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RADA16-I Hydrogel-Released CXCL12 Stably Promotes Stem Cell Migration

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Abstract

Introduction: CXCL12 (Stromal cell-derived factor 1α, SDF - 1α) plays an important role in the nervous system development and neural repair, and is gradiently expressed in the central nervous system, mediating proper neural progenitor cell (NPCs) migration and survival. Due to a relatively short half-life and restriction of the blood-brain barrier (BBB) in systemic bioactive factor delivery, the delivery of CXCL12 and other bioactive factors has become a great challenge in research and clinical application.

Aims: In order to observe the change of concentration grade and release time of CXCL12we studied the dynamic CXCL12 prolonged release pattern with the injectable, nontoxic, modifiable and degradable RADA16-I peptide hydrogel. The hydrogel resulted in a relatively stable CXCL12 concentration and provided more suitable microenvironment for stem cell survival and migration *in vitro*. The following major experiments were conducted: 1. Anti-adhesion and neural protection with RADA16-I were performed in rabbit skull trauma model; 2. Fluorescent semi-quantitative CLSM was used for analysis of CXCL12 released from CXCL12 hydrogel mixture, and ELISA for detecting CXCL12 release kinetic curve was utilized; 3. Effective concentration was estimated through the peak concentration of CXCL12 load and was determined with trans-well migration assay.

Results: 1. RADA16-I alone potentially played a role in anti-adhesion and neural repair. Furthermore, RADA16-I hydrogel slow-release system smoothly released chemokine CXCL12 *in vitro*.

2. Stable CXCL12 released system was more effective to be used for tissue repair compared to quickly declined CXCL12 without hydrogel system.

3. The peak concentration (50 ng/ml) of CXCL12 load released from hydrogel was the functional concentration for the induction of directional migration in mouse neuronal progenitor cells (mNPCs).

Conclusions: Slow-released hydrogel system and mixture, RADA16-I and CXCL12, offered effective grade level for cell migration. This system is potentially beneficial for neural tissue protection and neural repair in clinic.

Keywords: RADA16-I; CXCL12; Hydrogel; Migration; NPCs

Introduction

It is well known that neuro-regeneration depends on the proliferation, differentiation, migration, and survival of neural progenitor cells (NPCs). Endogenous NPCs proliferate and migrate into the injured region to promote tissue repair [1]. From observation of animal models, the endogenous NPCs begin to proliferate after migrating into the injured area, but are mostly going to apoptosis. On the other hand, the numbers of endogenous NPCs that proliferate and migrate are limited during neural injury [2]. Inflammation and edema further affect the survival of endogenous NPCs before NPCs are connected to the infarcted area. In addition, most of the transplanted NPCs are not able to play biological functions in this unfavorable local microenvironment [3]. Therefore, improving local microenvironment after neural injury to promote the proliferation and migration of NPCs has become an urgent problem. Exogenous NPCs transplantation should become a potential treatment option with updated microenvironment.

Studies have found that the levels of chemokines affect the prognosis of stroke [4,5]. Among these factorsstromal cell derived factor (CXCL12) is one of the most widely studied chemokines that

plays an important role in neural remodeling and NPCs survival, which induces NPCs migration and promotes NPC survival by activating to CXCL12 specific receptor CXCR4/CXCR7. The former studies have revealed that CXCL12 promoted neurogenesis and angiogenesis during the post acute phase of neural injury without eliciting an inflammatory response.

Therefore, it is crucial to keep the effective concentration of CXCL12 and a suitable micro-environment at the injury site for neural repair. Direct injection of CXCL12 on the site of injury will go through diffusion and protein degradation by different metalloproteinases (MMPs) [6]. In addition, CXCL12 has a shorter half-life and is restricted by blood brain barrier (BBB). Recently, the short self-assembling peptide hydrogel was used as a new release biomaterial for the study of tissue regeneration. RADA16-I (AcN-RADARADARADARADA-CNH) is a novel injectable self-assembled peptide. During the past decades, many new biomaterials, including biodegradable amphiphilic polymers, have been tested for tissue regeneration, and some of the biomaterials have been used to study the release of CXCL12 [7]. The self-assembling peptide nanofiber hydrogel is a suitable three-dimensional cell culture material, which has shown to promote cell migration, and stimulate cell proliferation and differentiation of mammalian skin, cartilage, bone, brain and nerve regeneration in animal studies. It has been demonstrated that neural stem cells were encapsulated successfully in hydrogel matrices of self-assembling peptide nanofibers and presented with marked differentiation into projection neurons, astrocytes and oligodendrocytes [8]. In animal study, hydrogel RADA16-I has been a very effective treatment for tissue damage repair and a good hemostasis effect that accelerates wound healing. With hydrophobic and hydrophilic amino acid quickly and orderly intertwined, the spontaneous assembly that is similar to the extracellular matrix of the three-dimensional reticular fiber structure was created [9]. Under physiological conditions, hydrogel RADA16-I's original solutions were adjusted to neutral pH or that added physiological salt concentration accelerated the gel process [10,11]. Compared to traditional biomaterials, the RADA16-I hydrogel was treated by ultrasonic treatment, which was changed into liquid state by ultrasonic treatment, and semi solid gel was formed after using the saline solution, allowing for injection locally. In addition, in this study, the mixture was composition of polypeptide (0.5%) and water (99.5%). Thus, the metabolites are non-toxic injectable to the human body [12]. Based on the biological characteristics of RADA16-I, a new type of functional self-assembling peptide nano-fiber scaffold material has been widely used in the field of bone, nerve, heart and blood vessel regeneration in pre-clinical trials. After all, this study hypothesized that the hydrogel loaded CXCL12, which regulates microenvironment in the neural injury, improved the efficiency of neurogenesis and angiogenesis.

