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Hyaluronic acid hydrogel immobilized with RGD peptides for brain tissue engineering

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Abstract In this paper, hyaluronic acid hydrogels with open porous structure have been developed for scaffold of brain tissue engineering. A short peptide sequence of arginine–glycine–aspartic acid (RGD) was immobilized on the backbone of the hydrogels. Both unmodified hydrogels and those modified with RGD were implanted into the defects of cortex in rats and evaluated for their ability to improve tissue reconstruction. After 6 and 12 weeks, sections of brains were processed for DAB and Glees staining. They were also labeled with GFAP and ED1 antibodies, and observed under the SEM for ultrastructral examination. After implanting into the lesion of cortex, the porous hydrogels functioned as a scaffold to support cells infiltration and angiogenesis, simultaneously inhibitting the formation of glial scar. In addition, HA hydrogels modified with RGD were able to promote neurites extension. Our experiments showed that the hyaluronic acid-RGD hydrogel provided a structural, three-dimensional continuity across the defect and favoured reorganization of local wound-repair cells, angiogenesis and axonal growth into the hydrogel scaffold, while there was little evidence of axons regeneration in unmodified hydrogel.

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Introduction

Neurological problems arising from traumatic brain injury (TBI) occur from physical deformation of the brain, which leads to complex biochemical and molecular cascades. Most experimental therapies for TBI currently use pharmacological agents in an attempt to reduce the sequelae of injury in the acute phase and to enhance the function of remaining brain tissue during the chronic stage of the injury process [1]. These treatments do not focus upon the regeneration or replacement of damaged or necrotic nervous tissue. Brain tissue engineering may be a promising option for repair of brain traumatic injury. Tissue engineering in the post-injury brain has several potential advantages over pharmacologic and cell strategies, including the ability to bridge structural gaps for reconnection of neuronal processes, targeting of a spectrum of biochemical and molecular alterations chronically, and eliciting specific cell behavior through scaffold modification. Moreover, providing a scaffold for neurite outgrowth may assist in implant integration and serve as a bridge for endogenous cell migration and axonal elongation.

The scaffold is a critical factor for brain tissue engineering. Various materials, including collagen [2], polycarbonate [3], polylactide [4], poly(2-hydroxyethyl methacrylate) (PHEMA)[5, 6] and poly[N-(2-hydroxylpropyl) methacrylamide] (PHPMA) hydrogels [7, 8], have been used as scaffold in experimental brain lesions in animal models, with various results. One of the approaches for developing scaffolds for tissue engineering is to mimic the structure and components of the extracellular matrix (ECM) of the tissue to be regenerated. Hyaluronic acid (HA) has long been recognized as an important component of the brain ECM [9], and it plays a critical role in the formation of the brain [10]. A few years ago an HA -based brain ECM model was developed [11]. HA was shown to promote nerve

regeneration in peripheral nerve conduits [12], indicating that it may be a suitable candidate for a tissue engineering scaffold in the brain. This prompted the current study, which is the first using an HA hydrogel as scaffold for brain tissue regeneration.

A short peptide sequence of arginine–glycine–aspartic acid (RGD) was attached into HA hydrogel in an attempt to promote cellular infiltration and the ingrowth of regenerating CNS axons. RGD sequences are natural components of ECM macromolecules [13, 14] and play a major role in promoting tissue organization and regeneration, including nervous tissue, because of the existence of ligand–receptor recognition systems that operate through cell surface integrin receptors [15, 16]. These molecules, when chemically attached to the backbone of hydrogels, have been shown to be biologically active [17, 18]. In present study, HA hydrogel was immobilized with the bioactive peptide RGD as the supporting matrix for brain repair. Host tissue reaction and growth was examined. Our experiments showed that the HA-RGD hydrogel provided a structural, three-dimensional continuity across the defect and favoured reorganization of local wound-repair cells, angiogenesis and axonal growth into the hydrogel scaffold.

Materials and methods

Hydrogel preparation

HA was crosslinked to form hydrogels by the protocol as previously reported [19]: HA sodium salt was dissolved in 100 ml distilled water to a concentration of 1 mg/ml. 1.2 g Adipic dihydrazide (ADH) was added to the solution. The pH of the solution was adjusted to 3.5–4.75 using 0.1N HCl. 0.6 g carbodiimide ethyl *N,N*-dimethylaminopropyl carbodiimide reagent (EDCI) was then added to the solution. After stirring thoroughly the mixture was allowed to gel at room temperature. The hydrogels were washed thoroughly with phosphate buffer solution (PBS, pH = 7.2) five times (5min/per times) in 48 h and lyophilized.

