Anaphylactic shock caused by haemocoagulase injection in China

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Abstract. Haemocoagulase injection is a mixture of purified enzymes isolated from the venom of *Bothrops atrox*, which is used for the prevention and treatment of haemorrhage. It is a relatively safe pharmacological agent that does not require a skin test prior to administration. However, following a literature search, 14 reported cases of anaphylactic shock caused by haemocoagulase injection were identified, including one lethal case in China. Using SDS-PAGE and protein identification, four primary components in haemocoagulase injection were characterized, including one metalloproteinase, which may be a thromboplastin-like enzyme, and two serine proteinases, which may be thrombin-like enzymes. Administering concentrated haemocoagulase injections failed to provoke a positive skin reaction in allergic patients. Basophil activation tests revealed that haemocoagulase injections did not upregulate cluster of differentiation 63 or C-C chemokine receptor type 3 expression. These findings suggest that haemocoagulase injection may cause fetal anaphylaxis. Although it is difficult to determine a clear conclusion without being able to evaluate the patients that underwent haemocoagulase injection-induced shock, it is unlikely that the venomous components of haemocoagulase injection cross-react with common allergens in allergic patients. It is possible that haemocoagulase injection-induced anaphylaxis is caused by its additive components, such as mannitol and succinylated gelatin.

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Introduction

Haemocoagulase injection is a mixture of purified enzymes that are isolated from the venom of *Bothrops atrox*, a viper native to South America. Haemocoagulase is able to promote blood coagulation via two different enzymatic activities: One, induced by thromboplastin-like enzymes, accelerates the conversion of prothrombin to thrombin, whereas the other induces a direct transformation of fibrinogen to fibrin monomer via thrombin-like enzymes; the fibrin can be subsequently converted by thrombin into a fibrin clot (1). Haemocoagulase injections do not contain any neurotoxins or other toxic substances from unpurified snake venom (1). The haemostatic effect of intravenous haemocoagulase injection becomes apparent 5-10 min post-administration and lasts for up to 24 h, whereas the blood coagulation action of intramuscular or subcutaneous administration occurs 20-30 min post-administration and continues for up to 60 h (1). Haemocoagulase injections are used for the prevention and treatment of hemorrhages in various branches of medicine, including gynecology, urology, gastroenterology and dentistry (1).

According to the manufacturer's clinical instructions, the primary side effect of haemocoagulase injection is allergic reaction, which is also listed as a side effect of the majority of allergy treatments, such as antihistamines (1). However, it is almost unavoidable for trace amounts of other snake venom contaminants to remain in haemocoagulase, although these amounts are considered to be safe and there is no requirement for skin tests to be performed as standard (1). Trace amounts of snake venom components may not be toxic, but may be allergens and cause severe anaphylactic reactions in sensitive individuals (2-4). For example, *Bothrops atrox* venom contains allergens that are able to selectively bind to specific immunoglobulin E (IgE) antibodies in the serum of patients who are allergic to this venom (2,3). Furthermore, we propose that patients who are allergic to various common allergens may be cross-reactive to the components of haemocoagulase injections. At present, haemocoagulase injection-induced anaphylaxis has not been reported outside of China; therefore, the aims of the present study were to summarize the anaphylactic cases caused by haemocoagulase injection in China and to investigate the venom components of haemocoagulase injection that may cause anaphylaxis.

Materials and methods

Cases. A total of 11,179 articles were identified by searching 'haemocoagulase' translated into the Chinese language in the China Knowledge Resource Integrated database (www.cnki.net; 9,346 articles), VIP database (qikan.cqvip.com; 1,154 articles) and Wanfang database (g.wanfangdata.com.hk; 679 articles). A total of 1,370 of the returned articles were duplicates; therefore, they were excluded. The remaining 9,809 articles were thoroughly evaluated and a total of 13 articles describing 14 cases of anaphylactic shock in China caused by haemocoagulase were identified (5-17). The articles were retrieved from scientific journals published in Chinese between 1997 and 2009 (Table I). The ages of patients in each study ranged from four months to 75 years.