In this study, we established a sustained and prolonged delivery system of CXCL12 with RADA16-I self-assembling peptide hydrogel. This should be used as a novel therapeutic supportive tool to tissue regeneration in clinical settings.

Materials and Methods

Preparation of RADA16-I hydrogels

RADA16 (AcN-RADARADARADARADA-CONH2) (BD PuraMatrix™) Peptide hydrogels were purchased from BD bioscience (Becton, Dickinson and Company, Shanghai, China) and were used without further dilution. The functionalized peptides were sonicated for 30 minutes to disassemble peptide aggregates and centrifuged to remove air bubbles before usage (Centrifugal force 1500 g, 5 minutes).

New Zealand white rabbit temporal lobe injury model

All rabbits underwent surgical procedure for temporal lobe injury. Briefly, a healthy New Zealand white rabbit was fixed on the stereotaxic instrument after anesthetized with 10% chloral hydrate (5 ml/Kg body weight). A temporal incision was created and the skull was exposed. A piece of skull over the temporal cortex was excised with a drill and the bone window was about 1 cm. Afterwards, dura incision was performed and the forebrain was exposed. The cerebral cortex was contused with syringe needle and electrocoagulation hemostasis, and afterwards, hydrogel was applied to the damaged surface. For the control group, gelfoam was applied with the same procedure and the skin was sutured with 4-0 surgical sutures.

Determination of CXCL12 release in complete medium and RADA16-I hydrogels

Qualitative determination of CXCL12 uptake by cLSM: To visualize the CXCL12 release and distribution within hydrogels, CXCL12 was labeled Alexa Fluor 647. CXCL12-647 was diluted to a final concentration of 50 μg/ml in 0.1% BSA and was mixed with RADA16-I hydrogels. A total of 50 μL mixed solution was added into a glass bottom dish and was then stimulated for self-assembling by adding 1 ml PBS in the dish. The release experiments were performed at 37°C with 5% CO_2 atmosphere, in which the supernatant CXCL12-647 fluorescence intensity was measured at different time points (0 h, 1 h, 6 h) by a confocal scanning laser microscopy (Zess Company). This procedure was repeated for at least 3 times.

Enzyme-linked immunosorbent assay (ELISA) for quantitative analysis of CXCL12 uptake and release in NPC complete medium and RADA16-I hydrogels

We prepared the NPC complete medium solution with 100 ng/ml CXCL12, and then added 1 ml in a 24-well plate and cultured in 5% CO_2, 37°C. We then collected 100 μl of supernatant samples at set time points from the 1 ml complete medium for ELISA test, and added back 10 μL of fresh complete culture medium to the supernatant.

For the experimental group, CXCL12 diluted in PBS (10 or 15 μg/ml) was added to 40 μl RADA16-I and was added 1 ml of complete medium to stimulate self-assembling. To determine the release kinetics of CXCL12 in NPC complete medium, the incubation medium was collected at defined time points (3, 6, 24, 96 and 168 h), and an equal volume of fresh medium was added back to the mixture hydrogels. All collected medium was frozen at -80°C until assayed in duplicate using the ELISA CXCL12 Quantikine kit (R&D Systems, Minneapolis, USA) in accordance to the manufacturer`s instructions.

Mouse NPCs collection and cell culture

The mouse cerebral cortex of each embryo at gestational day E13.5 was dissected and dissociated into single cells by triturating the tissue with a 1 ml pipette. The tissue was filtered through a cell filter with 40 μm pores and centrifuged at 1000 rpm for 5 min. The supernatant was removed and the cells were resuspended at a concentration of 10^6 cells/ml in culture dish. Cell proliferation was performed in serum-free basal medium (NeuroCult, Stem Cell Technologies, Inc, Canada) with 1% penicillin-streptomycin (Gibco), human basic fibroblast growth factor (FGF-2), and human epidermal growth factor (EGF) [13]. To

passage NPCs, the neurospheres were resuspended in accutase and mechanically dissociated by 1 ml pipette.

