Thrombin-loaded alginate-calcium microspheres: A novel hemostatic embolic material for transcatheter arterial embolization

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ABSTRACT

Transcatheter arterial embolization (TAE) is the best non-laparotomy choice for solid visceral organs rupture and bleeding nowadays. In our previous study, a new biodegradable macromolecule material thrombin-loaded alginate-calcium microsphere (TACM) was prepared and its characteristics were investigated preliminarily. In this study, we further investigated the biocompatibility of TACMs, as well as physical characteristic, application method and effect of TACMs with thrombus (embolic agent). The in vivo results attested that TACMs were non-irritating and non-genotoxic with desired biocompatibility, although brought about a slight and temporary inflammation. Application research showed that the function of thrombin was inhibited by common contrast agents, and it was impracticable to add contrast agents in TACMs with thrombus for tracing under X-rays in TAE. Then, a novel delivery method was developed. In addition, stress resistance test indicated that the TACMs with thrombus was significantly stronger than single autologous thrombus, the optimized ratio of TACMs to whole blood was 2:3 for forming mixed thrombus. Finally, large animal experiment revealed that the novel embolic agent – TACMs mixed thrombus was effective and safe in treating hemorrhage of solid abdominal viscera by TAE.

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1. Introduction

Blunt trauma of solid visceral organs (such as spleen and liver) leads to severe hemorrhage, which is often life-threatening to patients [1]. Laparotomy is the traditional therapy for such emergencies over the past few decades [2–4]. With advantages of minimal invasive, high success rate and less operation-related serious complications, the interventional techniques were rapidly developed in recent years. Transcatheter embolization has been provided as an alternative for patients with solid visceral organs injury and hemorrhage [2,5]. In particular, for patients with stable hemodynamics, transcatheter arterial embolization (TAE) has

become the best non-laparotomy choice for bleeding caused by solid visceral organs rupture [6,7]. Embolic agents, employed in TAE procedure, should block the blood flow of injured artery and reduce tissue perfusion, thus serve as a key determinant for successful hemostatic embolization [8,9].

At present, the embolic agents commonly used in TAE include coils, gelfoam, cyanoacrylate, and Onyx, etc. Coils are a kind of permanent embolic agent, which have a variety of shapes and sizes. They are made from precious metal (platinum, tungsten, etc.) with complex craftsmanship. These characters lead to inconvenience of employing different size of coils according to diameter of target vessel, as well as expensiveness and special relieving equipment. In addition, disadvantages of coils include incomplete occlusion, low rate of thrombus formation, and chronic inflammation or even damage of visceral organs caused by permanent coils embolization [10,11]. Gelfoam is derived from animal protein composed of various amino acids and can be absorbed by human tissues. The advantages of low price and good adoptability make gelfoam the first homeostatic agents and being employed in surgery for many years. Till now, gelfoam is the only commercially biodegradable embolic material approved to be used in vivo [11]. It is mainly used

to stop bleeding temporarily or devascularize a tumor prior for surgical removal. Degradation of gelfoam is influenced by many factors, including diameter of particles and dosage, resulting in complete degradation from days to months [12,13]. So increased risk of arterial rebleeding may occur if gelfoam is absorbed prior to complete hemostasis [14].

The α-n-butyl cyanoacrylate (α-NBCA) is a commonly used liquid embolic agent for hemostasis of solid viscera, such as liver and spleen [15,16]. Due to its powerful adhesion and rapid polymerization, α-NBCA is prone to polymerize within the catheter. Moreover, heat released during polymerization may cause injury of artery intima [17], or even severe inflammation, pain, or tissue ischemia [18]. Onyx, another liquid embolic agent developed recently, which is mainly used to embolize brain aneurysm or arteriovenous malformation. Although Onyx can also be used in embolic hemostasis of solid viscera [19,20], disadvantages of rapid coagulation, requirements of special catheter, demands of skillfulness, as well as severe artery spasm make it an inferior choice for interventional physician [21]. Therefore, no ideal embolic material for interventional management of patients with injury and bleeding of solid visceral organs was developed until now.

The development of alginate microspheres loaded with therapeutic drugs has become a hot spot in interventional filed nowadays [22,23]. Alginate is a naturally occurring anionic polymer typically obtained from brown seaweed. It contains blocks of (1, 4)-linked-β-D-mannuronate (M) and α-L-guluronate (G) residues. Alginate microspheres are widely used in medical and pharmaceutical research owing to its advantages of ability of being loaded with drugs, targeted delivery, safety, non-toxicity, controllable degradation, broad adaptability and convenient usability [24–27]. Thrombin, an effective hemostatic drug, catalyzes conversion of plasma fibrinogen to fibrin and promote the platelet aggregation, resulting in rapid thrombus formation and hemostasis [28]. It can be used to stop bleeding of superficial irregular wound or treat pseudoaneurysm by ultrasound guided injection (UGTI) [21]. Unfortunately, autologous thrombus formed by thrombin catalysis is soft, weak, prone to exfoliate or autolysis, resulting in inadequate hemostasis and failure of TAE frequently [29].

In previous study, we have prepared thrombin-loaded alginate-calcium microspheres (TACMs) using electrostatic droplet technique [30]. The TACMs is a kind of novel embolic material with the advantages of both conventional embolic microspheres and thrombin for treating blunt trauma and hemorrhage of solid viscera by TAE. The preliminary physical, pharmacological and biological characteristics of TACMs have been investigated by our group, as well as the embolization hemostasis for solid abdominal viscera using TACMs mixed thrombus. In this research, biocompatibility of TACMs, as well as the physical characteristic, the clinic application method and the embolic effect of TACMs mixed thrombus were validated and further demonstrated.

2. Materials and methods

2.1. Materials

Sodium alginate (purity ≥ 98%, viscosity 100cp, G/M = 0.38) was purchased from Bright Moon Seaweed Group Co. Ltd. (Qingdao, China). Thrombin was supplied by First Biochemical Pharmaceutical Co. Ltd. (Shanghai, China). Calcium chloride was purchased from Aladdin Reagent Co. Ltd (Shanghai, China). The contrast agents ioversol, iopromide, iodixanol and meglumine diatrizoate were purchased from Tyco Healthcare (Canada), Bayer Schering Pharma AG (Germany), GE Healthcare (Ireland) and Hansen Pharmaceutical Co. Ltd (Yiyang, China), respectively. Fibrinogen was purchased from Sigma-Aldrich Co. LLC (MO, USA). TNF-α ELISA Kit and IL-1β ELISA Kit were purchased from R&D (United Kingdom).

The TACMs used in this study were prepared by electrostatic droplet technique under mild conditions [30]. In brief, 10 mg/ml mixed solution was prepared by dissolving thrombin in 3% (w/v) alginate- normal saline (NS) solution, and then extruded through a needle into 2% (w/v) CaCl₂ solution by electrostatic droplet generator (YD-06, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, China) to form 350 μm TACMs (Fig. 1).

All the protocols for animal experiment in this research were approved by the institutional animal care and use committee (IACUC) of the General Hospital of Shenyang Military Region. All procedures of animal experiment were in full compliance with recommendations on animal studies of the Helsinki Declaration of World Medical Association.

