

HEMORHEOLOGY INDEX CHANGES IN A RAT ACUTE BLOOD STASIS MODEL: A SYSTEMATIC
REVIEW AND META-ANALYSIS

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Abstract

Background: Blood stasis has received increasing attention in research related to traditional Chinese medicine (TCM) and integrative Chinese and Western medicine. More than 90% of research studies use hemorheology indexes to evaluate the establishment of animal blood stasis models rather than pathological methods, as hemorheology index evaluations of blood stasis were short of the consolidated standard. The aim of this study was to evaluate the accuracy of hemorheology indexes in rat models of acute blood stasis (ABS) based on studies in which the ABS model had been confirmed by pathological methods.

Materials and Methods: We searched the Chinese National Knowledge Infrastructure database (CNKI), Chinese Medical Journal Database (CMJD), Chinese Biology Medicine disc (CBM), Wanfang database, and PubMed for studies of rat blood stasis models; the search identified 18 studies of rat ABS models induced by subcutaneous injection of epinephrine combined with an ice bath. Each included study received a modified Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) score list and methodological quality assessment, then data related to whole blood viscosity, plasma viscosity, platelet aggregation rate, erythrocyte aggregation index, and fibrinogen concentration were extracted. Extracted data were analyzed using Revman 5.3; heterogeneity was tested using Egger's test.

Results: A total of 343 studies of rat blood stasis were reviewed. Eighteen studies were included in this meta-analysis; the mean CAMARADES score was 3.5. The rat ABS model revealed a significant increase in whole blood viscosity (medium shear rate), whole blood viscosity (high shear rate), plasma viscosity, platelet aggregation rate, erythrocyte aggregation index, and fibrinogen concentration compared to controls, with weighted mean differences (WMD) of 2.42 mPa/s (95% confidence interval (CI) = 1.73 - 3.10); 1.76 mPa/s (95% CI = 1.28 - 2.24); 0.39 mPa/s (95% CI = 0.24 - 0.55); 13.66% (95% CI = 9.78 - 17.55); 0.84 (95% CI = 0.53 - 1.16); and 1.22 g/L (95% CI = 0.76 - 1.67), respectively. Subgroup analysis showed that whole blood viscosity, plasma viscosity, and the platelet aggregation rate test methods were more sensitive when measured at 0-24 h than at 24-72 h after induction of blood stasis.

Conclusions: Rat blood stasis studies have incomplete experimental design and quality controls, and thus need an integrated improvement. Meta-analysis of included studies indicated that the unified hemorheology index of whole blood viscosity (medium and high shear rate), platelet aggregation rate, erythrocyte aggregation rate, and fibrinogen concentration might be used for assessment of rat ABS models independent of pathology methods.

Key words: Blood stasis; hemorheology; traditional Chinese medicine; meta-analysis; rats

Introduction

Blood stasis is a common pathological mechanism of traditional Chinese medicine (TCM), and a common

pathological basis of cardiovascular disease. The concept of blood stasis includes two types: poor blood stasis, and blood flow outside meridians. This paper focuses on poor blood stasis. The TCM classic reference *Neijing (The Internal Canon of Medicine)* did not propose the concept of "blood stasis," but repeatedly mentioned "blood coagulation and weep" to highlight the pathological mechanisms of blood stasis. Around 220 AD, the *Treatise on Fevers (shang han lun)* officially proposed "blood stasis" as an important pathological condition. In the 14th century, *Prescriptions for Universal Relief (pu ji fang)* stressed that blood stasis plays a major role in the development of chronic diseases. TCM physicians Ye Tianshi in the 17th century and Wang Qing Ren in the 18th century highlighted the significance of blood stasis in the development and progression of diseases.

Blood stasis has attracted much attention in TCM and integrative Chinese and Western medicine research. Animal models of acute blood stasis (ABS) have been widely used to investigate the pathological changes of blood stasis. Blood flow retardation in the microvasculature, pathological performance of blood stasis, and increasing thrombus formation in vitro constitute the pathological features of blood stasis. More than 90% of studies use hemorheology indexes such as whole blood viscosity, plasma viscosity, erythrocyte aggregation indexes, and platelet aggregation rate, to evaluate the establishment of an animal blood stasis model rather than a pathological method (Gao et al., 2003). Hemorheology index evaluations of blood stasis lack consolidated standards and pathological support, but they are characterized by a variety of hemorheology indexes and easy application, and have been used as common evaluation criteria for studies focusing on therapeutic evaluations and pharmacology of blood stasis (He et al., 2005). Our study aimed to investigate the relationship between hemorheology indexes and rat models of ABS, based on the studies in which the ABS model had been confirmed by pathological methods.

