

Experimental study of temperature-sensitive chitosan/ β -glycerophosphate embolic material in embolizing the basicranial rete mirabile in swines

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Received July 1, 2014; Accepted February 19, 2015

DOI: 10.3892/etm.2015.2479

Abstract. The aim of the present study was to evaluate the feasibility of the non-adhesive temperature-sensitive liquid embolic material, chitosan/ β -glycerophosphate (C/GP), in embolizing the basicranial rete mirabile (REM) in a swine model of cerebral arteriovenous malformation (cAVM). A total of 24 domestic swines were used as the experimental animals, among which 12 pigs underwent direct embolization of one side of the REM, while the other 12 pigs underwent embolization of the bilateral REM following anastomosis of the carotid artery and jugular vein. A super-selective microcatheter was introduced into the REM during the embolization procedure, and the C/GP hydrogel was injected until an image of the REM disappeared in the angiography examination. Further angiography examinations were performed after 2 and 6 weeks, and histological examination of the REM was performed after 6 weeks. Of the 24 domestic swines, 23 cases underwent successful thrombosis. Convulsions occurred in one case and that pig died during the embolization procedure. Following embolization, the angiography observations revealed that the embolized REM was no longer able to be developed, and adhesion of the microcatheter tip with the embolic agent did not occur. In addition, no apparent revascularization was observed in the angiography examinations performed at weeks 2 and 6. Therefore, the current preliminary study indicated that use of the non-adhesive temperature-sensitive embolic material was feasible for the embolization of cAVM; thus, C/GP may be used as an ideal embolic material for the treatment of cAVM.

Introduction

Cerebral arteriovenous malformation (cAVM) is the major cause of intracranial hemorrhage and seizures in young and middle-aged adults, and can seriously threaten their quality of life and life expectancy (1,2). Due to the minimal harm inflicted on patients, endovascular embolization therapy has become an important method for the treatment of cAVM, and the selection of an embolic material is a major determinant towards the treatment efficacy.

The ideal cAVM embolic material should have a number of characteristics. Firstly, the material should be liquid, since this enables easy control, good dispersibility and ensures a sufficient injection time. Secondly, the material should exhibit a permanent embolism effect. Finally, the material should be non-toxic. Therefore, a non-adhesive liquid embolic material is ideal. In recent years, this particular material had received considerable attention in the field of endovascular treatment (3).

Therefore, the present study investigated chitosan/ β -glycerophosphate (C/GP) as the base material. The C/GP solution is liquid at room temperature, but forms a hydrogel at 37.0°C (body temperature). In addition, the pH of the C/GP solution is compatible with the physiological requests. These two materials have been previously demonstrated to be safe and non-toxic (4); however, the combination of C/GP has not been utilized in previous studies as a non-adhesive temperature-sensitive liquid embolic material to embolize cAVM. In the current preliminary study, the embolization efficacy of C/GP towards the basicranial REM was investigated in a swine model of cAVM, and the pathological changes of the REM tissues were assessed 6 weeks after the embolization. The aim of the present study was to provide a novel liquid embolic material for use in cAVM treatment.

Materials and methods

Preparations prior to the animal experiment surgery. In total, 24 domestic swines (Duroc pigs from the Animal Experimental Center of Jilin University, Jilin, China) were selected as the experimental animals. All animal experimentation protocols

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Key words: intervention, cerebral arteriovenous malformations, chitosan/ β -glycerophosphate, basicranial rete mirabile, embolism

were conducted in accordance with the policies set by the Chancellor of Jilin University's Animal Research Committee (Jilin, China) and the National Research Council (USA) Committee for the Update of the Guide for the Care and Use of Laboratory Animals (5). The pigs weighed between 25 and 30 kg, were aged 3–4 months and no restriction was set on gender. The animals were preoperatively fasted for 12 h, after which 0.5 mg atropine hydrochloride (Shanghai Sangon Biological Engineering Co. Ltd., Shanghai, China) was intramuscularly (IM) injected 30 min prior to the surgery. In order to prevent infection, 320,000 IU penicillin (Shanghai Sangon Biological Engineering Co. Ltd.) was also IM injected 30 min prior to the surgery. For anesthesia, ketamine hydrochloride (5 mg/kg body weight; Fujian Gutian Pharmaceutical Industry Co. Ltd., Fuzhou, China) was IM injected, and sumianxin II (0.3 ml/kg; Shanghai Sangon Biological Engineering Co. Ltd.) was also IM injected. When the swine became unconscious and the eyelash reflex disappeared, 1% ketamine hydrochloride in glucose solution was intravenously injected in a dropwise manner, and the injection rate was adjusted accordingly during the surgery to maintain the animals under satisfactory anesthesia. Vital signs of the animals, including the blood pressure, heart rate and breathing rate, were monitored with a multipurpose polygraph (Guangdong Biolight Meditech Co. Ltd., Zhuhai, China).