Activation of hydrogel scaffold and peptide immobilization

The hydrogel was activated with 1,1′-carbonyldiimidazole (CDI) (Sigma- Aldrich, St. Louis, MO) using the protocol described in the previous report [20]. The hydrogel was washed in acetone for several times then in dry acetone for three times. A CDI solution in dry acetone (20 mg/mL) was then incorporated into the hydrogel. After 10 min reaction with gentle stirring, the hydrogel was washed with dry acetone, five times with 5 mL each, to remove the uncoupled CDI. The peptide RGD from laminin chain purchased from Sigma (St. Louis, MO) was dissolved in sterile 100 mM $NaHCO_3$ (pH = 8.5) at the concentration of 50 μg/mL.

Peptide coupling to CDI activated hydrogel was performed for 36 h with gentle shaking at room temperature. Then the hydrogel was washed thoroughly using PBS five times in 48 h and lyophilized. The re-swelled hydrogel for animal implantation was kept in sterile 0.1 M PBS at 4°C.

Assay for the immobilized peptide

In order to estimate the amount of peptides immobilized to the hydrogel, the starting concentration of the pre-immobilization mixture and the end concentration of post-immobilization mixture were determined with the BCA protein assay kit. The amount of peptides immobilized to the hydrogel was calculated using the formula P = (A–B)V/C, where A = concentration measured before immobilization, B = concentration after immobilization, C = weight of hydrogel in dry state and V = the volume of the solution.

Microstructure study of hydrogel

To investigate the morphology of the microstructure, a piece of the scaffold was lyophilized. Freshly broken section was chosen and sputtered with a gold film, then observed under a scanning electron microscopy (SEM, JSM-6460LV) with an accelerating voltage of 20 kV.

The porosity was evaluated by the absorption of the water and mercury intrusion. A block of weighed dry gel (W_{dry}) was immerged into PBS. After balance for three hours, the hydrated gel was bolted off the free water on the surface and weighted (W_{wet}). The hydrated ratio was calculated by the formula:

$$H = (W_{wet} - W_{dry})/W_{wet}.$$

A mercury intruder of AutoPore IV (Micromerities Instrument Co. USA) was applied to measure the distribution of pores diameter and specific surface area of hydrogels in dry state.

Implantation surgery

All procedures involving animals and their care were conducted in conformity with the National guidelines for experimental animal in China published in 1998. The hydrogels was soaked in 75% alcohol solution about 40 min for sterilization, then rinsed with 0.01M PBS for three times to remove alcohol components. Experiments were performed on 8-week-old Sprague-Dawley female rats which weighed approximately 220–250 g. Animals were divided into three groups: group I implanted HA hydrogels (unmodified, 12 rats), group II implanted HA hydrogels modified with laminin (12 rats),

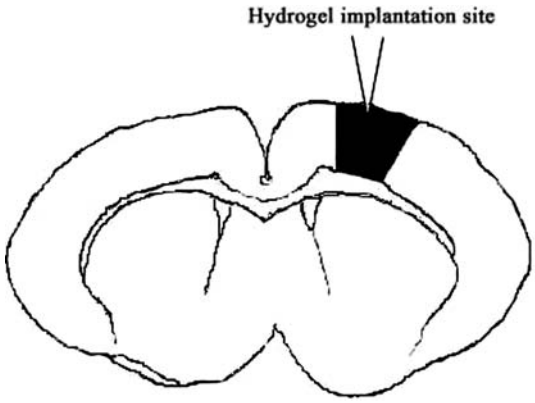

Fig. 1 Scheme of lesion site HA hydrogels were implanted in the right hemisphere shown as a shaded area in the coronal section

and group III injured only (control, 6 rats). Animals were anesthetized by an intraperitoneal injection of 6% chloral hydrate solution (0.6 ml/100 g), and then placed in a stereotactic apparatus. The head and the neck were shaved and thoroughly cleaned. Under aseptic conditions, a midline incision was made and a bone flap of about $3 \times 5\,mm^2$ on the left of the midline was removed. The dura was incised and peeled away in order to expose the parietal cortex. A block of cortical tissue ($2.0 \times 3.0 \times 2.0\ mm^3$) was removed and a lesion cavity was made in the left frontal cortex region. A piece of hydrogels sized to the dimensions and shape of the cavity was placed into the lesion site scheme of lesion site as shown in Fig. 1. Haemostasis was controlled with thrombin soaked gelfoam. The bone flap was returned and the scalp sutured to close the wound. The rats were given antibiotics (ampiciline 200–400 mg/kg) at the end of the surgical procedure.