Many methods are used to establish rat ABS models. The model of Qi stagnation and Cold condensation (QSCC), a common cause of acute blood stasis in TCM, used subcutaneous injection of epinephrine combined with an ice bath. This method accounted for a larger number of studies than others. This rat ABS model was established by two subcutaneous injections of epinephrine (8 µg/kg per injection, at an interval of 4 hours). Two hours after the first injection, the rats were subjected to a 5-min soak in ice water. The rat ABS model was established after the second injection.

Our study focused on rat ABS models induced by subcutaneous injection of epinephrine combined with an ice bath. Meta-analysis of whole blood viscosity, plasma viscosity, platelet aggregation rate, erythrocyte aggregation index, and fibrinogen concentration in the rat ABS model were conducted, and we estimated the accuracy and standardization of those indexes.

Materials and methods

Studies were included if they met the following criteria: (i) controlled studies that used an animal ABS model induced by subcutaneous injection of epinephrine combined with an ice bath; (ii) utilized rats as experimental subjects; (iii) assessed at least one of the following outcomes: whole blood viscosity, plasma viscosity, platelet aggregation rate, erythrocyte aggregation rate, or fibrinogen concentration; and (iv) applied pathological methods to a confirmed ABS model. Studies were excluded if (i) the ABS model was induced using other methods in combination with subcutaneous epinephrine injection combined with an ice bath, or (ii) data were presented in a way not suitable for meta-analysis.

Search strategy

Studies were identified through electronic databases, including the Chinese National Knowledge Infrastructure (CNKI), Chinese Medical Journal Database (CMJD), Chinese Biology Medicine disc (CBM), Wanfang database, and PubMed. The time period searched included all articles published before May 2016. The search terms used were as

follows: "blood stasis," "stasis of blood," "xueyu," "yuxue," and "rat." Limitations included "other animals."

Study selection

The identification of eligible publications was conducted by one investigator (JXZ) based on inclusion and exclusion criteria; a second author (YL) was consulted if articles were questionable. The citations of included studies were read in detail to examine their agreement with inclusion criteria.

Methodological quality

Methodological quality assessment of eligible publications was conducted independently by two authors (YF, SDL). The Collaborative Approach to Meta-Analysis and Review of Animal Data from Experimental Studies (CAMARADES) was applied for methodological quality assessment criteria (Zeng et al., 2012), and adjusted criteria included 10 dimensions: sample size estimation, randomization application, ABS model in the theory of Qi stagnation and Cold condensation, model verification, negative side-effects of anesthetics on the cardiovascular system, experimental temperature control, application of blinding methods in results, experimental animal protection principles, publication through peer review, and conflicts of interest. CAMARADES was frequently used to determine the methodological quality of experimental animal studies (Macleod et al., 2004; Crossley et al., 2008).

Data extraction

A data extraction sheet was made, and two reviewers (JXZ, YL) conducted the extraction independently. Any disagreements were resolved by consultation. The following information was entered in the extraction sheet: study year, study design, animal strains, animal weight, animal cleanliness class, breeding facilities, randomization and group, anesthetic class, model replicating methods, and ethical principles. Data for whole blood viscosity, plasma viscosity, platelet aggregation rate, erythrocyte aggregation index, and fibrinogen concentration from each study were recorded. The data were recorded or calculated using mean and standard deviation.

Statistical analysis

Data of included studies were entered into Review Manager 5.3 (Cochrane Collaboration, Oxford, UK). A P-value < 0.05 was considered significant. Cochrane's Q-statistic and I^2 statistics were calculated and reported to assess the degree of heterogeneity (Higgins et al., 2002). If $P > 0.05$ and $I^2 < 50\%$, the study was considered homogeneous and a fixed effects model was applied. If $P < 0.05$ and $I^2 > 50\%$, the study was considered heterogeneous and a random effects model was applied. The random effects model was selected because it can be used to assume heterogeneity, it incorporates intra- and inter-study variability, including the potential impact on effect magnitude from publication variations, and it is suitable for the analysis of heterogeneous studies (Swardfager et al., 2013). The weighted mean difference (WMD) was used to analyze continuous data from the same determination method, and the standardized mean difference (SMD) was used for a pooled analysis of continuous data from different determination methods (Harris et al., 2008). 95% CIs were calculated. STATA 12 (StataCorp, LLC, College Station, TX, USA) was used to assess publication bias. The publication bias of included studies was assessed using Begg's funnel plot and Egger's publication bias plot based on Begg's rank correlation test and Egger's linear regression test respectively (Begg et al., 1994; Egger et al., 1997). Two subgroups were divided by sampling time: 0-24 h and 24-72 h.