2.2. Skin irritation test

The assessment of skin irritation potential is an important part of preclinical safety assessment for new chemical products [31] and is largely based on animal experiments [32]. In this study, four healthy New Zealand rabbits (2.4–2.7 kg, male) were used to evaluate the skin irritation of TACMs. Briefly, 1 ml of TACMs was incubated in 5 ml NS at 37 °C for 24 h to get 100% extracts. Then, animals received intracutaneous injection of 0.5 ml extract on the left back skin next to backbone. Meanwhile, 0.5 ml of NS was injected intracutaneously on the right back skin for control. The cutaneous reaction surrounding the injection site was evaluated by the reported criteria (Table 1) at the time points of 24 h, 48 h and 72h [31].

2.3. Micronucleus test

Micronucleus data in vivo is related to blood and/or bone marrow, and is employed in a variety of protocols [33]. The dose group ranged from 5 to 15, the number of doses in each group often ranged from 3 to 8 (including controls) and the exposure regimens often
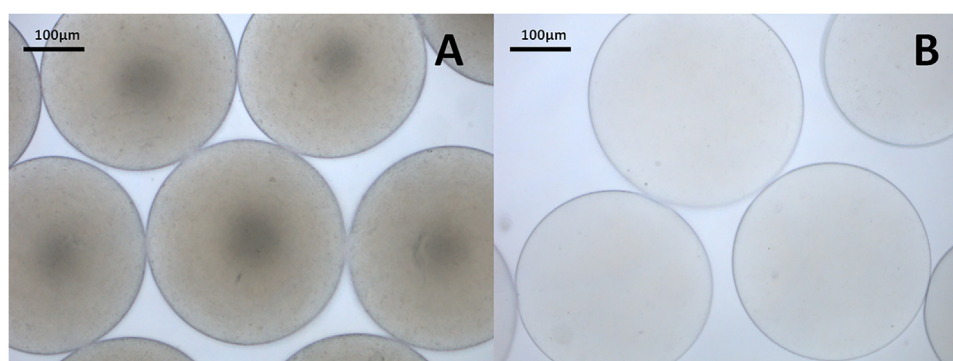

Fig. 1. Optical microscopy micrograph of thrombin-loaded alginate-calcium microsphere (A) and alginate-calcium microsphere without thrombin (B).

Table 1
Criteria of classification of the cutaneous reactions.

Cutaneous reaction	Score	Results
None	0	Normal
Sporadic or patchy erythema	1	Irritation
Moderate confluent erythema	2	Irritation
Severe erythema and edema	3	Irritation

varied from a single dose to repeated dosing up to182 days (26 weeks) [33,34]. In this study, genetic toxicity of TACMs was evaluated by micronucleus test of bone marrow of thirty C57 BL/6J mice (male, aged 5 weeks, 16–18 g). All mice were randomly divided into TACMS groups (including 3 subgroups), negative control group and positive control group. TACMs extract was prepared by incubating TACMs in NS at 1:5 in volume for 24 h. Mice in the three TACMs subgroups (6 in each) were injected with 10 ml/kg/d, 25 ml/kg/d and 50 ml/kg/d extract liquid into the abdominal cavity, respectively. The mice in negative control group and positive control group (6 in each) were injected with 25 ml/kg/d NS and 25 ml/kg/d Cytoxan, respectively. All the animals were repeated dosing up to 56 days (8 weeks). At the predetermined time point, mice from each group were sacrificed by cervical dislocation, and two femur marrow smears of each mouse were harvested. The smears were air-dried, fixed by formaldehyde, stained by Giemsa, and then observed by optical microscopes. Two thousand polychromatic erythrocytes (PCE) of each smear were observed with image analysis and the PCE with micronucleus were counted. The PCE micronucleus formation rates of different groups were analyzed statistically by SPSS 20.0 software.

2.4. Cytokine production

Peripheral blood mononuclear cells (PBMC) were used to evaluate the induction of cytokine production by TACMs in this research. The peripheral blood was collected from healthy volunteers. The protocol was approved by Ethical Committee of the General Hospital of Shenyang Military Region. PBMCs were isolated from buffy coats of peripheral whole blood by density gradient centrifugation using Ficoll Paque Plus and the purity above 90% was acceptable. PBMC were cultured in RPMI-1640 (Gibco, USA) with 10% fetal bovine serum (Gibco, USA) and 1% penicillin/streptomycin. The PBMC (10^6/ml) were added in 24-well plates with a volume of 1 ml per well and incubated with TAMCs (2.5, 5, 10, and 20 mg/ml) for 24 h. Lipopolysaccharide (LPS, 200 ng/ml; sigma, America) was used as a positive control and the pure PBMC as a negative control. Lipopolysaccharide (LPS) of 1 mg were reconstituted by adding 1 ml cell culture medium and further diluted to 10ug/ml by cell culture medium. 200ul LPS solution were then added to 24-well plates as positive control. Samples were centrifuged after incubation, and cytokines (TNFα, IL-1β) in the cell supernatant were measured using ELISA Kits (R&D, United Kingdom).

2.5. Feasibility evaluation of adding contrast agents to TACMs mixed thrombus for tracing

Contrast agents are the most commonly used medium in cardiovascular intervention. The majority of contrast agents contains iodine [35]. They are mainly applied to enhance the visibility of blood vessels and organs when being exposed under X-rays. In addition, the contrast agents are also frequently used to mix with embolic agent for tracing and avoiding mistaken embolization in treating tumor or stopping bleeding. Because TACMs mixed thrombus was invisible under X-rays, we firstly evaluated the feasibility of adding contrast agents to TACMs mixed thrombus as tracer during preparation.

Fibrinogen solution was prepared by dissolving fibrinogen powder in 0.9% (w/v) NS and the solution pH value (7.0–7.4) was adjusted by 0.05 mol/L sodium dihydrogen phosphate solution. The standards of thrombin-NS solution with gradient concentration (0.5u/ml, 1u/ml, 2u/ml, 4u/ml, 8u/ml, 16u/ml, 32u/ml and 64U/ml) were prepared. Then, 0.1 ml thrombin standard was added into 0.9 ml 0.1% fibrinogen solution which was in a test tube of 1 cm diameter and preheated by water bath at 37 °C for 5 min. After that, the test tube was water-heated sequentially and the initial setting time of fibrin was recorded timely. The experiment was repeated 5 times and a linear regression equation was finally obtained. The logs of activity unit of standard and initial setting time were defined as X and Y ($y = -0.701x + 2.344$, $R^2 = 0.985$), respectively.