SDS-PAGE. A total of 10 vials of haemocoagulase injection containing *Bothrop atrox* venom abstract, mannitol, succinylated gelatin and calcium chloride (20 ml; lot number, 921007; Solco Basle Ltd., Basel, Switzerland) were concentrated to 200 μl using Amicon Ultra-15 centrifugal filter units (3,000 Da cutoff; EMD Millipore, Billerica, MA, USA). SDS-PAGE was performed under reducing conditions. Concentrated haemocoagulase injection contents (20 μl) were loaded onto a 15% polyacrylamide gel. Protein bands were visualized following a standard Coomassie blue stain and silver staining procedure. Protein standards used for SDS-PAGE were: Rabbit muscle phosphorylase b (97.2 kDa), bovine serum albumin (66.4 kDa), chicken egg ovalbumin (44.3 kDa), bovine carbonic anhydrase (29 kDa), soya bean trypsin inhibitor (20.1 kDa) and chicken egg lysozyme (14.2 kDa; all Sangon Biotech Co., Ltd., Shanghai China).

Mass spectrometry (MS) analysis and protein identification. Silver staining was used to visualize one-dimensional SDS-PAGE gels, protein bands were excised and transferred to appendorf tubes for destaining and in-gel digestion with modified trypsin solution (Promega Corp., Madison, WI, USA) (18). Following digestion, tryptic peptides were extracted from the gel pieces using 5% acetonitrile (Sangon Biotech Co., Ltd.). Extracted peptides were diluted to 20 ul/sample using 0.1% formic acid. Electrospray ionisation (ESI)-MS/MS analysis of protein and protein identification were conducted as previously described (19). Briefly, a nanoflow liquid chromatography (LC) system and an LCQ DECA Ion Trap Mass Spectrometer (both Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used to perform LC-ESI-MS/MS identification of proteins. Extracted peptide samples were centrifuged at 13,500 x *g* for 20 min at 4˚C, and the supernatant was collected and loaded on the analytical column (RP-C18; 0.18x100 mm; Thermo Fisher Scientific, Inc.). Buffers A (2% v/v acetonitrile, 0.1% formic acid) and B (100% v/v acetonitrile, 0.1% formic acid) were used to elute peptides at a flow rate of 200 nl/min for 1 h. The data-dependent 'triple-play' method was used to analyze peptide ions as follows: i) full MS scan (m/z; 400-1,800), ii) ZoomScan (scan of the major ion with higher resolution), iii) MS/MS of the major ion. The SEQUEST (www.proteomicsresource.washington.edu/protocols06/sequest.php) or MASCOT programs (www.matrixscience.com) were used locally for protein identification, by database searching against snake expressed sequence tag (EST) databases (EST, bothrops bothrops_20100621; downloaded from NCBI, www.ncbi.nlm.nih.gov/) and snake protein sequences (downloaded from NCBI, www.ncbi.nlm.nih.gov) with the following parameters: Monoisotopic peptide masses, 2 Da; peptide mass tolerance, 0.8 Da; fragment mass tolerance, one missed cleavage; modifications allowed for oxidation of methionine and carboxyamidomethylation of cysteine. The criteria for positive peptide identification for a doubly-charged peptide were a correlation factor (Xcorr) >2.5, a delta cross-Xcorr >0.1 (indicating a statistically significant difference between the best and second best reported match), ≥1 tryptic peptide terminus and a high preliminary scoring. The correlation factor threshold was set at 3.5 for triply-charged ions, whereas the threshold was set at 2.0 for singly-charged peptides. Matched peptides were confirmed via visual examination of the spectra.

Skin prick test (SPT) and specific IgE determination. A total of 10 vials of haemocoagulase injections (20 ml) were concentrated and separated using Amicon Ultra-15 centrifugal filter units (3 and 10 kDa; EMD Millipore) to 200 μl of sample 1 (>10 kDa), 200 μl of sample 2 (≥3 and ≤10 kDa) and 1,600 μl of sample 3 (<3 kDa). Patients had not been taking medication, including antihistamines, steroids or other drugs, for at least two weeks prior to SPT of the haemocoagulase samples. Patients were excluded if they were exhibiting dermatographia or active skin disorders. SPT tests were performed on 10 allergic patients following routine methods with the aforementioned samples (Table II) between August and October 2010. The skin prick reaction was read 15 min post-administration (20,21). The diameter of the reaction wheal was measured; a diameter ≥3 mm larger than the negative control was considered to be positive. Specific IgE was determined using the UniCAP system (Phadia; Thermo Fisher Scientific, Inc.). The present study was approved by the Faculty Committee on the use of human subjects in research in The First Affiliated Hospital of Nanjing Medical University and all participants gave written, informed consent prior to study participation.