In vitro NPC characterization

For immunofluorescence staining, mNPC cultures were washed and then fixed using 4% paraformaldehyde (PFA), and permeabilized with 0.4% triton-X in PBS. After blocked by 1% BSA in PBS, mNPCs were incubated with primary antibodies including chicken anti-nestin (1:5000; Novus) and rabbit anti-SOX_2 (1:200abcam) for overnight, and then incubated with corresponding secondary antibodies for 1 h at room temperature. Nuclear DNA was labeled with 40, 6-diamidino-2-phenylindole (DAPI; Sigmae Aldrich) for 2 min after the secondary antibody at room temperature. Covered slips were mounted on glass slides with mounting medium (Sigmae Aldrich). Triple immunostaining was examined by a Zeiss META 710 confocal microscope.

Transwell migration assay

To determine the biological function of the sustained release of CXCL12 from hydrogels, we placed the hydrogels loaded with CXCL12 in the lower compartment of the tanswell system. NPC migration was evaluated using an 8-mm pore size transwell system (Costar) coated with fibronectin (Sigma-Aldirich) at 5 ng/ml and Poly-D-lysine (PDL, Sigma-Aldirich) at 100 mg/ml in PBS overnight. Briefly, NPCs were dissociated into single cells and resuspended in Mouse NeuroCult Proliferation Medium at a density of 10^5 cells/ml. A total of 10^4 passaged NPC in 100 µl were seeded in the upper chamber overnight at 37°C in 5% CO_2. After incubation, the final concentration of 100 ng/ml CXCL12 was added in the lower chamber. After 12 h, 4% paraformaldehyde was fixed, DAPI was stained for 15 minutes, and then washed with PBS for 3 times. The adherent cells on the top side of the filter were removed by gently swabbing with a cotton tip, and pictures were taken in the live cell imaging system, counting the cells migrating to the upper subventricular. The experiment was repeated for at least 3 times. In addition, the samples of the 10 µl experimental group and control group were collected before immunostaining and were frozen at -20°C.

Statistical analysis

Significant differences between the two groups were evaluated using a two-tailed Student t-test. The values were expressed as means ± standard deviation. A P-value less than 0.05 was considered statistically significant (GraphPad Prism). Each cell experiment was repeated for at least 3 times.

Results

Injury brain gross morphology examination

All groups of rabbits underwent craniotomy. In the control group, round wound was observed, with a diameter of about 1 cm, and the whole brain tissue was adhered to the dura mater. From the morphological examination of the cortical injury, the gelatin injection group showed severe contour distortion of the cerebral cortex. Healing was mostly achieved by scar tissue. In the hydrogel injection group, round wound injury diameter was about 0.5 cm. There was surface coverage and whole brain tissue (meninges) without adhesion. The brain tissue wound beneath the surface was smooth, and the color was yellowish (quasi hydrogel). This may be the result of the characteristics of sol-gel transition, in which the hydrogel can well fill into the position and shape of the damage, providing the 3D extracellular matrix to the injured cells and participation in anti-inflammatory response (Figure 1).

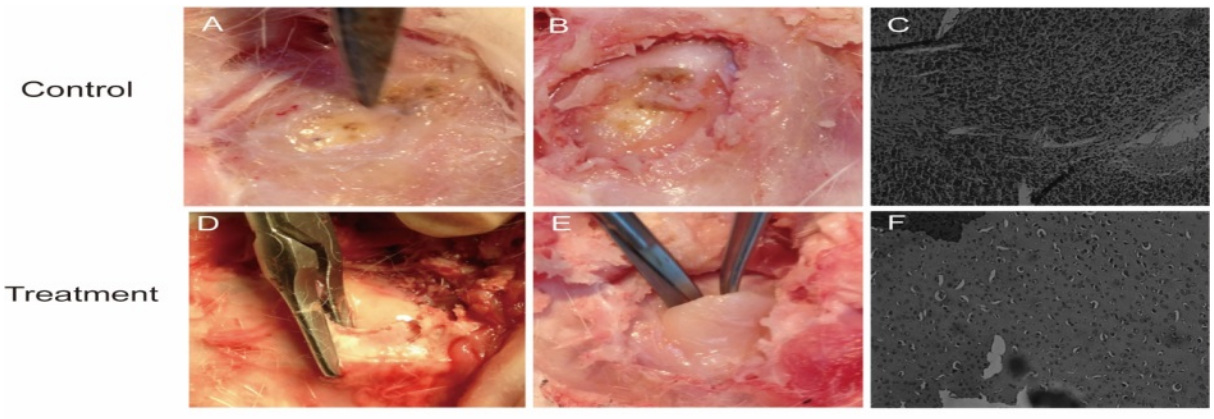

Figure 1: Gross morphological examination of rabbit brain injury model. Control group (A, B, C): Gelatin sponge injection; Treatment group (D, E, F)Hydrogel injection.