Histological assay

The rats were sacrificed at 6 or 12 weeks after implantation surgery with a lethal dose of pentobarbital (100 mg/kg, i.p.) and transcardically perfused with 100 ml of 0.9% NaCl and then with 300 ml 4% paraformaldehyde in 0.01 M PBS.

The cerebrum of the rats was fixed for 48 hrs in the solution of 4% paraformaldehyde containing 30% sucrose at 4°C. Sections of 30 μm thickness were cut in a cryostat for cytohistology. The brain sections were processed for haematoxylin-eosin (HE) and cresyl violet staining. Some were evaluated histologically using the Glees technique (silver staining) to detect nerve fibers. In order to reveal new blood vessels by the technique of DAB staining, some animals were directly overdosed with 6% chloral hydrate solution without being perfused with saline solution and paraformaldehyde in different time points. Then brains were post-fixed in 4% paraformaldehyde containing 30% sucrose for 72 h and 60 μm frozen coronal sections were collected. After washing in 0.01M PBS (3×5 min), sections were incubated in 0.05% DAB solution plus 0.3% H_2O_2 solution for 20 min at room temperature. Finally sections were washed in PBS (3×5 min), mounted on slides and coverslipped.

Immunohistochemistory

The immunostaining was performed with mouse anti-rat monocytes/macrophages (clone ED1; Sigma, 1:800) and anti-Glial Fibrillary Acidic Protein (GFAP, Dako, 1:100). A series of sections was incubated overnight at 4 °C with different primary antibodies (diluted to 1:200), all diluted in PBS containing 0.2% Triton X-100. After rinsing (3×10 min), sections were incubated at room temperature with appropriate secondary antibodies (either anti-mouse or anti-rabbit FITC; Sigma) diluted 1:50 in PBS solution. Sections were washed in PBS, mounted onto subbed slides, and then slipcovered. To quantify the tissue reactions adjacent to the implant, the number of positive ED1 ad GFAP cells in the interfacial area was counted in rows of squares along the implant surface.

SEM analysis

After 6 and 12 weeks implantation, the animals were anesthetized and perfused through the heart with solution of 4% paraformaldehyde plus 2% glutaraldehyde. Brains were processed for SEM studies.

Brains containing the hydrogel were dehydrated through ethanol solution of 70, 80, 90, 95, and 100% for 5 min each. Then tissues were air-dried at room temperature and mounted on stainless-steel stubs. The tissues were processed and examined as described above.

Results

Porous structure study

The surface morphologies of the lyophilized cross-linked hyaluronate hydrogels were examined by SEM. Polymer scaffolds for tissue engineering must be highly porous with large surface/volume ratios to accommodate a large number of cells, and to allow the neovascularization of the matrix [21]. Fabrication of porous biomaterials has been achieved using several methods, including freeze-drying [22]. An advantage of this process is to produce pores of uniform pore diameter using only water as the porogen. The hydrogel had the porous structure

reflecting the ice crystal formation in the homogeneous dispersion of the hydrogel. SEM examination of features of the hydrogel in dry state showed that the hydrogel was porous and contained an interconnected pore network (Fig. 2). The micrographs indicated the presence of highly

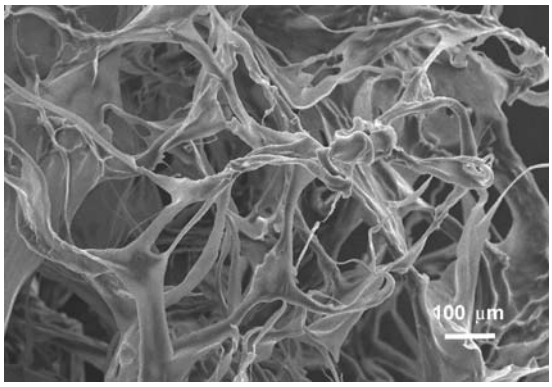

Fig. 2 SEM micrograph of the HA-RGD hydrogel scaffold shows the interconnected pore structure and various pore sizes

porous structure in the cross-linked HA derivatives with average pore diameters ranging from 10 to 50 μm. The ratio of the water absorption was about 98 $\pm$ 2% in weight, which showed the high porosity of the gel. Such a high porosity may provide enough space for migration of neural cells.

Assay for the immobilized peptide

The concentration of peptides in PBS was determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL), in order to calculate the amount attached to the HA hydrogel surface. The calculated attached quantity was 25 μg/mg hydrogel (in dry state).

