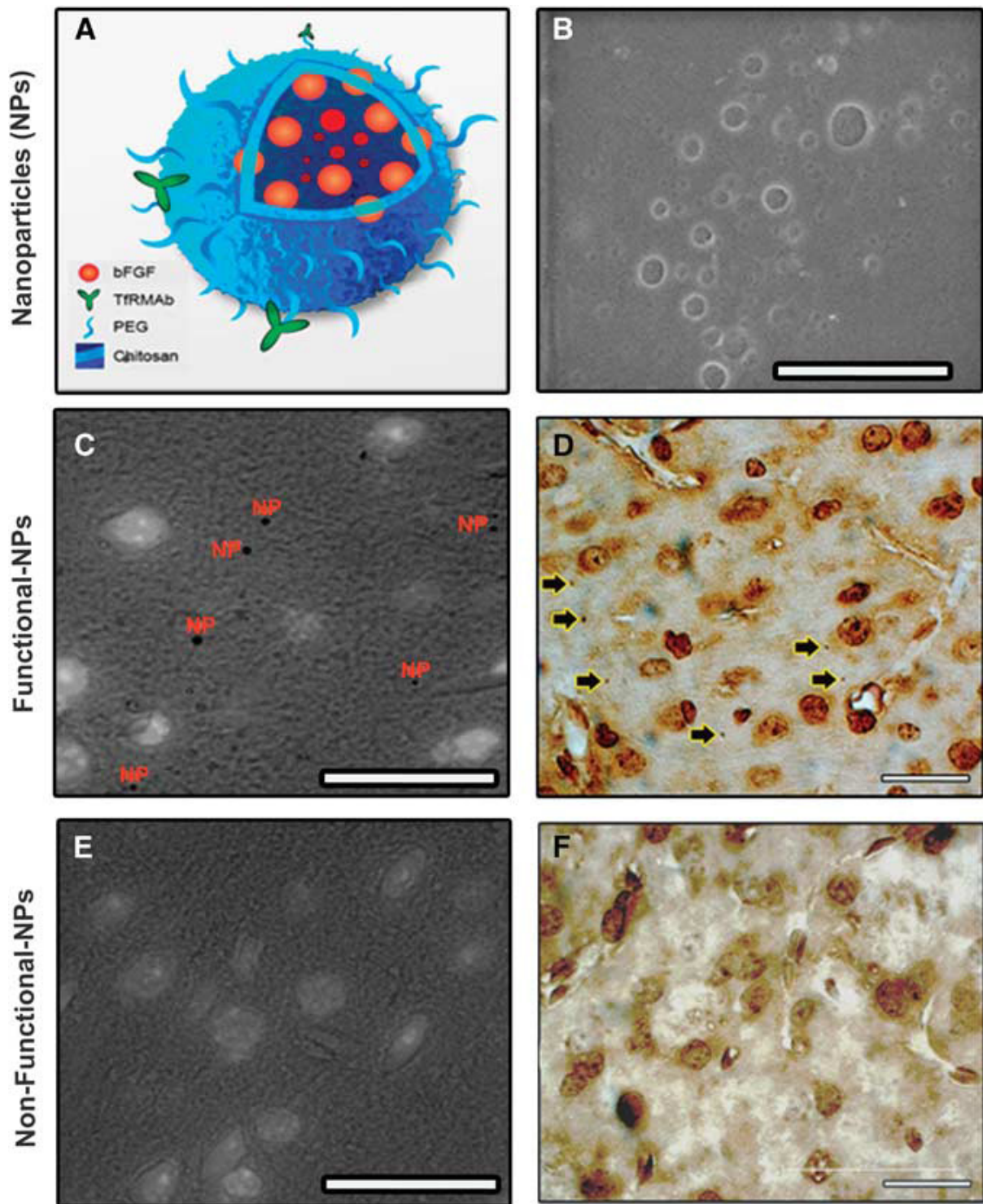


Figure 3: Numerous NPs were penetrated to the brain (cortex) parenchyma 1 h after systemic administration of the functionalized NPs. NPs were prepared by loading peptides (here, bFGF) to chitosan-polyethyleneglycol-biotin graft polymers and, were functionalized by

conjugating streptavidinylated anti-transferrin receptor-1 monoclonal antibody with streptavidin-biotin binding (A). The transmission electron micrograph in B illustrates a group of NPs in suspension; scale bar 2 μm . C; NPs in brain tissue were detected by binding streptavidin–horseradish peroxidase complex to free biotin molecules on the NP surface (NPs). D; Human bFGF molecules on the surface of NPs were also visualised with anti-human bFGF antibody (arrows). Non-functionalized NPs did not penetrate to parenchyma (E, F). Anti-human bFGF antibody has cross reactivity to the mouse bFGF, hence, labeled the cortical cells, especially their nuclei, and caused a light background staining (D, F). In C and E, bright field images illustrating DAB labelling were superimposed with fluorescent images exhibiting Hoechst 33258-labeled nuclei of the same area to show cells nearby NPs. Scale bars: 20 μm .