Basophil activation test (BAT). Cluster of differentiation (CD) 63 and C-C chemokine receptor type 3 (CCR3) expression on the surface of basophils is considered to be the indicator of basophil activation (22,23). Therefore, the influence of haemocoagulase samples 1, 2 and 3 on basophil activation was examined using a Buhlmann Flow2 CAST kit (Bühlmann Laboratories AG, Schönenbuch, Switzerland) according to the manufacturer's protocol. Briefly, 50 μl of haemocoagulase samples 1, 2 and 3 were added to 50 μl whole blood from allergic subjects. A 20-μl mixture of anti-human CCR3-phycoerythrin antibody and anti-human CD63-fluorescein isothiocyanate antibody (Bühlmann Laboratories AG) were added to cells and incubated for 15 min at 37˚C, as per the manufacturer's instructions; 50 μl goat anti-human IgE antibody was used as a positive control. Flow cytometry analysis of surface markers was performed at 488 nm using a FACSAria™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FACSDiva software (version 6.1.3; BD Biosciences).

Table I. Fourteen cases of anaphylaxis caused by haemocoagulase injection in China.

Gender, age (years)	Allergy history	Reason for usage	Route of delivery	Side-effect symptoms	Onset of symptoms (min)	Ref
Male, 16	Y	Upper gastrointestinal hemorrhage	IV	AP, CP, D, FC, CE, EP, H	2	5
Female, 75	Y	Upper gastrointestinal hemorrhage	IM	FC, CE, D, MN, PR, H	5	5
Male, 62	Y	Postoperative hemorrhage	IM	R, D, FC, CE, LM, H	8	6
Male, 26	N	Postoperative hemorrhage	IV	FF, C, D, SM, CP	2	7
Female, 0.3	Y	Postoperative hemorrhage	IV	FF, D, CP	3	8
Male, 30	N	Upper gastrointestinal hemorrhage	IV	CE, FC, D, CP	<30	9
Female, 20	N	Rupture of corpus luteum	IV	PR, D, MN, CE, FC	10	10
Female, 68	N	Postoperative hemorrhage	IM, IV	D, FC, UC, LC	<30	11
Male, 63	N	Upper gastrointestinal hemorrhage	IV	D, CP, PR, R, FC, EP, H, CP	5	12
Male, 61	N	Postoperative hemorrhage	IM, IV	HS, H, D, UC	<30	13
Male, 36	Y	Epistaxis	IV	R, D, EP, CP, H, CE, SF	<30	14
Male, 27	Y	Post-trauma hemorrhage	IV	FC, D, EP, LC, LM	<30	15
Male, 52	N	Intraoperative hemorrhage	IM, IV	H	2	16
Female, 26	N	Prophylactic use prior to operation	IV	R, V, FC, LC, D, UC, CP, EP, CE, SF, H	<30	17

IV, intravenous injection; IM, intramuscular injection; AP, abdominal pain; CP, cardiopalmus; D, dyspnoea; FC, facial cyanosis; CE, cool extremities; EP, excessive perspiration; H, hypotension; MN, mouth numbness; PR, pruritus; R, restlessness; LM, limb myasthenia; FF, face flush; C, cough; SM, skin maculopapule; HS, hyperspasmia; UC, unconsciousness; LC, lip cyanosis; SF, skin flush; V, vomiting; Y, yes; N, no.

Statistical analysis. All data were expressed as mean ± standard deviation and analyses were performed using SPSS version 17.0 software (SPSS, Inc., Chicago, IL, USA). Statistical significance was evaluated using a Student's *t*-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cases. Among the 14 cases of anaphylactic shock, eight were administered an intravenous injection (IV), two were administered an intramuscular injection (IM) and four were administered IV and IM. The onset of symptoms occurred between several sec and 30 min following injection. In one case, a 4-month old infant patient underwent anaphylactic shock 3 min following haemocoagulase injection (8). In another case, a 62-year-old man had no allergic reaction to his nasal cavity sponge containing haemocoagulase during surgery; however, the patient experienced anaphylactic shock 8 min following the administration of an IM haemocoagulase injection (6). Of the 14 cases, 13 patients were rescued and one resulted in mortality. In the lethal case, a 61-year-old man suffered from anaphylactic shock 30 min following IM and IV haemocoagulase administration (13) (Table I).