Histology assay

All the rats recovered from the anaesthesia one hour after the operation and behaved normally one week after operation. After transcardical perfusion, the skull was dissected by scalpel to expose cerebrum with the injury. No hydrogel implant was found to move from the implantation locus. Hydrogels implanted into the lesion of cortex still exist in the site after 6 weeks. All of brains that contained gels implants were well attached to the surrounding host cerebral cortical tissue. Six weeks after implantation, there was no cavum descended from tissue liquefaction and putrescence or envelopes formed by hyperplasia of connective tissue between the implants and host cortex in of hydrogels unmodified and modified. In the control group injured only, tissue defects still existed and any phenomena of cells and extracellular matrices infiltration could not be seen in this region.

No glial scar was found at the interface of host and hydrogel in both the untreated HA hydrogel and HA-RGD hydrogel. The porous network structure of the unmodified hydrogel could be clearly seen by SEM (Fig. 3a) after

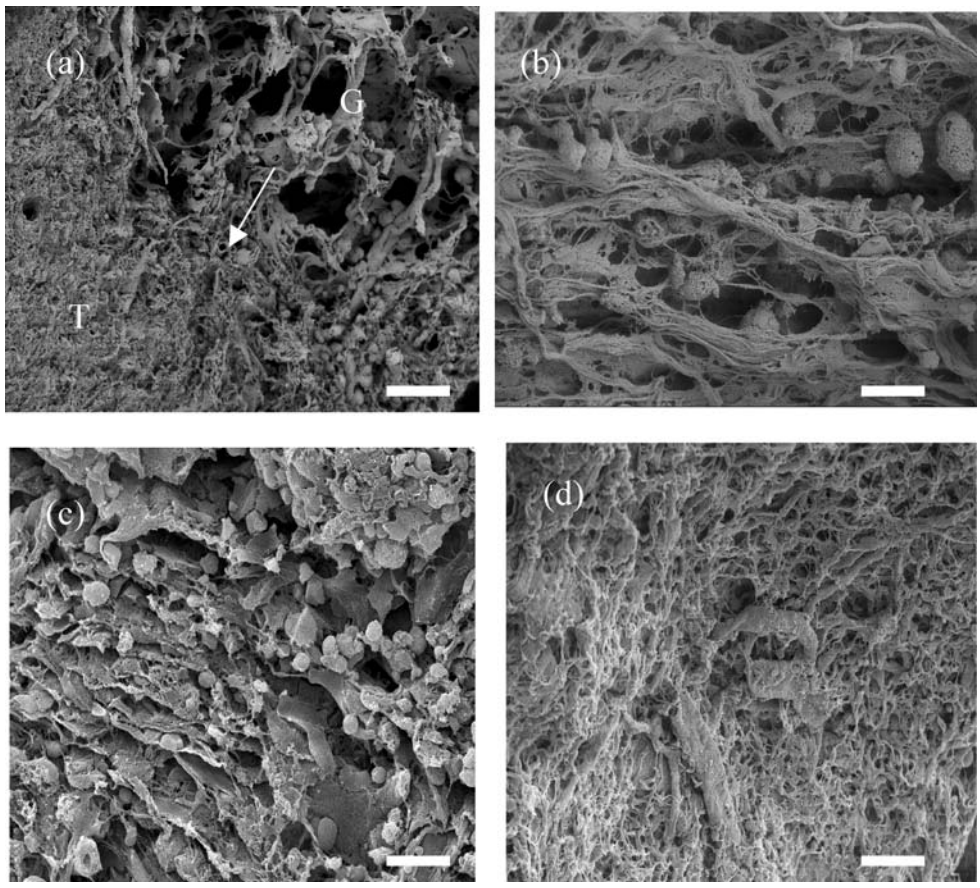

Fig. 3 SEM examination of the implantation site showing the structure of hydrogels filled in the defects of the cortex. Hydrogels have merged with the normal tissue of the brain and reestablished the anatomical continuity of the tissue 6 weeks post implantation. Note the interface between the hydrogel and host tissue (a). The hydrogels the three-dimensional network (*arrowhead*) is invaded by ample cells (*arrows*) that migrate from the adjacent host tissue in the group of hydrogels modified (b) and unmodified (c). Bundles of like collagen (*arrows*) could be observed between invaded cells in the grafts under high power (d). Scale bar = 40 μm (a); 10 μm (b, c); 2 μm (d); G is the hydrogel implant. T is host tissue

6 weeks of implantation. It was difficult to clearly distinguish a sharp interface between the hydrogel and the brain tissue because of the gel integration into the host; there was no discontinuity between the polymer surface and the tissue. The total available surface of the hydrogel merged with the cut surface of the brain tissue. Numerous host cells had migrated into the HA-RGD hydrogels (Fig. 3c) after 6 weeks implantation. After 12 weeks implantation, degradation and loss of the structure of unmodified hydrogels was observed and new extracellular matrix including collagen fibers secreted by the host cells were found to have formed in the network (Fig. 3b). The structure of the HA-RGD hydrogel could hardly be discriminated and newly regenerated blood vessels could be observed (Fig. 3d, arrow).