Results

Identification of eligible studies

Database searches identified 343 references. Thirty-two references were excluded due to duplication and irrelevance, 312 descriptive studies remained. One hundred eighty-seven studies were excluded due to model replicating methods, another 64 studies were excluded because they lacked pathological assessment, and another 36 studies were excluded because they were not relevant to the study aims. An additional 7 studies were excluded due to inappropriate results forms or study design. Finally, 18 studies were included in this meta-analysis.

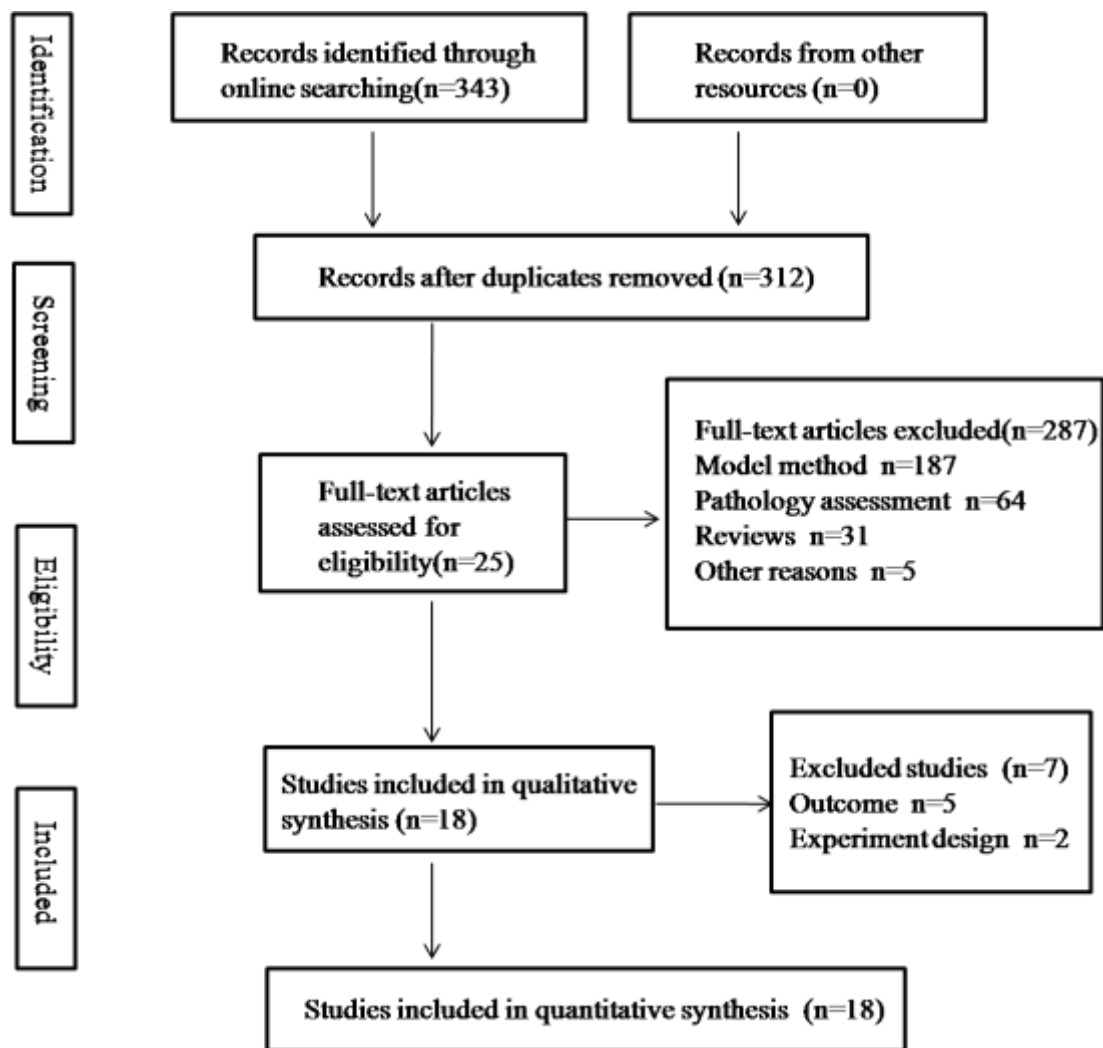


Figure 1: Flow diagram showing the number of records identified, screened, extracted, and included in the final analysis.