Establishment of the swine cAVM model. Under the microscope (Leica Microsystems Co. Ltd., Beijing, China), the porcine REM vessels resembled human arterioles. A cross-section image revealed that the REM vessels had the same histological structures as human arterioles, including a vascular intima, a smooth muscle layer, a vascular adventitia and connective tissues among the vessels. The diameters of the vessels varied between 70 and 275 μm , with the average diameter of an REM vessel being 154 μm (6). Massoud *et al.* (7) previously reported that the average diameter of an REM vessel, which varies due to differences in size and weight of the swine, was 328 μm , while the average vessel diameter in cAVM was 265 μm ; these numbers are very similar. According to the REM situation, the experiment was divided into two groups. In the low-flow group, one side of the REM was maintained in the natural state and microvascular anastomosis was not performed (Fig. 1A). In the high-flow group, the ascending pharyngeal artery (AP) of one side of the REM was set as the blood-supplying artery, and through microsurgical techniques, the distal end of the contralateral common carotid artery (CCA) was connected with the proximal end of the internal jugular vein (IJV) via microanastomosis, which subsequently changed the direction of the blood flow. An angiography examination of one side of the CCA revealed that the blood flow went from the AP, through the REM, into the anastomosed AP on the opposite side, and then through the CCA, the anastomotic stoma and finally into the IJV (Fig. 1B).

Preparation of the C/GP solution. A 1-ml sample of concentrated hydrochloric acid (37.5%), containing 0.012 mol hydrochloric acid, was added to deionized water to reach a total liquid volume of 120 ml. The formulation was prepared immediately prior to usage. With regard to the preparation of 2% (w/v) chitosan, the chitosan used was produced by Biosyntech, Inc. (Laval, QC, Canada), with the following specifications:

Molecular weight, 308 kDa; degree of deacetylation, 94%; viscosity, 140 mPa.s. In total, 5 g chitosan was dissolved in 0.1 M hydrochloric acid, and well-mixed at room temperature to produce a final solution of 250 ml. The solution was subsequently sterilized at 121°C with high pressure steam for 20 min, followed by storage at 4°C for future use. An 8% (w/v) β -GP solution was produced by Sigma-Aldrich (Oakville, ON, Canada). In total, 8 g β -GP was diluted with deionized water to form a 100-ml solution, which was subsequently disinfected and sterilized with a 0.2- μm filter (Chengdu Ailaite Technology Co. Ltd., Chengdu, China). The solution was finally stored at 4°C for future use. The 2% (w/v) chitosan and 8% (w/v) β -GP solutions were mixed at an equal volume (1:1) and then tantalum powder (Sigma-Aldrich) or Omnipaquem (General Electric Pharmaceutical (Shanghai) Ltd., Shanghai, China) was added. The final mixture was refrigerated at 4°C. The solution was prepared immediately prior to usage.