In order to assess the host brain cellular response to the presence of hydrogel, immunohistochemical probing of GFAP and ED1 was employed. These surface markers are indicative of the amount of reactive gliosis attributed to reactive astrocytes and phagocytic microglia/macrophages, respectively. An intensely stained GFAP network of astrocytes was found within implanted RGD-containing polymers and unmodified hydrogels. However, the host cellular reaction to RGD containing hydrogels was significantly greater than that seen in unmodified hydrogels. Large numbers of $GFAP^+$ astrocytes

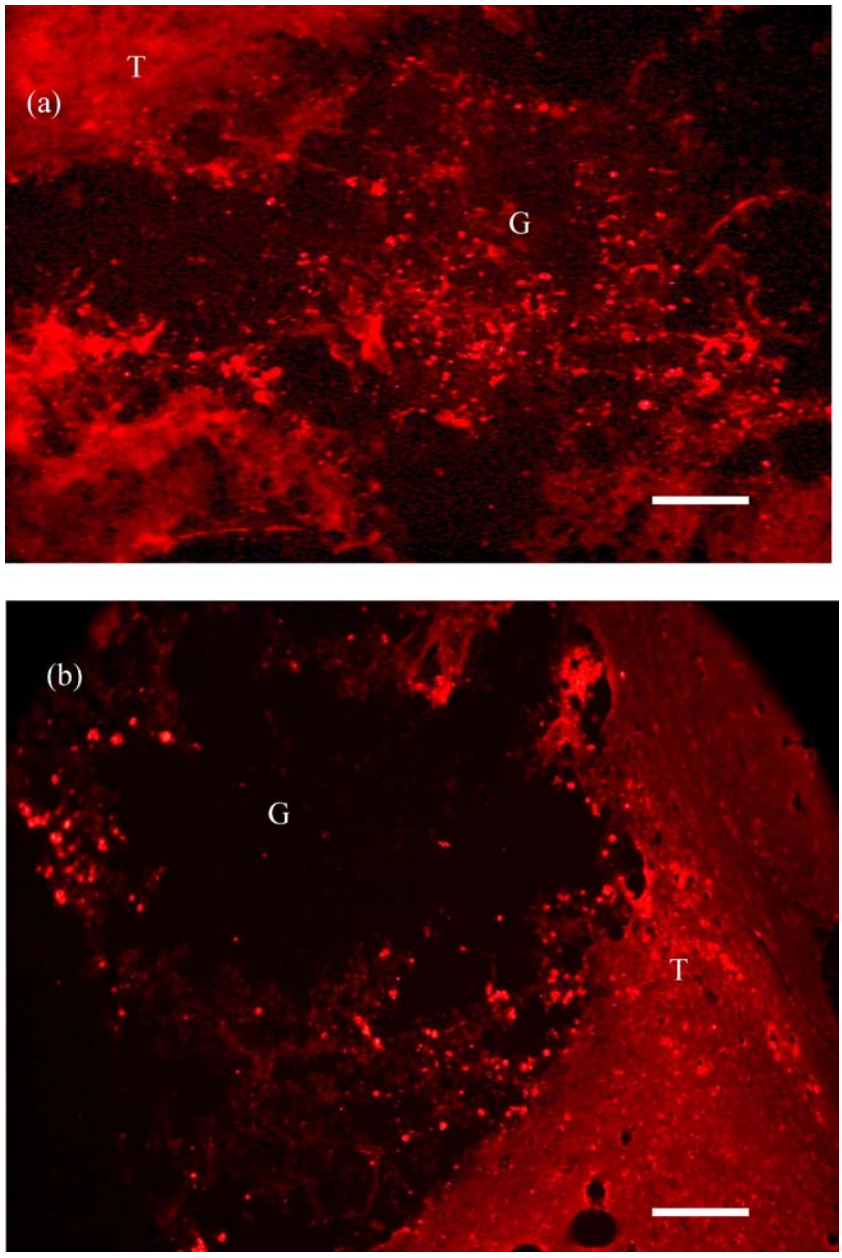

Fig. 4 Photomicrographs of immunostaining section of the brain implants with hydrogel to show the GFAP positive cells that migrated into the hydrogel. (a) HA-RGD hydrogel (b) Unmodified hydrogel. G is the hydrogel implant. T is host tissue. The bar is 100 μm