CXCL12-647 release in CXCL12-647 loaded hydrogel

The interface between the supernatant and hydrogel demonstrated that CXCL12 was successfully loaded into the hydrogels. Fluorescence images showed that CXCL12-647 was evenly distributed in hydrogel, demonstrating that the hydrogel structure was homogeneous and had good stability (Figure 2A-2C). The CXCL12-647 loaded hydrogel can release CXCL12-647 (Figure 2D) by detecting the CXCL12 fluorescence intensity of the 647 fluorescence channel released at different time points by hydrothermal colloid release and supernatant. The average fluorescence intensity of CXCL12-647 at the interface of the hydrogel and PBS was the strongest at 0 h, and decreased

significantly at 1 h (Figure 2A-2B). There was a statistically significant difference between the two groups, suggesting that CXCL12-647 loaded hydrogels had a rapid release of CXCL12-647 within 1 h (Figure 2D). CXCL12-647 at the hydrogel and fluid interface quickly released protein molecules into the supernatant solution. In the porous structure formed by the gel, the protein molecules in the larger diameter of the pores first diffused out quickly. Whereas the protein molecules in the tangent pores were released slowly. Compared to the PBS group (Figure 2D), the hydrogel could be used as a "storage pool" for CXCL12 to meet the needs of time.

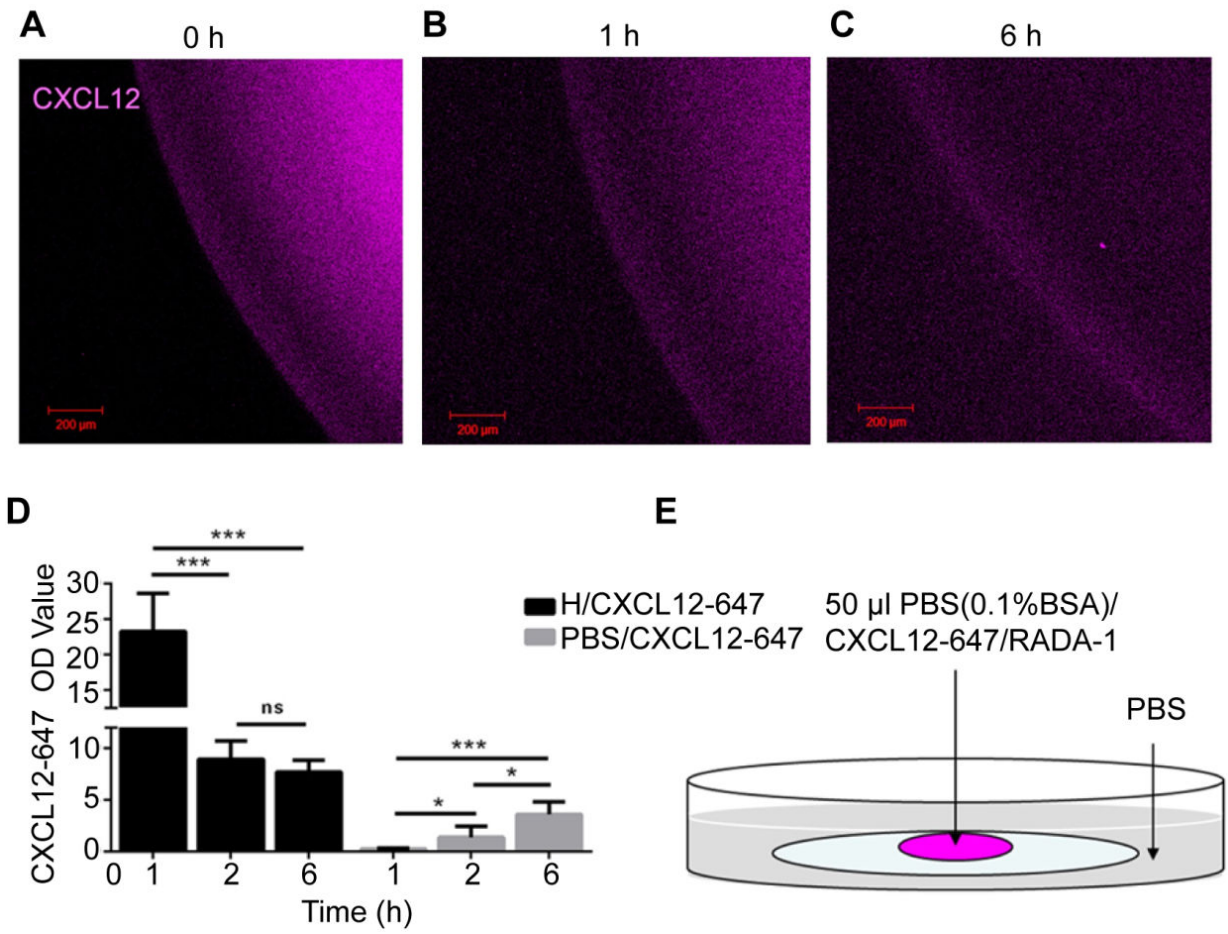

Figure 2: Quantitative CXCL12-647 release experimentsA-Cvisualization of Alex-647-labeled CXCL12 uptake and distribution in the hydrogel at different time points; DAverage optical density of Alex-647-labeled CXCL12 in hydrogel and PBS at different time points; EExperimental model of CXCL12-647 release in CXCL12-647 loaded hydrogel; Scale bar200 μm.