Procedures of the C/GP REM embolization surgery. Under anesthesia, the animals were laid in a supine position on a double C-arm digital subtraction angiography (DSA) table (Siemens Co. Ltd., Beijing, China). A 6F catheter sheath (Terumo Holding Co. Ltd., Shanghai, China) was punctured into and kept in the femoral artery, which was beneath the concave area of the vastus medialis and pectineus. A Y-shaped valve and a three-way irrigation system (Terumo) was used for the bolus injection of 70 U/kg heparin. A 5F catheter (Cook Medical, Bloomington, IN, USA) was selectively inserted into one side of the CCA (low-flow group: direct embolism on one side of the REM) or the contralateral CCA towards the anastomotic vessel (high-flow group; embolism on two sides of the REM following successfully built cerebral arteriovenous malformation models with common carotid artery and internal jugular vein anastomosis). A high-pressure syringe (Medrad Inc., Indianola, PA, USA) was used to inject 7 ml contrast agent Ultravist 300 (Bayer AG, Leverkusen, Germany) for the DSA examination, with the injection flow rate set as 4 ml/sec. The DSA examination was performed to assess the condition of the AP, REM and associated arteries. The 6F flat guiding catheter was then replaced in order to enable the head of the catheter to reach the proximal end of the REM-AP. Subsequently, a Tracker 18 (Target Therapeutics, Inc., Fremont, CA, USA) microcatheter was guided by the tip-modified microwire to selectively enter the REM through the AP. After manually injecting the contrast agent for the DSA examination, the C/GP solution was injected via the microcatheter. The injection flow rate was 0.6–1 ml/min, and the injected dose was 1.5–2 ml; thus, the injection time varied between 1.5 and 3.0 min. In order to observe the degree of embolization, DSA was conducted with the injection of contrast agent following fluoroscopic observation of the embolization of embolic agent and the flow direction with hand push contrast agent ‘smoking’ during the injection of embolic agent. If the REM did not exhibit complete thrombosis, the embolic agent was continuously injected until the REM was shown to be totally thrombosed and without any visualization on the angiography examination. Following the embolization therapy, the microcatheter was slowly withdrawn. Postoperative angiography examinations were performed at 2 and 6 weeks after embolization to assess for signs of revascularization.

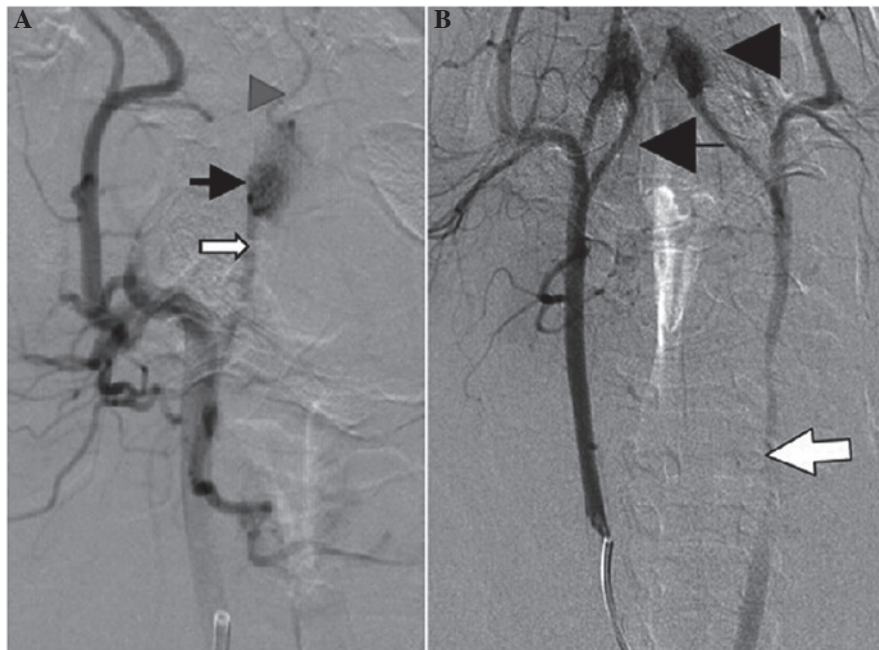


Figure 1. (A) An angiography examination of the common carotid artery (CCA) was performed in the low-flow group. From the image, it is clear that the ascending pharyngeal artery (AP; white arrow) sent out blood flow to the rete mirabile (REM; long black arrow), while finally aggregating in the intracranium and forming the internal carotid artery (triangle). (B) A one-side CCA angiography revealed the successful establishment of the cerebral arteriovenous malformation (cAVM) model in the experimental group three days after the surgery. The blood-supplying vessel was the AP (long black arrow), the basicranial REM was the cAVM mass (black triangle), and the draining vessel was located at anastomotic point of the microscopically anastomosed CCA and internal jugular vein (white arrow).