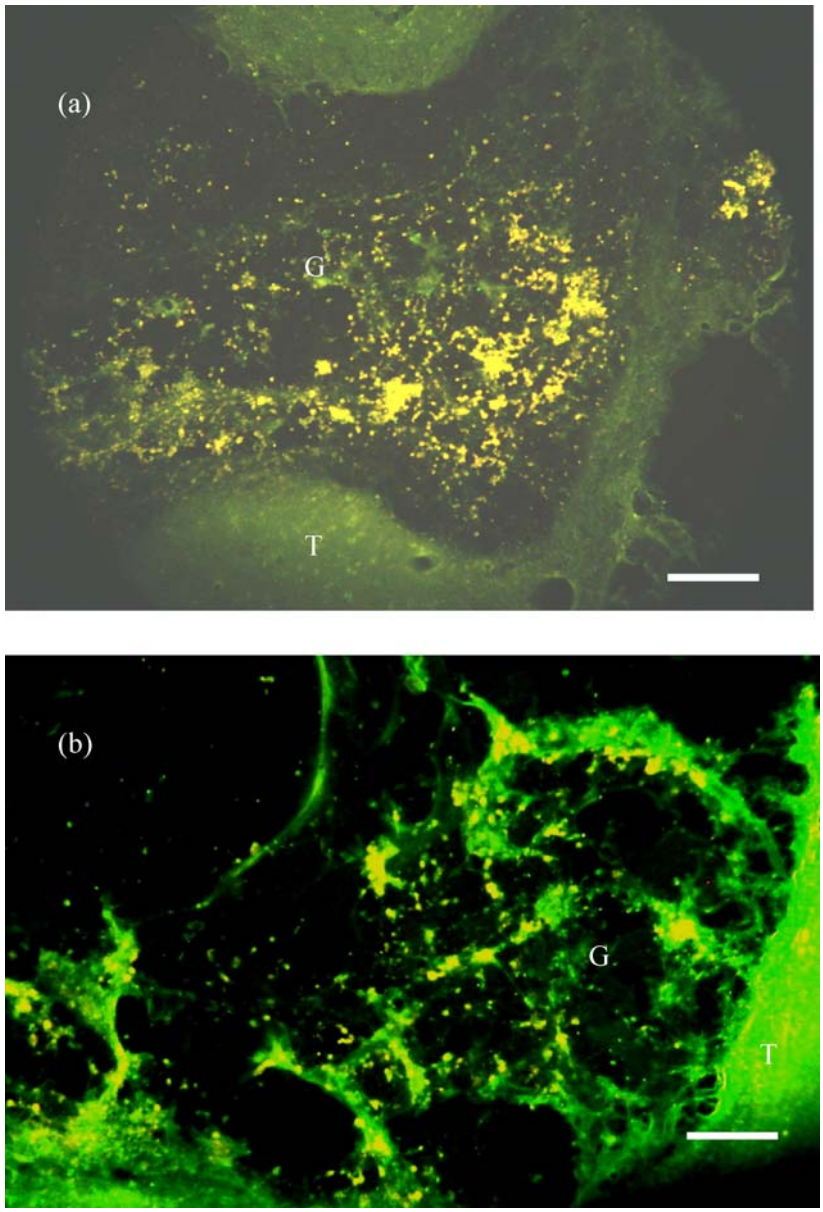

Fig. 5 Photomicrographs of immunostaining section of the brain implants with hydrogel to show the ED1 positive cells that migrated into the hydrogel. (a) HA-RGD hydrogel (b) Unmodified hydrogel. G is the hydrogel implant. T is host tissue. The bar is 100 μm

had migrated into these modified HA hydrogels, producing a cellular network (Fig. 4a and b). ED1 macrophages were also found in large numbers inside these implanted hydrogels. The number of ED1 macrophages found within the hydrogel pieces was also greater than that in unmodified hydrogel (Fig. 5a and b). However, the semiquantitative analysis we have used did not allow us to obtain a precise figure for the number of GFAP and ED1 positive cells located within the hydrogels.

Angiogenesis in the grafts was studied in the 6 week explants using DAB staining. When DAB granule combined with endogenesis peroxidase in blood cells, the colour would be black under the redox reaction. Then blood vessels profile could be observed indirectly. 6 weeks later, some newblood vessels were existed in the edge and epicenter of the implanted hydrogels on coronal brain sections with DAB staining in groups of hydrogels unmodified and modified. These vessels had no difference with the normal in tortuous figure and size (Fig. 6a and b).