The contrast agents ioversol, iopromide, iodixanol and meglumine diatrizoate, which were most commonly used in clinic, were tested in this study. Each of contrast agents was added in NS according to the volume ratio of 1:0, 1:1, 1:2 and 1:3. The test solutions
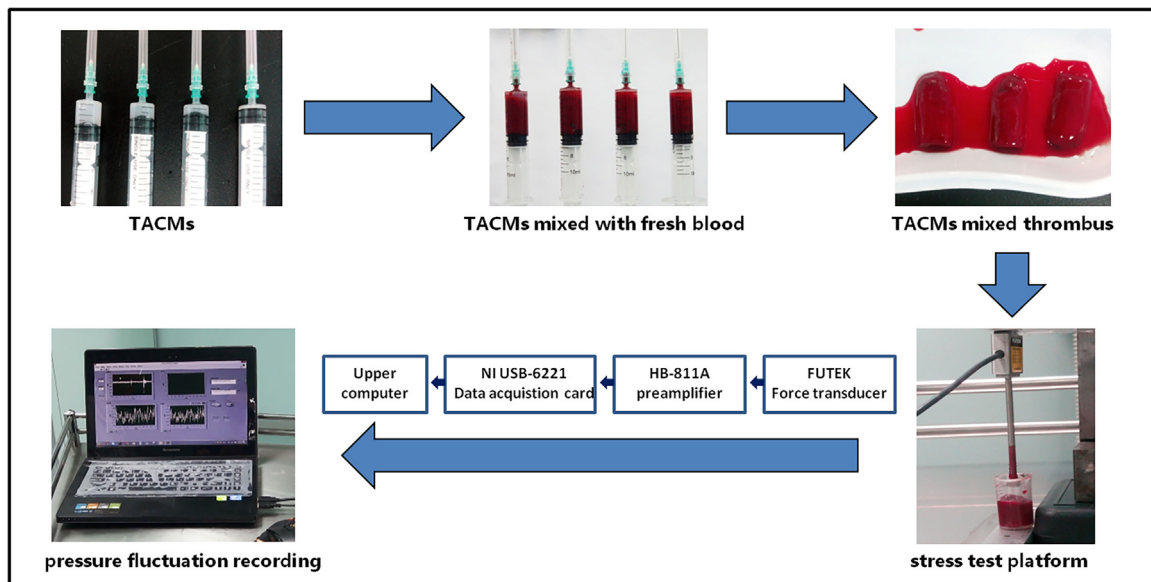

Fig. 2. Stress resistance test of TACMs mixed thrombus and schematic diagram of high sensitivity stress resistance measurement system.
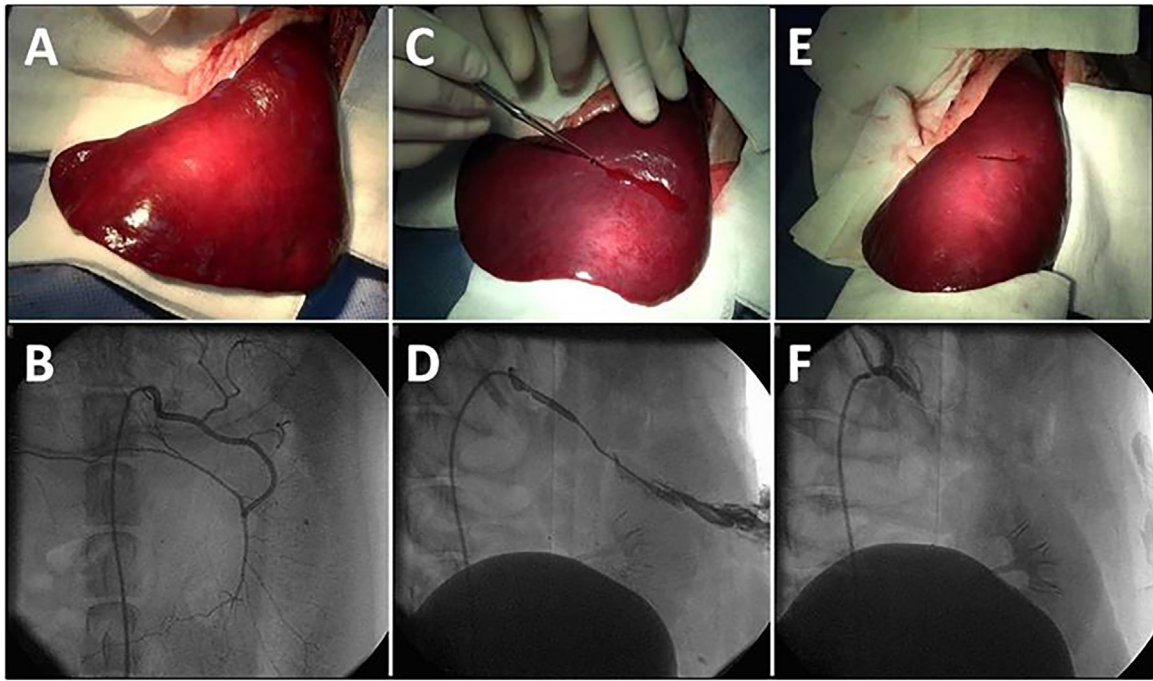
Fig. 3. Establishment of splenic injured and hemorrhagic models of Beagle and the interventional embolization using TACMs mixed thrombus. (A) the exposed and externalized spleen; (B) angiography of the normal splenic artery; (C) the hemorrhage model of spleen made by scalpel; (D) the leakage of contrast agent from incision at 5 min after hemorrhage model established and pressed by sterile gauze; (E) bleeding stopped completely after embolization using TACMs mixed thrombus; (F) angiography after TACMs mixed thrombus embolization.
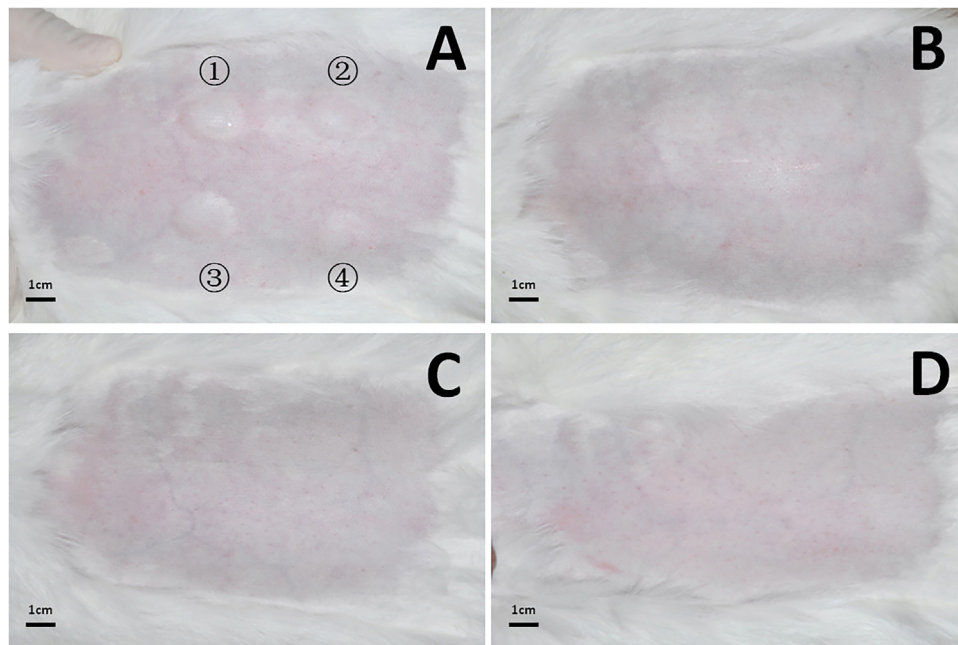

Fig. 4. Skin irritation test of TACMs extract after intracutaneous injection on the back of New Zealand rabbit. (A) TACMs extract (①and②) and NS (③and④) were injected respectively on the left and right back skin next to backbone; (B, C, D and E): images of injection sites after 24 h, 48 h, and 72 h, respectively.

were prepared by adding thrombin in the contrast agents and NS mixed solution (32 U/ml). Subsequently, the initial setting time of each solution above was tested by the same procedure of testing standards. The activity retention of thrombin in all test articles was calculated based on linear regression equation, respectively.