The CXCL12 was rapidly decreased in the complete medium

In the natural state, due to the rapid degradation character and a very short half-lifeCXCL12 could not meet the needs for a long period of time. In 100 ng/ml CXCL12 complete medium solution, the concentration of CXCL12 decreased to less than 50 ng/ml at 1 h and below 10 ng/ml at 6 h, and the CXCL12 concentration that was measured later was lower. Actually, CXCL12 had to be supplied next day *in vitro*. In this experiment, the initial concentration of 1 ml was 100 ng/ml of CXCL12 solution, and the concentrations of CXCL12 at different time points was measured by ELISA to evaluate the concentration reduction. This study confirmed that CXCL12 concentration decreased significantly and rapidly without sustained release. There was a need for a slow release of CXCL12 drug delivery system to solve or to delay the occurrence of slow-release phenomenon in order for CXCL12 to better function in inducing neural progenitor cells. Firstly, the nearby hydrogel and liquid interface the protein molecules released to the supernatant solution was quicker. In the porous structure formed by hydrogels, the protein molecules in the larger pores first spread rapidly, and the protein molecules in the tight pores were released later and at a slower pace. The average fluorescence intensity of CXCL12-647 in the supernatant near the junction of hydrogel and supernatant solution increased with time in 0-6 h, and there was statistical difference. This test demonstrated that CXCL12-647 loaded hydrogel could release its loaded CXCL12-647, confirming the releasability of RADA16-I hydrogels, and laying the foundation for further study of CXCL12 loaded hydrogels.

Quantification of CXCL12-mediated NPCs migration in transwell assays

NPCs derived from E13.5 mouse cortices were characterized through immunocytochemistry. After initial enrichments in mouse NeuroCult NSC Proliferation Medium containing EGF and bFGF, the NPCs cultures expanded in the form of neurosphere. Notably, both neurospheres and adherent cultures derived from neurospheres had specific immunoreactivity with both nestin and SOX2 antibody (Nestin and SOX2 are NPC markers) [14]. The prevalence of nestin and SOX2 double positive cells suggested that the cells used in the transwell assays were indeed NPCs (Figure 3).

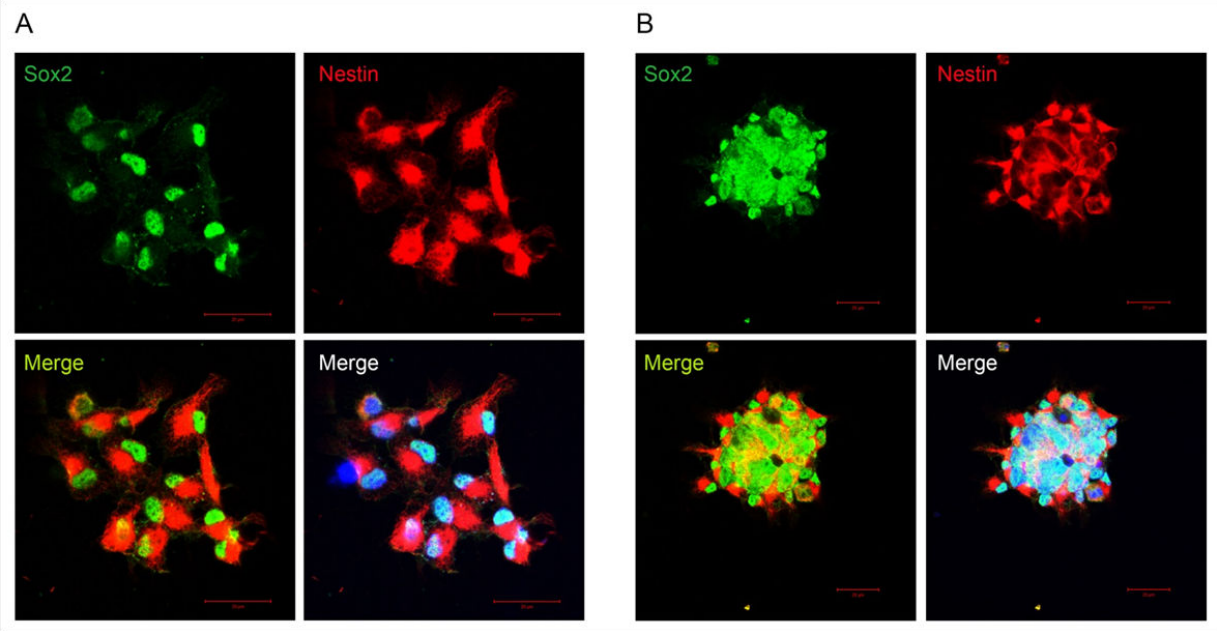

Figure 3: Identification of mouse neural progenitor cells. A: Expression of neural stem cell marker protein in mouse primary cultured neural progenitor cells: SOX2 (green) and Nestin (red), DAPI (blue). B: Expression of neural stem cell marker protein in mouse primary cultured neurospheres. Scale bar 20 μm.