Silver staining sections in group of RGD modified hydrogels revealed the presence of numerous argyrophillia processes regrowth into the reconstructed lesion. The processes were seen not only in the edge of the grafts, but in the epicenter of lesion. However, the phenomenon was not observed in sections of other two groups (Fig. 7a and b).

The tissue responses to HA hydrogels implanted into the lesion of CNS in three groups were summarized in Table 1.

Table 1 The tissue responses after HA hydrogel implanted into the brain defects in three groups

Group	Rats	Glial scar	Integration	Cells infiltration	Angiogenesis	Neurites regrowth
Group I HA hydrogel unmodified	12	−	+	+	+	−
Group II HA hydrogel modified	12	−	+	+	+	+
Group III injured only (control)	6	+	−	−	−	−

−: Presents negative, +: Presents positive

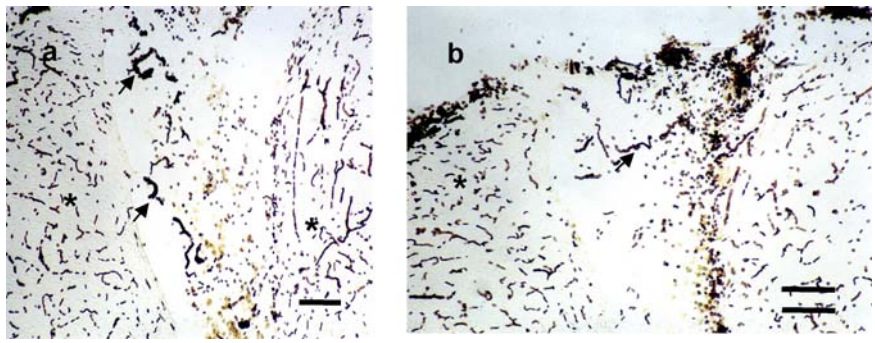

Fig. 6 DAB stained section to show the regenerated micro vessels in the hydrogel at 6th week implantation. The arrows show the regenerated capillary. The bar is 40 μm

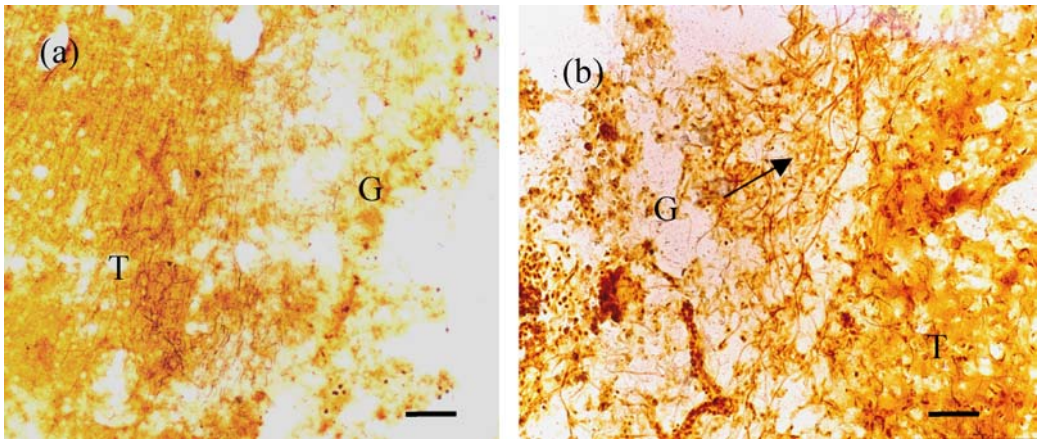

Fig. 7 Photomicrographs of Glees stained section of the brain in 12th week postoperationly for implants with unmodified hydrogel (a) and with RGD modified hydrogel (b). The arrows in the figures point to the neonatal neurofibrils. G is the hydrogel implant. T is host tissue. The bar is 20 μm

Discussion

The main objective of this study was to evaluate the ability of HA hydrogel to repair of CNS trauma. In recent years, tissue engineering was expected to provide methods for repairing tissue injury or defects. Tissue engineering has succeeded in many kinds of tissues and organs, such as skin, muscle tendon and blood vessel [23]. Similarly, many projects were designed to mend CNS defects and some encouraging results were obtained [24].