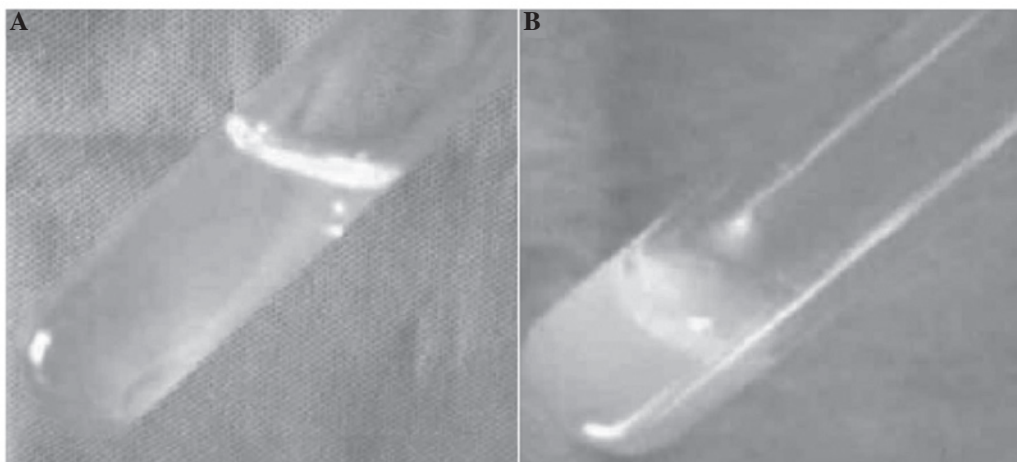


Figure 2. (A) Chitosan/ β -glycerophosphate (C/GP) material was liquid at temperatures of $<37^{\circ}\text{C}$. (B) C/GP material was a gel at 37°C .

Post-processing following the embolization treatment. At 6 weeks after the embolization, the animals underwent an angiography examination, after which a lethal dose of sodium pentobarbital (Shanghai XiTang Biological Technology Co. Ltd., Shanghai, China) was intravenously injected. The brain tissues, including the REM, were completely removed. The specimens were fixed in 10% formalin for 48 h, washed three times with 0.01 M phosphate-buffered saline solution and placed in 37°C incubators for preservation. The specimens were then fixed in neutral formaldehyde solution for a week. Following conventional dehydration, paraffin embedding, slicing (slice thickness, 5 mm) and hematoxylin and eosin (HE) staining, the specimens were histologically analyzed.

Results

Gelatinization time and the ratio of material. The average gelatinization time of the 1:1 C/GP solution at 37°C was 120 sec. As a developer, 1 g tantalum powder or 1 ml Omnipaquem was added to each 10-ml sample of C/GP solution. The C/GP material was liquid at temperatures of $<37^{\circ}\text{C}$ and became a gel at 37°C (Fig. 2A and B).

Postoperative observations. In the low-flow group, all 12 swines survived the surgery, without wound infections and hemiplegia. In the high-flow group, 11 swines survived the surgery; however, one pig died of convulsion and respiratory

depression during the embolization procedure. In an additional case, the pig exhibited postoperative contralateral facial paralysis, masticatory muscle weakness and extreme weight loss following embolization of the REM and external carotid artery.

Histological results. REM histological results were assessed 6 weeks after the embolization. The results revealed that the C/GP gel filled the REM vascular cavity and that the tunica intima was integral. Furthermore, no significant signs of inflammation were observed (Fig. 3). General histological observations revealed that the bilateral AP, the structures of the REM and the internal carotid artery were consistent with that observed by DSA. No abnormal changes occurred in the brain tissue of the low-flow group. The embolized side of the REM was completely embolized, and was comparatively harder and paler compared with the contralateral side of the REM. The emboli appeared jelly-like. The REM was easily stripped from the sphenotic foramen at the skull base. By contrast, the high-flow group exhibited a comparatively harder and paler REM, with the emboli appearing jelly-like.

Under the light microscope, the REM specimens revealed an even gel dispersion inside the vascular lumen, indicating that the vessel lumen was thrombosed completely, and the hydrogel was located inside the lumen. No signs of inflammation were observed around the vessels. In addition, the smooth muscle layer remained intact, the vascular intima was clearly visible, and no desquamation or vessel wall necrosis was found to have occurred.

Imaging results. DSA examination prior to the embolization revealed that the AP of the 24 swines originated from the CCA. The terminal branches of the AP formed an oval-shaped basicranial REM at the location of the cavernous sinus, and the REM reconverged into the internal carotid artery inside the cranium, which then formed the branches to supply the brain tissues. In addition, the two branches, ramusanastomoticus (RA) and arteria anastomotica (AA), extended from the porcine external carotid artery and were also involved in the blood supply of the REM.