Release kinetics of CXCL12 from RADA16-I hydrogels

The release profiles of different initially applied CXCL12 concentrations from the RADA-I peptide hydrogels are shown in Figure 4B. As illustrated from the curves, the release profile was characterized by an initial outbreak release and a sustained lower release over the course of one week. To explore whether the release profile was dose-dependent, the CXCL12-release was modulated through immobilization from solution of different concentrations of CXCL12 (10, 15 μg/ml). These results indicated that the self-assembling peptide system could be used to control for the release of CXCL12. The release time and plateau concentration of CXCL12 from the hydrogel showed an increased correlation with the concentration of peptide. The concentration of the release platform could be increased by increasing the initial concentration of CXCL12.

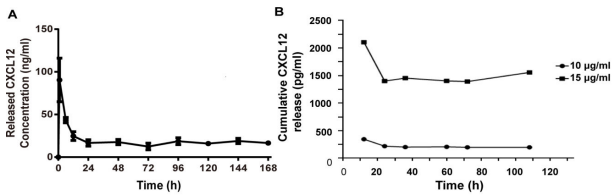

Figure 4: The release profile of different CXCL12 initial concentration in RADA16-I. A: Stable release of CXCL12 was detected until 168 hours (7 days); B: The stable release exhibited dose-depended manner.

The effective concentration of NPCs migration in Transwell assay

The above results demonstrated that RADA16-I hydrogel could release CXCL12 load with a concentration of 100 ng/ml for a short period of time, and maintain a concentration of 25 ng/ml for more than one week. Finally, it is necessary to verify whether CXCL12, which is subsequently released by RADA16-I hydrogel, has a biological function and has the ability to induce migration of neural progenitor

cells. The designed experiment was shown in (Figure 5A). After the trans-well system was coated with FN and PDL, the cells in the lower chamber were filled with 600 μl complete medium and the control group was replaced with 600 μl of the complete medium. The experimental group was replaced with 100 ng/ml CXCL12 complete medium solution, and migration was induced after 12 h. The control group and experimental group migrated to the lower uterine mouse neural progenitor cells. After staining, the number of mouse neural progenitor cells in each field of view was counted with a Live Cell Imaging System. Each sample had more than 6 eyes (random), and the experiment was repeated for 3 times. The result proved hat CXCL12 could induce the migration of neural progenitor cells in the trans-well experimental system. Furthermore, the initial concentration of 100 ng/ml CXCL12 complete medium solution was effective to induce migration of mouse neural progenitor cells. RADA16-I loaded hydrogel released the peak of CXCL12 concentration of about 100 ng/ml, which could meet the effective concentration of induced migration of mouse neural progenitor cells. Therefore, it can be verified by trans-well experimental system that the RADA16-released CXCL12 has the function of inducing migration of mouse neural progenitor cells (Figure 5).