Finally, the feasibility of adding contrast agents to TACMs mixed thrombus as tracer tracing was verified according to the activity retention of thrombin. The scheme of embolic agent (TACMs and whole blood mixed thrombus) delivering in vivo was confirmed.

2.6. Stress resistance experiment of TACMs mixed thrombus in vitro

To investigate the effect of TACMs on thrombus strength, the high sensitivity stress resistance measurement system developed by our team were applied to assess strength and stress of mixed thrombus with different content of microspheres in vitro.

The FUTEK force transducer, HB-811A low noise and weak voltage signal preamplifier, NI USB-6221 data acquisition card and
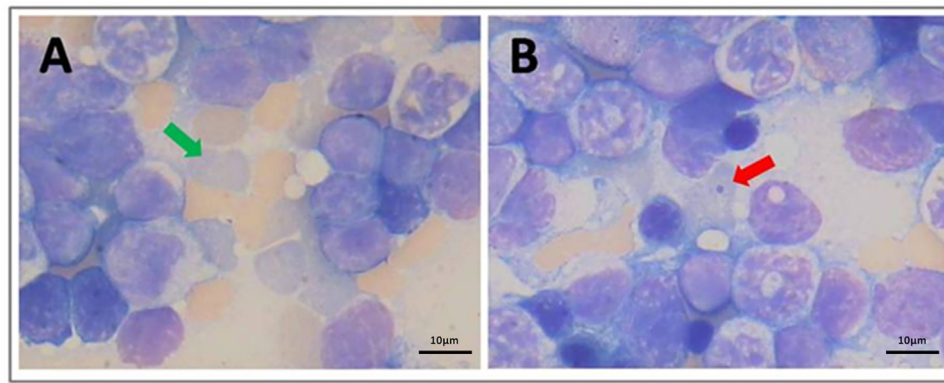
Fig. 5. Normal and micronucleated polychromatic erythrocyte (PEC) in femur marrow smear of C57 BL/6J mouse. (A) normal femur marrow smear (Green arrow: normal PEC); (B) femur marrow smear with micronucleus (Red arrow: micronucleated PEC). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
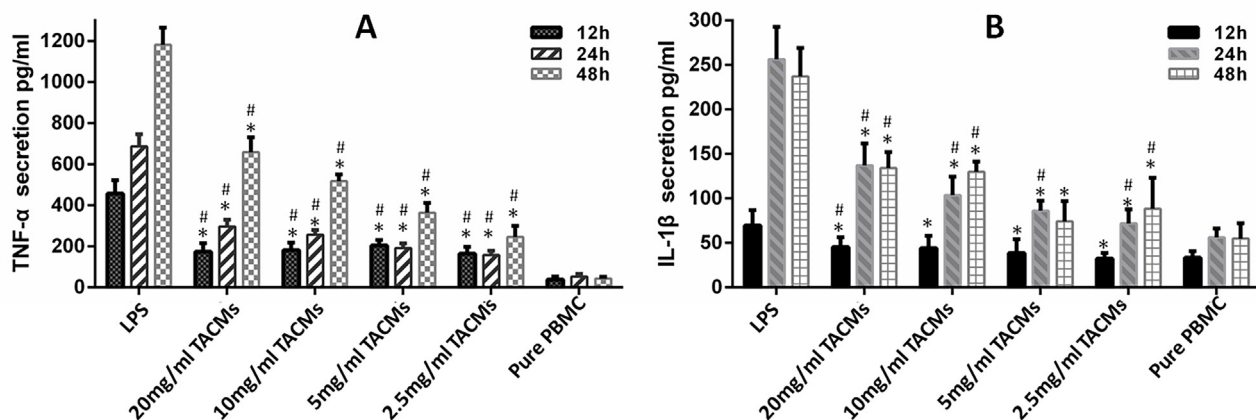

Fig. 6. Secretion of TNF-α and IL-1β in different TACMs groups, positive group (LPS) and negative group (pure PBMC). (A) the levels of TNF-α in experimental groups with different doses of TACMs and control groups at different time points; (B) the levels of IL-1β in experimental groups with different doses of TACMs and control groups at different time points (n = 8).* P < 0.05, experimental group vs. positive control group; # P < 0.05, experimental group vs. negative control group.

upper computer were connected together and switched on firstly (Fig. 2). Then, all equipments were calibrated. To improve sensitivity and accuracy, the measurement ranges of equipment were set reasonably. Pressure range was 0–100 g and data acquisition frequency was 1000 times per second.

The test procedures were as follows (Fig. 2): the fresh whole blood was collected from a healthy beagle (males, 22 kg) and then mixed uniformity with different doses (0, 0.5, 1, 2 and 3 ml) of TACMs to form 5 ml mixed thrombus in 10 ml plastic vials, respectively. The mixed thrombus was then taken and placed in stress test platform for analysis instantly. And then, the probe of force transducer squeezed the mixed thrombus straight downward at 1.5 mm/s.

The pressure fluctuation of mixed thrombus was recorded by upper computer during the process of probe squeezing the thrombus. Meanwhile, the deformation-pressure curve was graphed. Each kind of samples was retested for 5 times. The differences of TEG results among TACMs groups and control group were tested by one-way ANOVA.

2.7. Embolic effect and post-procedural complications of TACMs mixed thrombus

Large animal experiments were designed to investigate the embolic effect and post-procedural complications of TACMs mixed thrombus in splenic injured and hemorrhagic models of large animals. Sixteen beagles (male, 20–24 kg) were used and divided into

experimental group and operational blank control group randomly (8 for each group).

2.7.1. Preparation of embolic agent

Anesthesia was induced by 40 mg propofol which was injected in leg venous in 10 s. The anesthesia maintenance was achieved by continuous intravenous administration of 10 mg/kg/h propofol by micro-pump during the operation. The electrocardiogram was continually monitored during the operation. After that, the right common femoral artery was surgically exposed and a 6F sheath was placed. The embolic agent used in experimental group was prepared by mixing TACMs with whole blood of animal in vitro. In short, 3 ml whole blood was drawn from the artery sheath, followed by injection of 100U/kg heparin into the sheath. The whole blood was rapidly mixed with 2 ml TACMs in a 10 ml syringe to form TACMs and whole blood mixed thrombus as embolic agent.

2.7.2. Modeling splenic injury and embolization hemostasis in experimental group

The splenic artery was exposed by laparotomy (Fig. 3A), then splenic artery angiography was performed by 6F angiography catheter (Fig. 3B). An incision (4.0 cm in length and 1.0 cm in depth) was subsequently made by a scalpel to establish hemorrhage model (Fig. 3C), which was similar to grades of American Association for the Surgery of Trauma-Organ Injury Scale (AAST-OIS) II-IV[36]. The splenic incision was pressed by sterile gauze for 5 min to identify the continued bleeding and rule out the effect

Table 2
Formation rate of micronucleus.