Angiographic performance at weeks 2 and 6 after the embolization. In nine cases from the low-flow group, completely no development of the REM was observed in the angiographic examination following the C/GP embolization (Fig. 4A). Although three cases exhibited an occluded AP, the region of the AP near the original segment of the REM continued to display the RA after the embolization, and the AA supplied the blood to the REM (Fig. 4B). In addition, following the super-selective re-embolism using the Tracker 10 microcatheter towards the RA, visualization of the REM in the angiography examination did not develop completely (Fig. 4C). All the 12 swines in the high-flow group exhibited images showing successful establishment of the cAVM model prior to the embolization, while after the C/GP-dual embolization, the REM was not visible on the images. In addition, no revascularization was observed at weeks 2 and 6 following the embolization.

Perioperative situations. Injection of the embolic agent through the microcatheter exhibited no resistance, with easy bolus intravenous push. In addition, the time was controlled

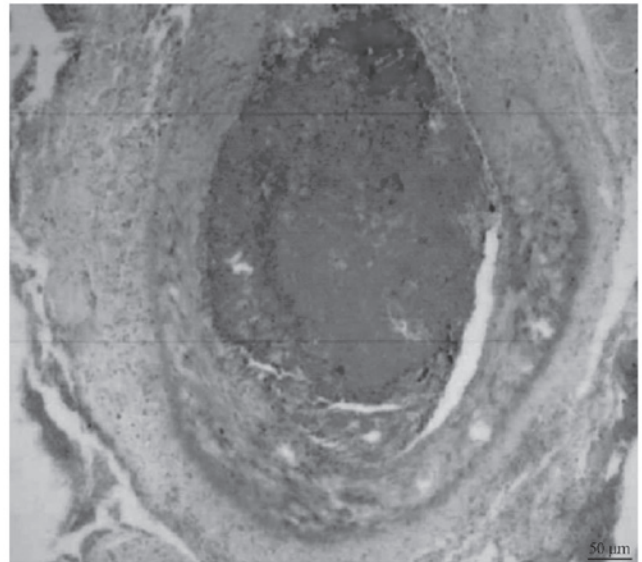


Figure 3. At week 6 after the embolization procedure, the histological results revealed that the chitosan/ β -glycerophosphate gel had filled the rete mirabile vascular cavity, the tunica intima was integral and there were no significant signs of inflammation.

between 1.5 and 3 min, with the total dosage varying between 0.8 and 1.5 ml. Following completion of the embolization and the withdrawal of the microcatheter, no adhesion occurred between the tips of the microcatheter and the embolic agent.

Discussion

cAVM is a common congenital cerebrovascular maldevelopment, in which the arteries and veins are directly connected without capillaries, which subsequently leads to a series of hemodynamic disturbances. The condition is most common in young adults aged between 20 and 40 years. The main clinical manifestations include recurrent intracranial hemorrhage, epilepsy, headaches and neurological deficits. The annual incidence of cerebral hemorrhage is 2-3%, with the mortality rate between 1 and 2% (8-11).

To date, the treatment of AVM has included lesion excision, endovascular embolization and stereotactic radiotherapy; however, each method has limitations (12,13). Among the various therapies, endovascular treatment exhibits the least trauma to the patient; however, the embolic material is the major factor that restricts the treatment efficacy. An ideal embolic material should have a number of characteristics. Firstly, the material should be sterile, non-toxic, non-carcinogenic, non-deformative and non-mutative. In addition, a liquid material is desirable, since this can be easily controlled, have good dispersibility and can ensure a sufficient injection time for surgical ease. Finally, the material should prevent revascularization from occurring easily, and should exert permanent thrombotic effects. However, the embolic materials currently used in clinical practice do not fully meet the aforementioned criteria.