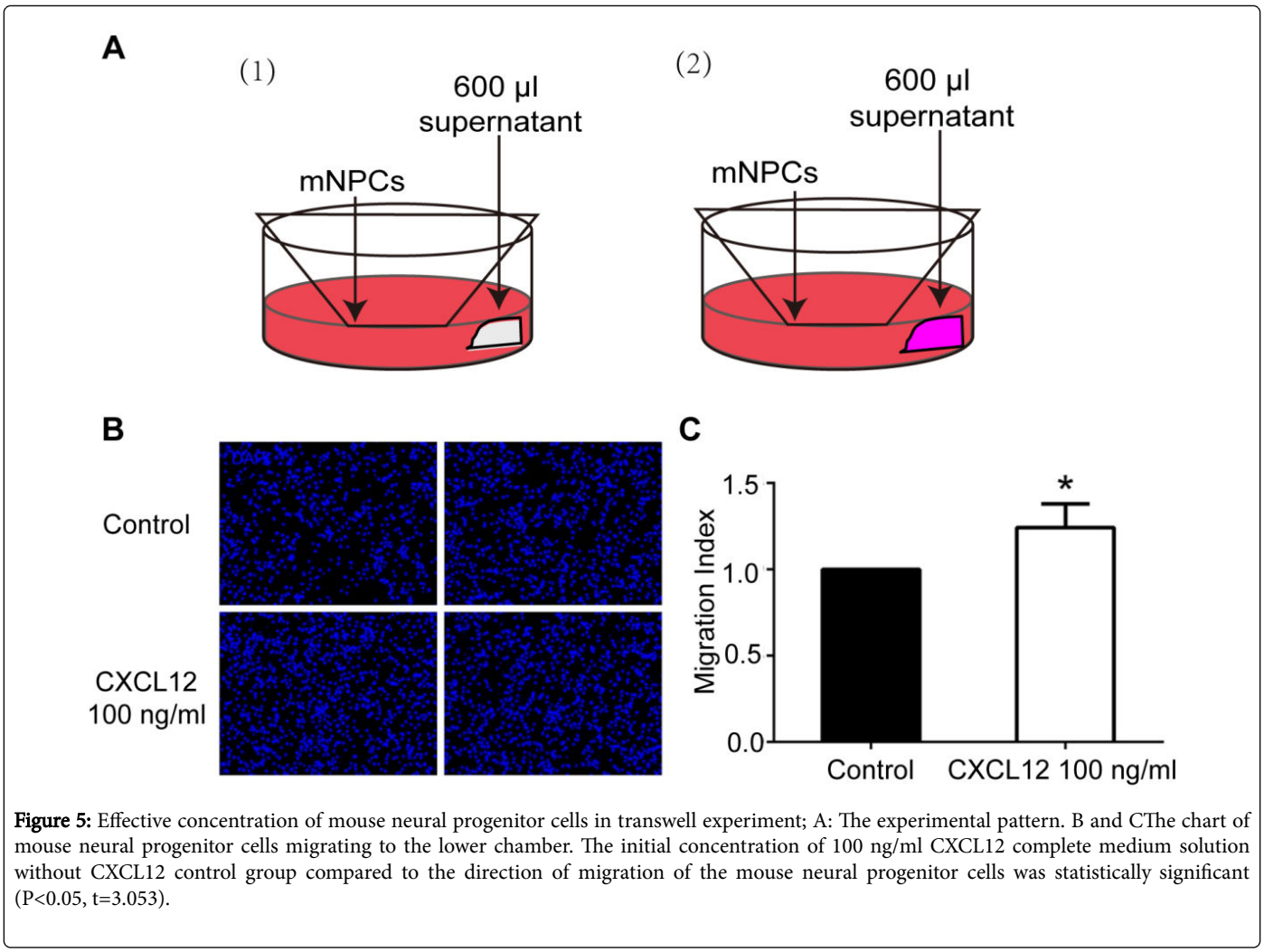

Figure 5: Effective concentration of mouse neural progenitor cells in transwell experiment; A: The experimental pattern. B and CThe chart of mouse neural progenitor cells migrating to the lower chamber. The initial concentration of 100 ng/ml CXCL12 complete medium solution without CXCL12 control group compared to the direction of migration of the mouse neural progenitor cells was statistically significant ($P<0.05$, $t=3.053$).

Discussion

In this study,we demonstrated the protective effect to injury brain tissue and for sustained release of CXCL12. Under normal physiological conditions, high levels of CXCL12 are secreted by ependymal cells, but in pathological cases, such as stroke and brain injury, endothelial cells and glial cells release a considerable amount of CXCL12. Many animal experiments have shown that CXCL12 does not only play a role as inflammatory factor, but also can mobilize neural stem cells and endothelial progenitor cells, promoting nerve repair and stroke prognosis. After a stroke occurs, the NPCs participate in the later process of nerve repair and tissue regeneration. The ability of neovascularization is dependent on the amount of homing NPCs. It has been reported that CXCL12 can regulate the expression of CyclinD1 and β-catenin, promoting proliferation and survival of mNPCs [15]. Thus, prolonged periods (>1 week) of additional CXCL12 are promising in order to promote long-term cell survival, stem cell attraction, and recovery of brain injury or ischemia. After the occurrence of brain injury or ischemia, long recovery time, BBB blocks, and unfavorable inflammatory microenvironment for stem cell survival, more sustained high levels of CXCL12 that help nerve tissue repair would be highly necessary.

RADA16 hydrogels have the potential to be applied to clinical settings with biodegradability. Depending on the size of the 1% polypeptide used, most of hydrogel were excreted in the urine by 3-4

weeks, with no residue in the brain and lung tissue [16]. The release of CXCL12 hydrogel concentration can reach a peak of 100 ng/ml in 1 h, and be maintained at 25 ng/ml for more than 1 week (Figure 6D). Compared to the control group (Figure 6A,C), there was a significantly improved CXCL12 concentration maintenance and duration. Although CXCL12 loaded hydrogel release curve is temporarily unable to meet the clinical application up to one month, hydrogel has a good modification property. Through further modification and adjustment, it can ultimately provide higher and longer duration concentration of CXCL12. Firstly, we can adjust the ratio of CXCL12 to RADA16-I hydrogel. To a certain extent, the higher content of CXCL12, the higher the relative peak value and longer duration of CXCL12.