Group	Number of femur marrow smears	Number of polychromatic erythrocytes	Number of polychromatic erythrocytes with micronucleus	Formation rate of micronucleus(‰)
TACMs				
10 ml/kg/d	12	24000	67	2.79
25 ml/kg/d	12	24000	59	2.46
50 ml/kg/d	12	24000	75	3.13
Negative control	12	24000	57	2.38
Positive control	12	24000	706	29.42*

* $p < 0.05$. TACMs: Injected TACMs extract liquid into the abdominal cavity; Negative control: Injected 25 ml/kg/d NS; Positive control: Injected 25 ml/kg/d Cytoxan.

of autologous clotting. The bleeding was confirmed by both naked eyes and angiography (Fig. 3C and D). Then, the embolization of splenic artery was performed at the predetermined embolism site. Briefly, the opening of 6F guiding catheter was located into the proximal artery of spleen (beyond the dorsal pancreatic artery) along the guide wire accurately and 1 ml TACMs mixed thrombus was released site-specifically by 'sandwich' delivery method. The total dose of embolic agent used in each operation was determined by two interventional experts and the injection was halted once the embolization was successful. The change of bleeding were monitored by both incision observations (Fig. 3E) and angiography(Fig. 3F). The time from modeling splenic injury successfully to complete stop of bleeding was recorded. At last, abdominal closure and femoral artery ligation were performed, and animals were sent back to the experimental animal center for observation.

The beagles in operational blank control group were processed with the same laparotomy, splenic artery interventional operation, abdominal closure and femoral artery ligation as experimental group. However, no splenic incision was made and no embolic agent was injected by guiding catheter in operational blank control group.

The main outcome measures of animals in two groups after operation were compared, including mortality, temperature, body weight and blood routine result at the time-point of 1d, 3d, 5d, 7d, 15d, 30d after operation. In addition, follow up angiographies of splenic artery were performed at 2 week, 1 month and 3 month after embolization for observing the perfusion and morphological change of embolized spleen. At 3 month, the animals in two groups were sacrificed by delivering the fatal dose of propofol. The embolized splenic artery in experimental group and normal splenic artery in control group were retrieved and fixed in 4% paraformaldehyde. Then, 4 μm thick paraffin sections were prepared and standard HE staining was performed to evaluate the integrality and the inflammatory reaction of vessel wall, as well as the peripheral lymph nodes.

3. Results and discussion

3.1. Skin irritation test

Before humans can be exposed to such substances, the tendency of skin irritation caused by new chemicals must be carefully checked. In this research, the New Zealand rabbits were used to evaluate the skin irritation of the extracts of TACMs. At 24, 48 and 72 h of administration, we found that the intracutaneous injection of TACMs extract did not induce any cutaneous reaction on the rabbit back skin, compared with negative control group (intracutaneous injection of NS) in all animals (Fig. 4A). The scores of cutaneous reactions were all zero. These results indicated that the TACMs was not a kind of skin irritated material (Fig. 4B–4D), and could be served as a safe embolic agent with excellent biocompatibility.
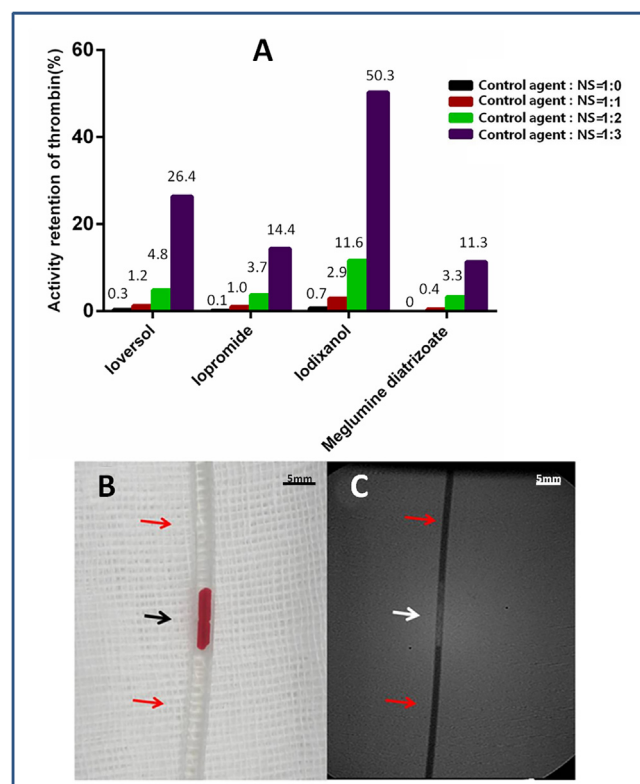

Fig. 7. Inhabitation of common clinic control agents on the activity of thrombin and sanwich delivering method. (A) TACMs mixed thrombus in transparent extension tube (Black arrow: TACMs mixed thrombus; Red arrow: control agent); (B) TACMs mixed thrombus in guiding catheter under X-ray (White arrow: TACMs mixed thrombus; Red arrow: control agent). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Micronucleus test

Genetic toxicity studies have been generally used in a qualitative method to assess whether a compound is genotoxic or not. In vivo erythrocyte micronucleus test assesses the ability of a tested compound to induce chromosomal damage in progenitor red blood cells in bone marrow [37]. The increased incidences of micronucleated PCE (reticulocytes, Fig. 5B) in bone marrow of chemical-exposed animals are indicators of chromosomal damage in the form of chromosomal breakage or chromosome loss at anaphase [33]. In this study, formation rates of micronucleus in different TACMs subgroups were similar to those in negative control group (Table 2, $P > 0.05$) and there were no significant increase of micronucleated PCE in all TACMs subgroups during the observation period, while formation rate of micronucleus in positive control (cyclophosphamide injection) was significantly higher than that in the TACMs groups, as well as in negative control group ($P < 0.05$). These results suggested that TACMs induced neither teratogenic-
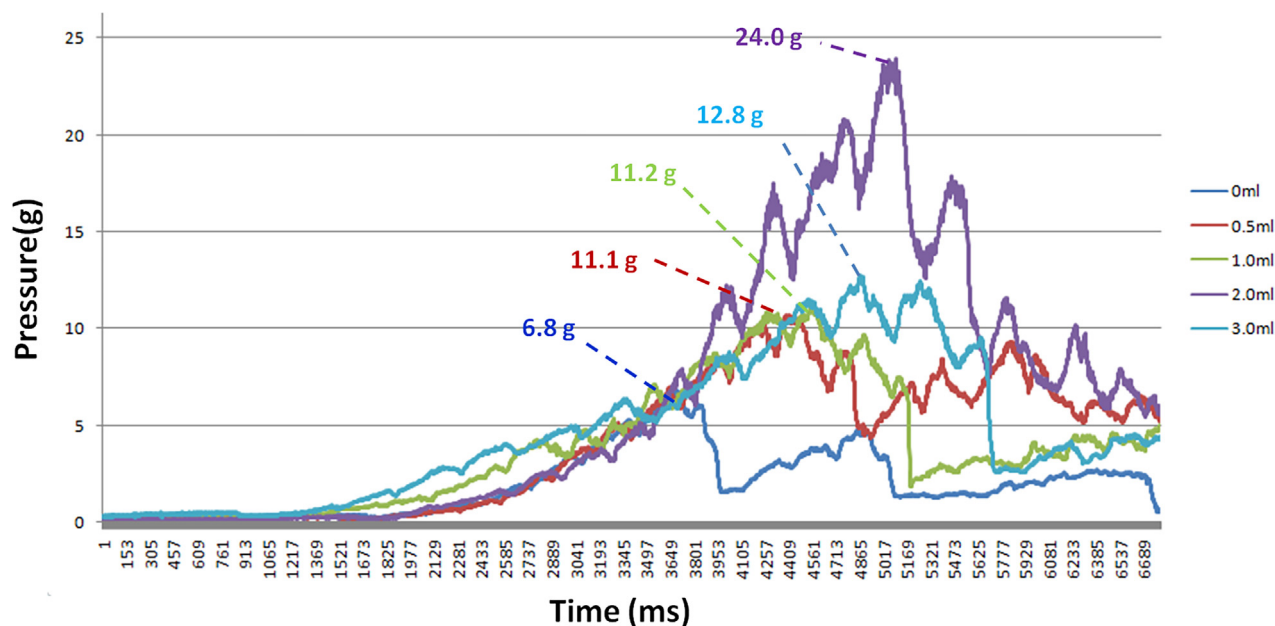
Fig. 8. Time-pressure curve of mixed thrombus with different doses of TACMs.