Various embolic materials are currently used in clinical practice. One particular embolic material is polyvinyl alcohol polymer (PVA) (14). However, a principal drawback of this material is the requirement of a large diameter catheter for delivery; thus, PVA is unable to penetrate and reach the ideal

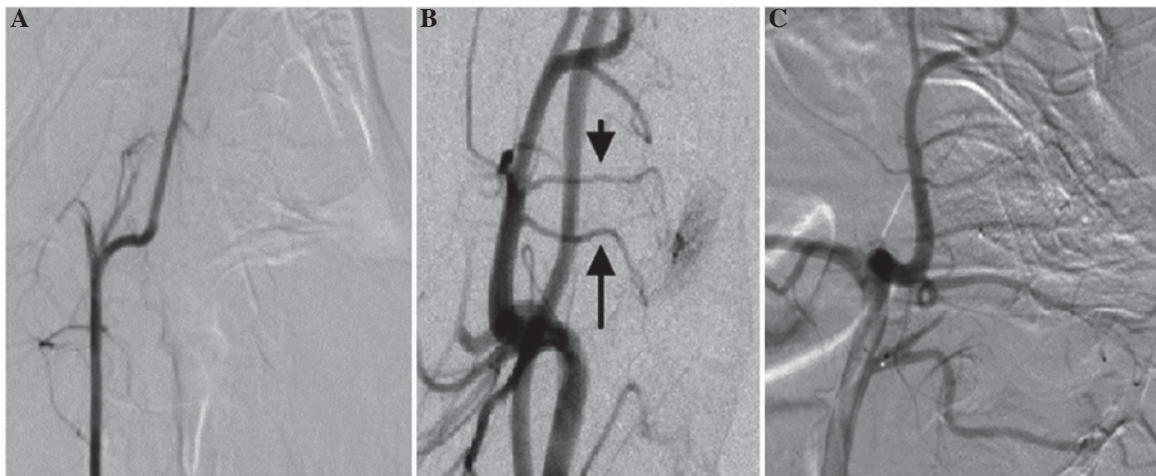


Figure 4. (A) The bilateral rete mirabile (REM) exhibited no angiographic images following the embolization. (B) Ramusanastomotica (RA; long arrow) and arteria anastomotica (AA; short arrow) branches were shown to supply the blood to the REM. (C) Super-selective RA and AA embolization.

location super-selectively. In addition, adhesive liquid embolic materials (15-18), including cyano acid isobutyl fatty acid and its derivatives, are most commonly used in clinical practice. However, their limitations include adhering easily to the high-molecular microcatheter, which subsequently blocks the catheter. In certain cases, this may result in the breakage of the microcatheter inside the cranium. Furthermore, non-adhesive liquid embolic materials (19,20) can be used, which do not adhere to the catheter wall. These materials include CAP, EVAL, Eudragit-E and HEMA-co-MMA. The most commonly used ONYX glue is a solution of EVAL, DMSO and tantalum powder mixed at certain percentages, which is necessary to achieve the correct embolization technique. However, dimethyl sulfoxide is an organic solvent that has been identified to exert potential reproductive toxic effects towards the vascular system (21).

Liquid embolic materials that are non-adhesive with non-organic solvents appear to have the better long-term developmental prospects, and hydrogel has such characteristics. Hydrogel is a class of polymer that swells due to the absorption of water. The hydrogel is able to retain water, while not dissolving. In addition, hydrogel has very similar properties to body tissues, while exhibiting little irritation to the surrounding tissues, as well as good tissue compatibility. However, typical hydrogel is in a liquid state at high temperatures and a solid state at low temperatures. Therefore, it may not be possible to successfully import hydrogel into the aneurysm cavity, and importation may lead to possible liquefaction of the emboli, resulting in associated syndromes. Accordingly, a pioneer study in 2000 by Chenite *et al* (22) was the first to describe a C/β-GP-based hydrogel. The authors used a C/GP solution as the gel system to develop a novel injectable neutral chitosan solution. This solution had a physiological pH, exhibited a liquid state at room temperature and was able to surround the live cells and therapeutic proteins when injected into the body. Furthermore, the solution was able to form a biodegradable gel at the injection site under body temperature. Molecules within the hydrogel formed chains through different intermolecular interaction forces, including van der Waals forces, hydrophobic interactions and hydrogen bonding.

Following comparison of chitosan gels prepared with different molecular-weight chitosans, a higher degree of deacetylation was considered to result in a faster gel-forming speed, while the molecular weight was shown to have no impact (23). When the concentration of chitosan and the degree of deacetylation remained unchanged, increasing the GP concentration resulted in the gel temperature decreasing and the pH increasing. In addition, a higher degree of deacetylation in the C/GP material exhibited a better biocompatibility (24).