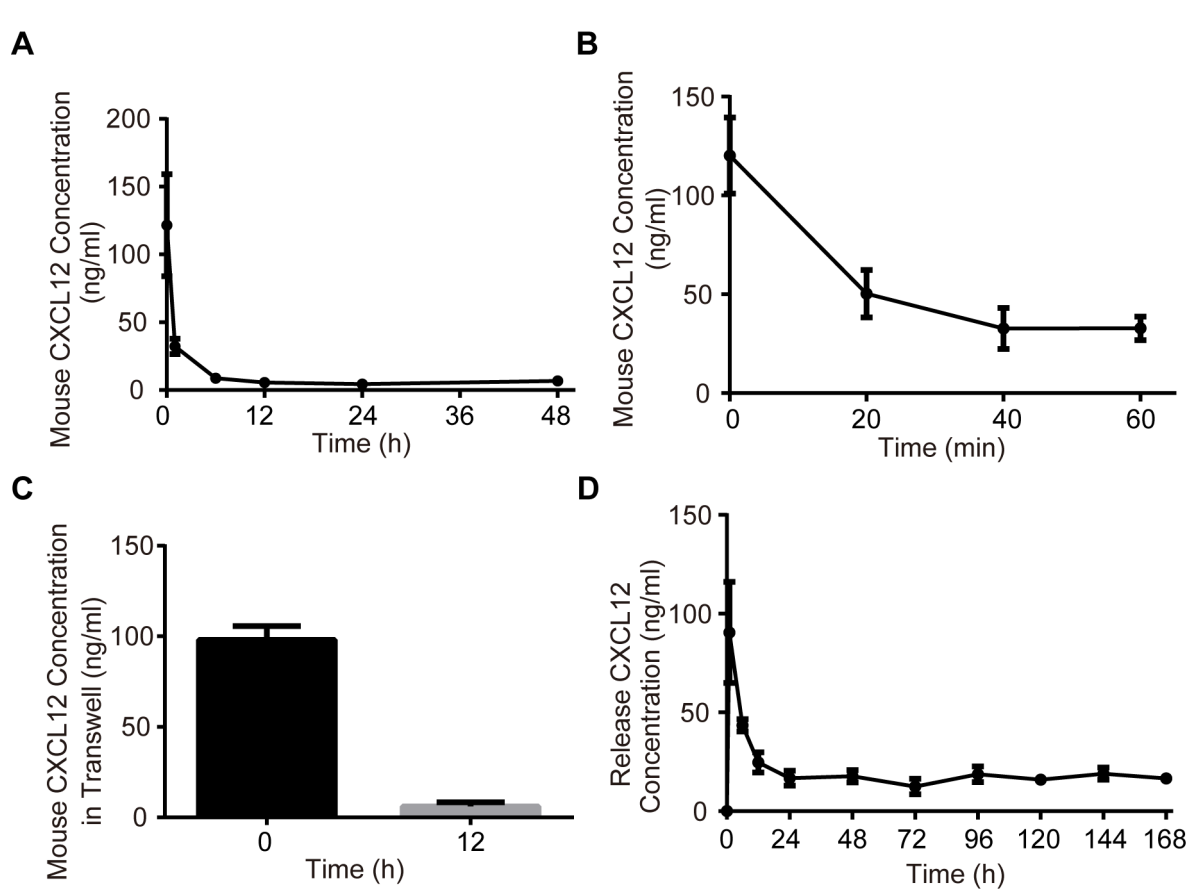

Figure 6: Release kinetics of CXCL12 from RADA16 -I hydrogels in medium solution; A: The concentration of CXCL12 was determined by ELISA in 100 ng/ml CXCL12 complete medium solution at the time of 0, 1, 6, 12, 14 and 48 h; B: The concentration change curve of CXCL12 in 1 hour; CCXCL12 concentration changes in transwell systems in 12 h; DRelease kinetics of CXCL12 in RADA16 -I hydrogels in 168 h.

Furthermore, RADA16-I is electrically neutral. The negatively charged aspartic acid is near the interior of the structure, while the positively charged arginine is near the outside; thus, exhibiting a positively charged nature. Modification of RADA16-I with RADA16-PFS is positively charged and RADA16-DGE is negatively charged [17]. While CXCL12 is negatively charged, according to the principle of the same charge repulsion and heterogeneous charge attractionCXCL12's release rate can be adjusted by modification of RADA16- I, and becomes more suitable for chemokine release mode in clinical application and scientific research. The initial burst effect may be the result of CXCL12 in the interface between the hydrogel and the sustained release medium, and passing through the BBB is expected to lead to high conversion rates.

Once in the circulation, the elevated plasma levels of active CXCL12 would raise the possibility for mobilization and migration of neural progenitor cells. A major, yet, still unsolved issue is that the CXCL12 loaded hydrogel needs to be injected into the hippocampal DG region of mice by stereotactic injection. The RADA16-I intracranial metabolic time is required to observe the duration of inflammation compared to direct injection of saline or 100 ng/ml CXCL12.

Conclusion

As a localized and precise long-term delivery of CXCL12 from the sustained release system, the self-assembled peptide hydrogel RADA16-I can be considered as a valuable option. However, the CXCL12 delivery dynamics are determined by the loading amount and include an initial burst release. We also examined hydrogel and its delivery of CXCL12 enhancing the migratory efficacy, which suggested that this system is potentially useful for tissue anti-adhesion and neural tissue protection in neural injury.

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