Table 3
Levels of TNF-αin experiment groups with different doses of TACMs and control groups at different time points (n = 8, pg/ml).

Group	12h	24h	48h
Positive Control (LPS)	456.2 ± 65.6	686.3 ± 59.3	1181.7 ± 83.0
20 mg/ml TACMs	174.1 ± 41.1*#	296.5 ± 33.2*#	657.9 ± 72.4*#
10 mg/ml TACMs	181.2 ± 36.7*#	256.0 ± 23.3*#	517.3 ± 32.6*#
5 mg/ml TACMs	204.5 ± 25.3*#	191.6 ± 22.8*#	363.6 ± 47.2*#
2.5 mg/ml TACMs	163.8 ± 34.1*#	157.4 ± 21.0*#	245.2 ± 54.5*#
Negative Control (Pure PBMC)	38.9 ± 13.2	52.1 ± 13.9	42.6 ± 8.4

LPS: Lipopolysaccharide; PBMC: Peripheral blood mononuclear cells; * P < 0.05, experiment group vs. positive control group; # P < 0.05, experiment group vs. negative control group.

Table 4
Levels of IL-1βin experiment groups with different doses of TACMs and control groups at different time points (n = 8, pg/ml).

Group	12h	24h	48h
Positive Control (LPS)	69.6 ± 17.3	256.3 ± 36.5	237.2 ± 31.9
20 mg/ml TACMs	45.4 ± 11.0*#	137.0 ± 24.6*#	176.2 ± 17.9*#
10 mg/ml TACMs	44.2 ± 13.6*	103.5 ± 20.9*#	129.8 ± 11.5*#
5 mg/ml TACMs	38.7 ± 15.5*	86.1 ± 11.3*#	74.2 ± 22.6*
2.5 mg/ml TACMs	32.3 ± 6.4*	71.9 ± 15.8*#	88.3 ± 34.7*#
Negative Control (Pure PBMC)	33.4 ± 7.3	56.0 ± 10.1	54.8 ± 17.2

LPS: Lipopolysaccharide; PBMC: Peripheral blood mononuclear cells; * P < 0.05, experiment group vs. positive control group; # P < 0.05, experiment group vs. negative control group.

ity, nor mutagenicity with broken DNA or aneuploidy, and met the biosafety requirement of embolic agent.

3.3. Cytokine production

Chronic inflammation may occur in tissues adjacent to TACMs when TACMs was introduced into blood vessel. It relies heavily on recruitment of monocytes and macrophages, as well as sequential release of proinflammatory mediators [38,39]. It has been reported that overproduction of cytokines contributes to persistent inflammatory response or tissue destruction around foreign materials [40]. The secretion of TNF-α and IL-1β from human PBMC increased in TACM group, but not in negative control group (Fig. 6, Tables 3 and 4), with an elevation of TACMs concentration. However, the secretion level in TACM group was relatively lower than that in LPS group. In addition, the secretion of TNF-α and IL-1β from human PBMC also showed time dependence generally.

The rise of cytokine secretion from PBMCs could be explained by release of thrombins from TACMs, and thrombins could activate cytokine secretion from monocytes as a kind of proinflammatory substance [41–43]. In our previous research, we have found that the inflammatory cells were present and cellular infiltration was observed around the TACMs in vivo, which indicated the degradation of microspheres [30]. Meanwhile, the amounts of inflammatory cells declined with the degradation of TACMs and no microsphere and inflammation were identified finally [30].

3.4. Feasibility evaluation of adding contrast agents to TACMs mixed thrombus for tracing

The TACMs mixed thrombus was invisible under X-rays, so we performed a study on the feasibility of adding contrast agents to TACMs mixed thrombus for tracing under X-rays. The enzyme reactive center and the structure are the precondition of catalytic action of thrombin. The protein spatial structures of thrombin are held by chemical bond, such as hydrogen bond, hydrophobic and ionic bond. All these bonds can be destroyed by strong acid, strong base, heavy metal or high temperature, eventually resulting in the inactivation of enzymes. The results of our research showed that the four iodine containing contrast agents, commonly used in clinic, decreased the activity of thrombin in a concentration dependent manner (Fig. 7A). Iodixanol will bring the slightest influence among the contrast agents. Han et al. suggest that the iodine contrast agents can be combined with biological macromolecules and affect their normal function [44]. Moreover, some studies had indicated that radiographic contrast agents inhibited the thrombin function, i.e. inhibition of thrombin binding to fibrin [45]. So it was reasonable to avoid contact of TACMs with contrast agents with the greatest effort in the whole process of embolic agent preparation and delivery.