SDS-PAGE. The proteins in haemocoagulase injection were concentrated 100 fold (from 20 ml to 200 μl in samples 1 and 2). The concentrated haemocoagulase revealed no obvious bands following Comassie blue staining but exhibited four major bands following silver staining. The molecular weights of the four bands were 55, 44, 32 and 26 kDa (Fig. 1). In the original haemocoagulase injection the protein components were undetectable using the Coomassie Plus™ assay kit (Pierce; Thermo Fisher Scientific, Inc.) with spectrophotometry or silver staining on SDS-PAGE.

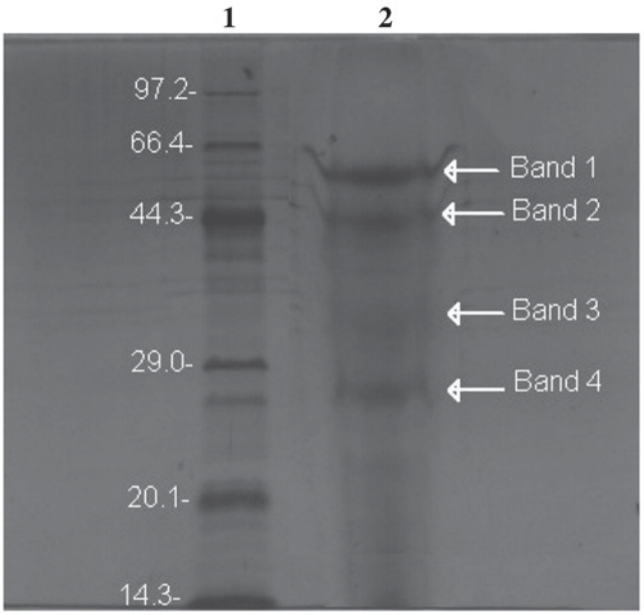

Figure 1. SDS-PAGE of haemocoagulase injection. Lane 1: protein markers including rabbit muscle phosphorylase b (97.2 kDa), bovine serum albumin (66.4 kDa), chicken egg ovalbumin (44.3 kDa), bovine carbonic anhydrase (29 kDa), soya bean trypsin inhibitor (20.1 kDa) and chicken egg lysozyme (14.2 kDa). Lane 2: Haemocoagulase in reducing conditions. Haemocoagulase exhibited four major bands via silver staining.

Protein identification. The four major bands of haemocoagulase were identified using LC-ESI-MS/MS following database searching against snake EST databases and snake protein

Table II. General characteristics of allergic patients.

Patient no.	Age, years	Gender	Disease history, years	Symptom season	Clinical diagnosis	Specific IgE measurement (grade)						
						DP	DF	Cat	AA	PH	Ragweed	GC
1	38	Male	15	Spring	AR	5	4	0	0	2	2	2
2	25	Male	9	Spring	AR	3	3	0	0	0	2	0
3	41	Male	13	Spring	AR	6	6	1	0	0	0	0
4	16	Male	2	Spring	AR	6	6	0	0	0	0	0
5	36	Male	6	Spring	AR	6	6	0	1	0	1	1
6	46	Female	20	Spring	AR	5	5	0	0	0	1	1
7	46	Male	8	Spring	AR	3	4	0	0	0	0	2
8	28	Female	5	Spring	AR	3	3	2	0	2	2	2
9	39	Female	10	Spring	AR	2	3	0	0	2	0	0
10	67	Male	40	Spring	AR	6	6	0	0	0	0	0

IgE, immunoglobulin E; AR, allergic rhinitis; DP, *dermatophagoides pteronyssinus*; DF, *dermatophagoides farina*; AA, *alternaria alternata*; PH, *platanus hispanica*; GC, German cockroach.

Table III. Characterization of the major bands of haemocoagulase injection by liquid chromatography-electrospray ionization-tandem mass spectrometry following database searching.

Band	Exp. Mr, kDa	Sequence matched	Homologous protein	Accession in NCBI database
1	55	R.ELMDLYLXXXPVWR.S	Recombination activating protein 1	ADD83390
2	44	K.KFVSGK.G; R.EDLQNQILR. R.MPGRWIPFESR.E	Snake venom zinc metalloproteinase	289592858
3	35	K.FICPNK.N; R.SVANDDEVIR.Y; E; R.SVANDDEVIRYPK.E; R.VTHTCIFASLQDTCTQCSAFR.R; R.YNAXECSITDEGSPRVTHTCIF ASLQDTCTQCSAF.R;	Snake venom serine proteinase	20376323
4	26	R.FPGKDPQEAK.F; R.SVPNDDEEIRYPK.E; K.WTEMVKIFGMVLFR.IR. RFAITLISTITSTVHSK.L; K.QPSIWDGLGNPASGRGLDSMTR. ER.KLCAGVLEGGIDTCSADSGGP LICNGQLQGIVSWR.GR. LNRPVNNSEHIAPLSLPSNPPSVG SVCRIMGWGTITPSK.A	Snake venom serine proteinase	Q5W958