Wang *et al* (25) were the first to apply a temperature-sensitive C/β-GP solution for renal artery embolization, and good results were achieved. Through the use of an enzymatic digestion method, the authors successfully prepared an aneurysm model in nine Chinese rabbits. Three weeks after the modeling, the embolic material was used to embolize the aneurysm model. With bilateral femoral artery entry, a balloon was inserted into the right femoral artery, while a microcatheter was inserted into the left femoral artery. After placing the microcatheter into the aneurysm cavity, a vascular map was constructed, and the balloon was directed across the aneurysmatic neck. The balloon was subsequently filled to completely block the aneurysmatic neck. Next, the embolic material was slowly injected via the microcatheter to completely thrombose the aneurysm. At 4 weeks after the surgery, an angiography examination was conducted and the rabbits were sacrificed. Tissue samples were collected for HE staining and pathological analysis was conducted. The angiography examination revealed that all nine rabbits had undergone a successful embolization of the *in vivo* aneurysm. The entire thrombosis process was clearly visible, and after thrombosis, the aneurysm was observed to be completely filled with the emboli material, and the angiography image had developed well. In addition, there was no ectopic embolization, tube gluing or catheter obstruction during the embolization process. The rabbits were in a good condition postoperatively, and had no special complications. The postoperative 4-week angiography revealed no revascularization.

In the present study, the chitosan specifications were as follows: Viscosity, 130 mPa.s; molecular weight, 80,400 Da; degree of deacetylation, 94%. A solution of 2% chitosan and

8% β -GP was mixed at different proportions. The optimal ratio was determined to be 1:1, from which the pH value was 7.28 and the mechanical strength was up to 14 kPa; thus, the cell survival rate was the highest at this ratio, reaching $89.0 \pm 2.7\%$. These characteristics demonstrated the safety and efficacy of this specific liquid embolic material. In addition, previous *in vitro* and *in vivo* animal experiments have verified the safety and efficacy of this material (26). The 1:1 C/GP solution had an average gelatinization time of 120 sec at 37°C ; thus, the solution was determined to be suitable for the embolization therapy of cAVM.

Chitosan is known to have good biocompatibility, antibacterial, antimicrobial, antiviral and anticancer properties, as well as being biodegradable (27-29). Furthermore, chitosan has been approved by the Food and Drug Administration (FDA) for application as a wound dressing. GP is an organic compound that is found in a natural form inside the human body. The FDA have approved the use of GP for intravenous therapy. In the present study, there was one case of mortality in the high-flow group. In this case, it was considered that the embolic agent may have been injected into the brain through the internal carotid artery, and the postoperative anatomical histology confirmed that the bilateral internal carotid arteries were ectopically embolized. An additional case exhibited ipsilateral masticatory muscle weakness, of which a possible explanation may be that ipsilateral embolization of the external carotid artery led to the ischemia of the ipsilateral face. The general histological examination following the embolization revealed no abnormal changes in the brain tissues. Furthermore, light microscopy examination revealed that the REM lumen was filled with the gel substance, the vascular intima was clearly visible, and the smooth muscle layers were intact. There was no desquamation or vessel wall necrosis, or any inflammation observed in the surrounding vessels, indicating that C/GP was non-toxic to the blood vessels. Following the addition of C/GP to the tantalum powder, the angiography images developed well; however, since the tantalum powder is metallic, it may destroy the slide blade for the histological section. If the C/GP solution was added to a liquid developer, such as Omnipaque, there was no such risk towards the section knife.

The speed of the C/GP injection was required to be maintained at an adequate pace, and it was essential that the injection speed was controlled. A fast injection speed may have resulted in the C/GP not depositing inside the cAVM prior to forming the hydrogel, while draining into the other major blood vessels. However, a slow injection speed may have lead to the premature deposition of hydrogel inside the catheter, subsequently blocking the catheter. In the present study, a flow rate of 0.6-1 ml/min was selected for the bolus injection, and each dosage was 0.8-1.5 ml, with the injection time set as 1.5-3 min. Using these parameters, good results were achieved.

In conclusion, the temperature-sensitive liquid embolic material, C/GP, exhibited a number of advantages, including its non-adhesive, complete REM embolization, non-toxic and biocompatible properties, in addition to its ability to easily pass through different specifications of microcatheters. Furthermore, C/GP was not shown to cause any adverse reactions and was found to be relatively soft following solidification. Therefore, the current preliminary study indicated that the application of C/GP was feasible for cAVM treatment.

As an ideal material for cAVM embolization, C/GP may have broad application prospects.

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