To meet the needs of reserving thrombin activity in microspheres and tracing delivered embolic material simultaneously, we developed a novel delivery method named "sandwich" method [30] (Fig. 7B and C). In brief, the catheter was successively filled
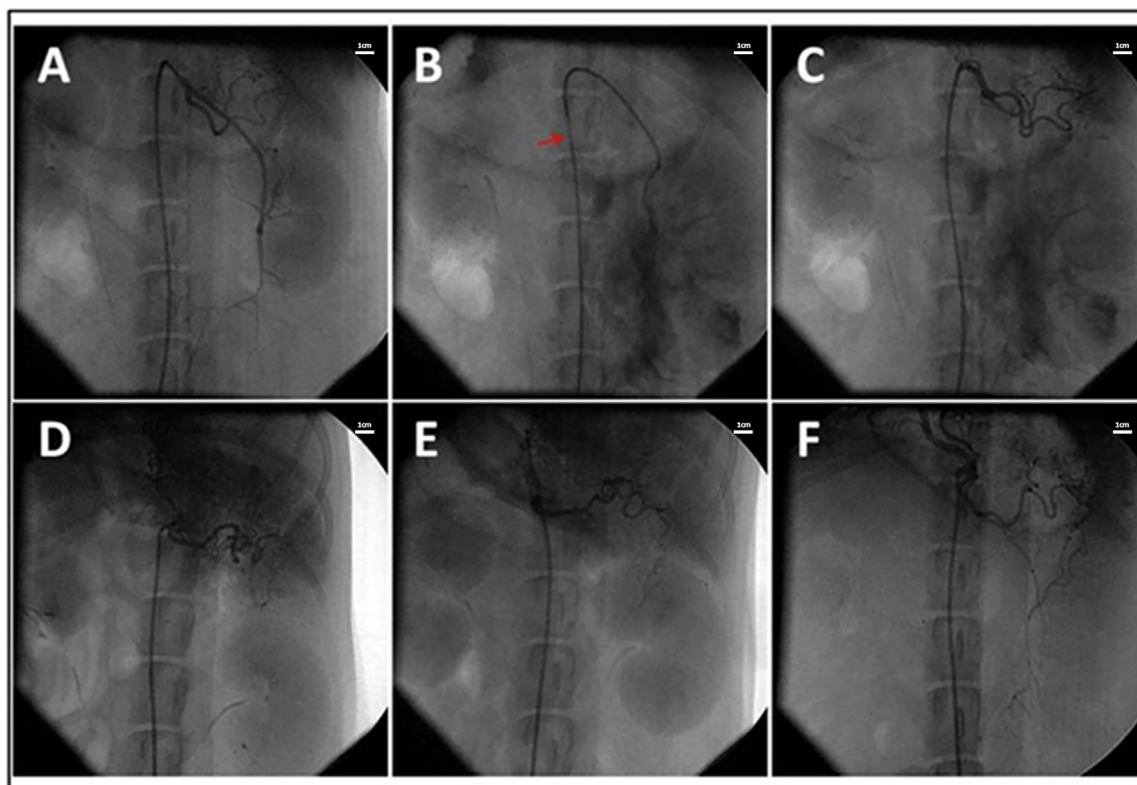
Fig. 9. Interventional embolization process and follow up angiography. (A) angiography of normal splenic artery; (B) targeting delivering of TACMs mixed thrombus (Red arrow) as embolic agent; (C) angiography at 30 min after embolization; (D, E, F) angiography 2-week, 1- and 3-month after TAE. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
The maximal stress resistance value of TACMs mixed thrombus with different doses of TACMs.

Dose of TACMs (ml)	0	0.5	1.0	2.0[*]	3.0
Maximal stress resistance value (g)	6.8	11.1	11.2	24[*]	12.8

[*] maximum of maximal stress resistance value.

with contrast agent, TACMs mixed thrombus and contrast agent in vitro. Then, the TACMs mixed thrombus were visible when delivering in vivo under the X-ray without contrast agent mixed. The "sandwich" method could not only clearly trace the track of mixed thrombus, but also separate TACMs from contrast agents to reserve the activity of thrombin in TACMs effectively.

3.5. The stress resistance experiment of TACMs mixed thrombus in vitro

The highest point on the stress curve of Fig. 8 represented the maximal strength of TACMs mixed thrombus. The results of stress resistance test showed that strength of TACMs mixed thrombus was significantly higher than simple autologous thrombus. Furthermore, the strength of mixed thrombus was related to composition of microspheres. As Table 5 showed, the thrombus strength increased significantly with incremental doses of TACMs. In addition, results of stress test further established an optimized ratio of 2:3 of the TACMs to the whole blood, under which mixed thrombus has the strongest stress resistance.

These results indicated that TACMs mixed with blood could form firmer thrombus than simple autologous thrombus. The strength of mixed thrombus as well as hemostatic effect of embolization should be improved by optimal ratio of TACMs to the whole blood.

This result provided clinical guidance for the next embolization hemostasis experiment in large animals.

3.6. Embolic effect and post-procedural complications of TACMs mixed thrombus

The embolic materials used in TAE can be categorized into permanent material (for progressive diseases, such as tumors) and temporary material (for self-limited diseases, such as traumatic lesions) [46]. Most of the commercially available embolic agents for hemorrhage of solid abdominal viscera are permanent and non-biodegradable [47,48]. It was reported that the long-term presence of the embolic materials in vivo provoked chronic inflammation, or even caused tissue damage [49,50]. So biodegradable embolic materials are preferred for avoiding complications and reserving the function of injured organs [51]. However, as the only commercially available biodegradable embolic material for blunt trauma of solid abdominal viscera, gelatin sponge particles can not precisely control the extent of embolization [52], and the ischemic complications are often related to the nontargeted embolization and occlusion of distal small arteries [53,54]. Of note, it was reported that the gelatin sponge particles, which occlude the injured artery successfully in TAE operation, may be rapidly absorbed in vivo, leading to rebleeding before final hemostasis [48].

In this study, we showed that preparation of TACMs mixed thrombus in vitro was convenient and time-saving. By applying "sandwich" method, the movement track of TACMs mixed thrombus in vivo and its localization in target vessels could be clearly displayed under X-ray (Fig. 9B). Because the TACMs mixed thrombus contained no organic solvent, there was neither any need for catheters made of special material, nor chemical stimulated spasm of vessel. By using commercially available catheters, TACMs mixed
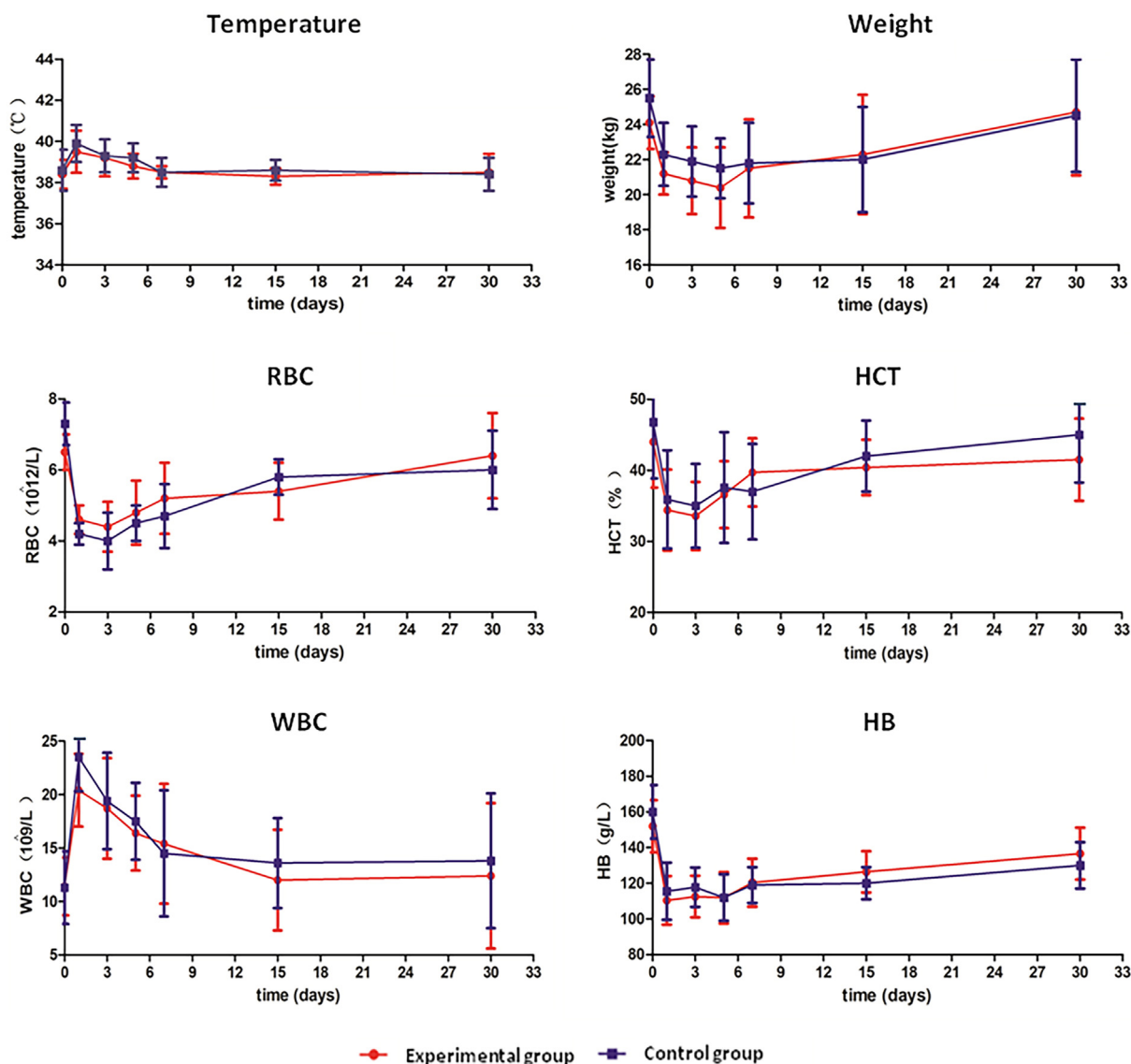
Fig. 10. General conditions of animals in experimental group and control group after operation.