Exp. Mr, experimental molecular weight; NCBI, national center for biotechnology information.

sequences deposited in NCBI. The protein in band 2 was characterized as a snake venom metalloproteinases and the proteins in band 3 and 4 were identified as snake venom serine proteinases. However, characterization via protein matching was unsuccessful in band 1 of the snake venom, with the strongest similarity demonstrated with recombination activating protein 1 in *Leptotyphlops distant*, a non-venomous blind snake (Table III).

SPT. None of the 10 allergic individuals exhibited a positive reaction to haemocoagulase and sample 1, 2 and 3 administration (data not shown). None of the 13 anaphylactic shock patients were recruited to undergo SPT.

BAT. Administration of haemocoagulase and samples 1, 2 and 3 did not induce a significant increase in CD63 and CCR3 double-positive cells compared with the negative control;

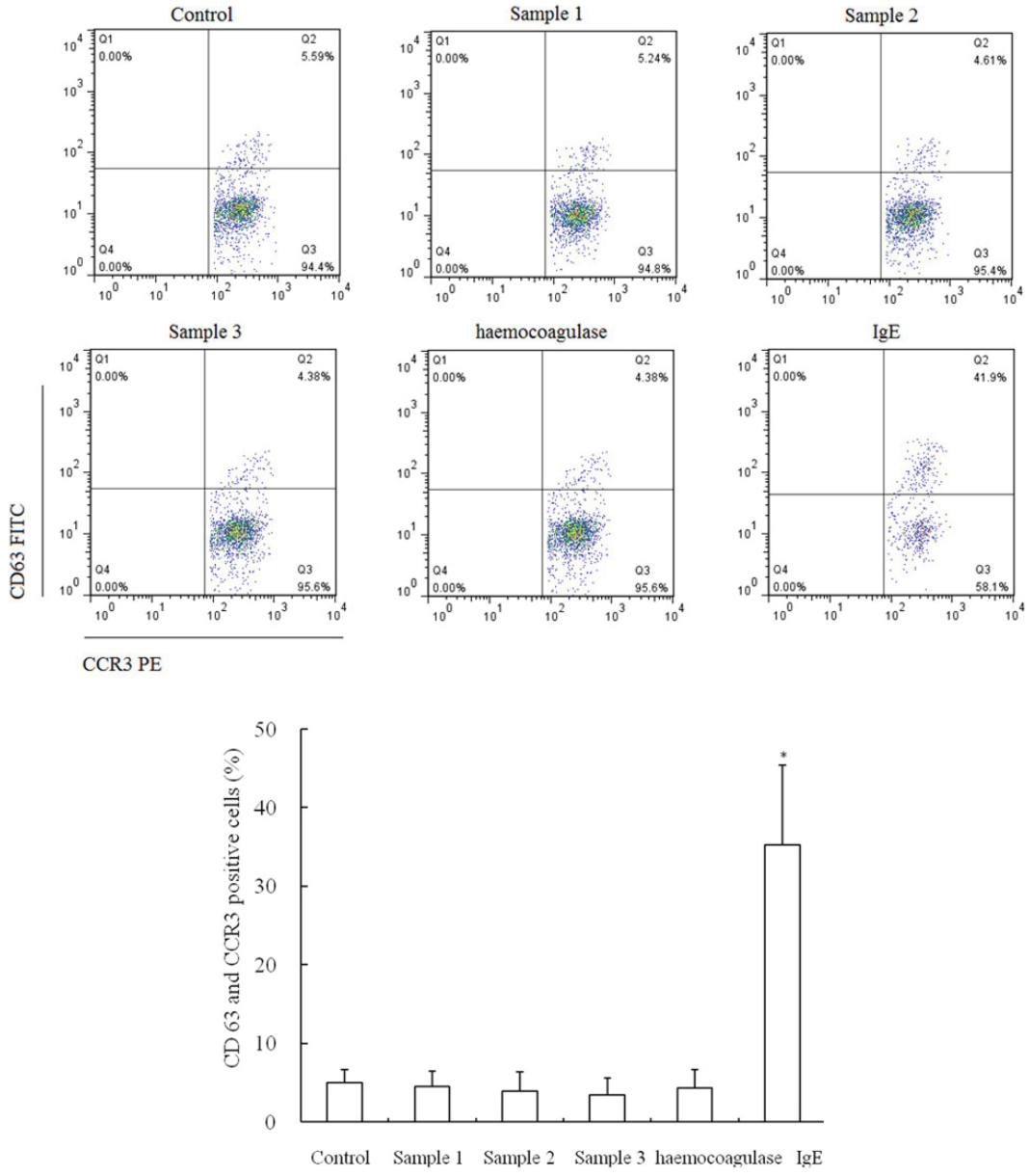

Figure 2. Effect of haemocoagulase and its components on expression of CD63 and CCR3 on basophils. Sample 1, 2 and 3 represent the molecular weights of the protein components >10, between 3 and 10, and <3 kDa, respectively. Values are presented as the mean ± standard deviation for 10 different allergic patients. CCR3, C-C chemokine receptor type 3; CD63, cluster of differentiation 63; FITC, fluorescein isothiocyanate; PE, phycoerythrin; IgE, immunoglobulin E. *P<0.05 vs. control.

however, the positive control, a goat anti-human IgE antibody, induced a 36.1% increase in CD63 and CCR3 double positive cells (Fig. 2).

Discussion

Haemocoagulase is widely used in all fields of medicine, is considered safe and does not require a skin test prior to administration (1). However, a literature search returned 14 reported cases of anaphylactic shock induced by haemocoagulase injection in China (4-16), which should be noted by clinical practitioners. Snake venom is a mixture of a number of toxic and non-toxic components; therefore, it is unavoidable that trace amounts of snake venom components may be found within haemocoagulase, which is purified from snake venom (1). These contaminants may not be toxic but may be allergens; for example, it has been reported that *Bothrops atrox* venom contains certain allergens that specifically bind to IgEs in the serum of patients allergic to these venom components (2,3). Furthermore, if patients are sensitive to allergens that are cross-reactive with the venom components in haemocoagulase injections, they may be at risk of anaphylaxis from haemocoagulase injection.