In this study, HA was made to be a hydrogel and implanted into defects of the cerebrum .This hydrogel was tested to assist the regeneration of CNS after trauma. Tissue integration of the hydrogel was observed at the ultrastructural level using SEM. The results of histological assay showed a large number of cells were widely dispersed into the implants. It was confirmed that migrated cells were mainly composed of $GFAP^+$ astrocytes and ED1 positive macrophages. In cortical lesion sites the host cellular reaction to RGD containing hydrogels was significantly greater than that seen in unmodified hydrogels. Large numbers of $GFAP^+$ astrocytes had migrated into these modified HA hydrogels, producing a cellular network. The extensive astrocytic colonization of RGD containing hydrogels compared with unmodified hydrogels strongly suggests that the presence of the peptide had a specific impact on astrocytic migration and adhesion. Astrocytes in culture respond chemotaxically to fibronectin [25] and they have been shown to express a variety of integrin receptors, including the $\alpha_1\beta_1$ receptor, the $\alpha_5\beta_1$ fibronectin receptor, the $\alpha_6\beta_1$ laminin receptor, and a receptor ($\alpha_v\beta_3$) which mediates attachment to vitronectin [26, 27]. These studies showed that the attachment of astrocytes to

fibronectin and vitronectin was RGD dependent. Thus the massive infiltration (and perhaps proliferation) of astrocytes within RGD containing hydrogels placed into the lesioned brain was in all probability mediated by functional integrins expressed by these macroglia. Comparatively large numbers of ED1 macrophages were also seen within RGD-linked polymers. Different macrophage populations are known to adhere to ECM substrata such as fibronectin and laminin *in vitro* [28, 29] and monocytes/macrophages express a variety of integrin receptors [30]. Thus the migration of ED1 macrophages into implanted hydrogels may have been enhanced by the presence of RGD recognition sequences in the polymers.

Severe injury of brain tissue results in the formation of a complex scar that prevents regenerating axons and glial cells from entering the lesion. Inducing regeneration in adult brain requires removing the scar tissue and developing a method to reform the tissue structure. HA can reduce the formation of scar at injury sites in the peripheral nervous system [31] and in other tissues [32–34]. It is for this reason that HA was chosen for our work in the CNS, in order to prevent scar formation that can impede the extension of regenerated axons and the migration of glial cells. In the current study, astrocytes migrated into the HA and HA-RGD hydrogel, but no astrocyte scar was found in immunostained sections and SEM study.

In the group of modified hydrogels, the results of silver staining showed numerous processes regrowth into the reconstructed lesion. However no evidence of neurite outgrowth was found in unmodified hydrogel. It is possible that some of the axonal ingrowth into RGD containing hydrogels was due to direct interactions between growth cones and the polymerized peptide sequences themselves [35, 36]. Commonly, however, axons were seen in close association with laminin1 cellular-like profiles. These profiles had a similar distribution and pattern to GFAP1/S1001 astrocytes and thus it is likely that astrocytes were the major source of this glycoprotein [37–39]. The association between axons and laminin1 astrocytes is consistent with studies which have shown that laminin can promote axonal/neurite outgrowth [40, 41]; however, it should be pointed out that axonal growth stimulated by reactive astrocytes can also occur by laminin-independent mechanisms [42, 43]. The possible influence of diffusible cytokines and neurotrophins released from astrocytes should also be considered [44, 45].

Angiogenesis, the formation of new capillary blood vessels, is essential processes in tissue development and regeneration. Oligosaccharide of HA fragments have been shown to induce angiogenesis in several tissues or organs, such as skin and ear, as well as within collagen gels in vitro [46–48]. One of the important steps of neovessel formation is the migration of vascular endothelial cells. With degradation of hydrogels, degradation products stimulated endothelial cells proliferation and migration after implanting into the defects of cortex. Proliferating endothelial cells are able to migrate into the hydrogel space and form a capillary sprout. Sprouts grow by continuing endothelial proliferation, gradually forming a lumen as they organize into tubules. Then new microvessels become mature when contiguous tubules anastomose with each other and a new basement member formed [49]. Blood vessels emerged in grafts and plentiful vessels observed in the regions adjacent to the lesion show that hydrogels implanted in CNS can induce angiogenesis.

One potential drawback of this study is the degradation time of the material. After 12 weeks, SEM study showed HA hydrogels implanted into the lesion was completely degraded by hyaluronidase. This relatively short time of degradation might be a disadvantage to tissue reparation. In the future work, two methods may be chosen to prolong the degradation of HA hydrogels. One is to improve the cross-linking density of hydrogels [50]. Another is to introduce sulphate groups along the hyaluronic acid chain [51].

In conclusion, we showed HA hydrogels currently synthesized had considerable neural biocompatibility after it was implanted into the lesion of CNS. HA hydrogels can provide a scaffold to support cells infiltration and angiogenesis, while inhibit the formation of glial scar. In addition, HA hydrogels modified with RGD can promote neurites extension. Our study showed that HA-RGD hydrogel may be a promising scaffold for further research in brain tissue engineering.

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