thrombus could be delivered to the target site of splenic artery quickly and conveniently, without any occlusion or stickiness of catheter. All interventional embolizations were performed successfully in experimental group without any complications related to the procedures, such as reflux of embolic agent. The leakage of contrast agent from the incision was not found anymore under X-ray after embolization (Fig. 9C). Meanwhile, direct observation showed the incision bleeding decreased gradually after splenic artery embolization effectively and stopped completely within 2.2 ± 1.6 min. The average dosage of embolic agent used in each operation was 1.0 ± 0.5 ml.

The follow up angiography indicated the establishment of collateral circulation of embolized splenic artery in short time (less than 2-week) after embolization (Fig. 9D). The blood perfusion and morphology of spleen parenchyma recovered gradually during the 3-month follow-up (Fig. 9F). Severe post-procedural complications, such as re-bleeding or total spleen infarction, which must be treated by open surgery, were not observed during follow-up. The spleen was also well reserved and the 3-month survival rate of animals in experimental group was 100%.

The general conditions of animals in experimental group and control group were monitored after operation (Fig. 10). There were no significant differences in body weight, body temperature, white blood cell (WBC) count, red blood cell (RBC) count, hematocrit and hemoglobin level between both groups. The transient fluctuations of physiological states were mainly contributed to surgical injury and stress, as well as the food-intake descending momentarily after TAE.

In experimental group, the histological observation of embolized splenic artery showed that TACMs were found at the pre-determined embolization site of splenic artery and encapsulated by connective tissue (Fig. 11A). The vessel wall of embolized splenic artery was intact and showed clear layers without inflammatory cell infiltration. In addition, there was no obvious lymphocyte proliferation in lymph nodes adjacent to the embolized splenic artery (Fig. 11C). All these results showed that TACMs could be considered as a good biocompatible and biodegradable embolic material, which neither destroyed the structure of vessel wall nor caused severe post-procedural complications, such as pseudoaneurysm.
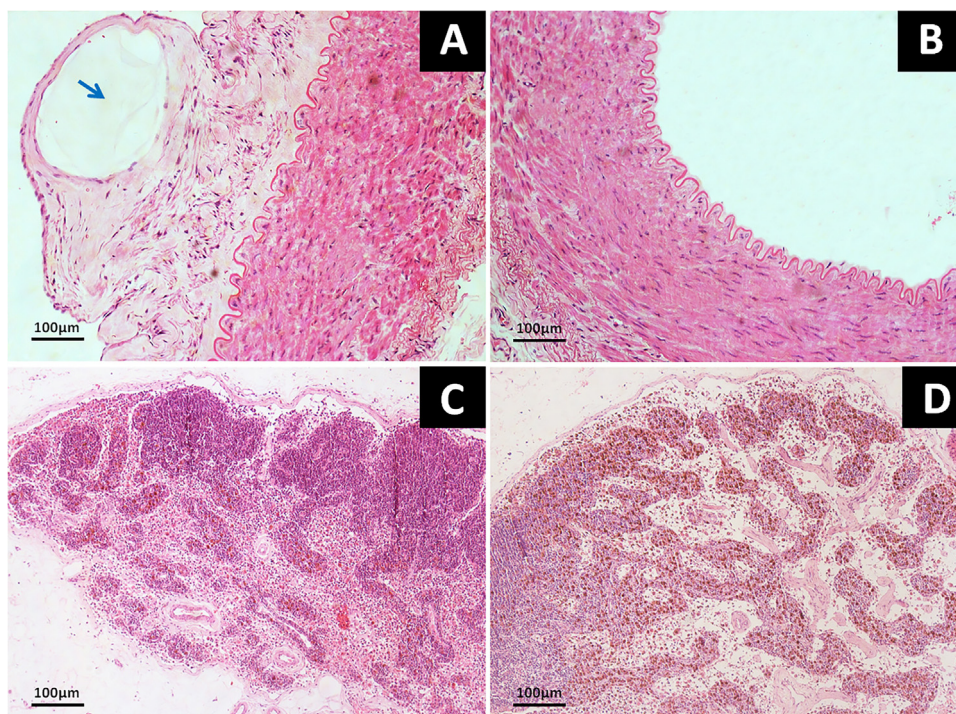
Fig. 11. Histological observation of embolized splenic artery and adjacent lymph node. (A) splenic artery embolized by TACMs mixed thrombus (Blue arrow: TACM); (B) normal splenic artery; (C, D) lymph nodes nearby the embolized splenic artery and the normal splenic artery. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The comparison of biocompatibility and embolic effect between TACMs mixed thrombus and the existing biodegradable embolic material used in clinical (such as gelatin sponge particles) will be further investigated in the near future.

4. Conclusions

Absence of ideal embolic agent for treatment of blunt trauma of solid visceral organs calls for development of novel embolic materials. In this study, biocompatibility of TACMs and the physical characteristic, application method, short- and long-term embolic effect of TACMs mixed thrombus were further investigated. Combined with the results of our previous researches, TACMs was a kind of biodegradable embolic material with good biocompatibility. The TACMs mixed thrombus, as a kind of novel embolic agent for TAE, had advantages of rapid and reliable embolic hemostasis, broad application and easy operation. The development and application of TACMs are promising in improving the effect and prognosis of transcatheter hemostasis for solid visceral rupture and hemorrhage.

Authors' contributions

YH and WY conceived of the study. JR, ML and FX participated in the original design. JR, ML, FX, JS, LZ, HZ, QZ, CP and FL performed the experiments. JR, XT, DL and XW drafted the manuscript and read it critically. All authors read and approved the final manuscript.

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