Four major protein components of haemocoagulase were identified in the present study. Among them were one metalloproteinase, which may be the thromboplastin-like enzyme that accelerates the conversion of prothrombin to thrombin, and two serine proteinases, which may be thrombin-like enzymes that convert thrombin into a fibrin clot (24). Snake venom metalloproteinase (25) and phospholipase A_2s (26-28) are able to

activate human mast cells via IgE-independent mechanisms and it has been demonstrated that serine proteinases, including mast cell tryptase (29), thrombin (30), trypsin (31) and chymase (32), provoke mast cell activation. Therefore, it is suggested that the anaphylactic shock caused by snake venom components in haemocoagulase may be mediated by the activation of basophils or mast cells. However, proteins with molecular weights >10 kDa and between 3 and 10 kDa in haemocoagulase injections did not affect the expression of CD63 and CCR3 in basophils. Therefore, two possibilities remain to explain the reported cases of haemocoagulase injection-induced anaphylactic shock. The first is that basophils from the allergic patients assessed in the present study were not sensitive to snake venom, suggesting that there is no cross reactivity between the snake venom components detected in SDS-PAGE and the specific allergens that these patients are allergic to. The other is that anaphylactic shock may be induced by additive components in the injection, including mannitol and succinylated gelatin. Previous studies have demonstrated that succinylated gelatin (Gelofusine®) may provoke peri-operative anaphylaxis during cardiopulmonary bypass (33,34) and mannitol may induce anaphylactic reaction (35). Currently, mannitol is used as a novel indirect osmotic bronchial challenge agent to aid the diagnosis and management of asthma and is thought to reflect underlying inflammatory processes in asthma (36).

In conclusion, it is difficult to arrive at a clear conclusion without the direct involvement of the patients that have previously experienced haemocoagulase injection-induced shock; however, the present study suggests that the venom components present in haemocoagulase injection are not cross-reactive with common allergens in allergic patients. Therefore, further studies are required to identify the cause of anaphylactic shock in these patients. Furthermore, it must be noted in clinical settings that haemocoagulase injection may cause fetal anaphylaxis.

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