Study characteristics and methodological quality

Eighteen studies published from 1999 to 2016 were eligible for meta-analysis. A total of 348 rats were included; Wistar rats accounted for 27.59% of the total number of rats used; Sprague-Dawley rats accounted for 72.41%. Female rats made up 33.92% of the number of rats used; male rats accounted for 61.49%, and sex was not disclosed for the remaining 4.6%. Four studies used rats massing 200 g and below, 1 study used rats massing 300 g and above, and 13 studies used rats massing 200 to 300 g. Studies applied the same method for modeling: two subcutaneous injection of 99

epinephrine combined with a 5-min ice bath midway between the two injections; the interval between the injections was 4 hours. One study used hematoxylin and eosin (HE) staining to assess rat ABS models, 1 study used blood perfusion quantity, 3 studies used the visual outcome of microcirculation, and 13 studies used an in vitro thrombus.

None of the included studies described the sample size calculation methods or randomization methods used. The anesthetic used included urethane in 3 studies, pentobarbital in 3 studies, chloral hydrate in 8 studies, and 4 studies that did not mention an anesthetic agent. None of the included studies described blinding methods used, animal ethics guidelines adhered to, or conflicts of interest. For methodological quality assessment, 3 studies scored 5 points, 3 studies scored 4 points, and 12 studies scored 3 points.

Table 1 Characteristics of included studies

Study author(s)	Rat strain and sex	Rat weight (g)	Number of control rats	Number of model rats	Anesthetic	Detection time after modeling (h)	Pathology assessment
Li et al., 2011)	Wistar male	150-200	8	8	Chloral hydrate	1	microcirculation + thrombus
Wang et al., 2011	SD male	350-450	10	10	Chloral hydrate	24	thrombus
Zhang et al., 2016	SD male	250-350	10	10	Chloral hydrate	24	thrombus
Shi et al., 2006	Wistar Half male half female	250-300	10	10	Chloral hydrate	24	thrombus
Bao et al., 2008	Wistar Half male half female	250-300	10	10	NA	72	thrombus
Deng et al., 2007	SD female	220-240	10	10	Chloral hydrate	20	HE staining
Wang et al., 2007	SD male	200-220	10	10	urethane	NA	thrombus
Guo et al., 2009	SD Half male half female	180-200	10	10	Chloral hydrate	12	Blood perfusion
Gao et al., 2013	Wistar Half male half female	230-270	10	10	urethane	12	thrombus
Si et al., 2007	SD NA	280-300	8	8	pentobarbital	12	thrombus
Yin et al., 2008	SD Half male half female	280-320	8	8	pentobarbital	12	thrombus

Li et al., 2010	Wistar Half male half female	250-300	10	10	Chloral hydrate	12	thrombus
Peng et al., 2012	SD Half male half female	220-280	8	8	NA	12	microcirculation
Guo et al., 2012	SD Half male half female	180-200	10	10	Chloral hydrate	12	thrombus
Xie et al., 1999	SD male	230-260	10	10	NA	NA	thrombus
Chen et al., 2012	SD Half male half female	248-288	10	10	urethane	24	thrombus
Zhang et al., 2014	SD Half male half female	200-220	12	12	pentobarbita l	24	thrombus
Li et al., 2001	SD male	120-160	10	10	NA	24	microcirculation

Note: NA = not available

Table 2: Methodological quality assessment of included studies

Study	①	②	③	④	⑤	⑥	⑦	⑧	⑨	⑩	Score
Li et al., 2011	0	0	1	1	0	0	0	0	1	0	3
Wang et al., 2011	0	0	1	1	0	0	0	0	1	0	3
Zhang et al., 2016	0	0	1	1	0	0	0	0	1	0	3
Shi et al., 2006	0	0	1	1	0	0	0	0	1	0	3
Bao et al., 2008	0	0	1	1	0	0	0	0	1	0	3
Deng et al., 2007	0	0	1	1	0	0	0	0	1	0	3
Wang et al., 2007	0	0	1	1	1	1	0	0	1	0	5
Guo et al., 2009	0	0	1	1	0	0	0	0	1	0	3
Gao et al., 2013	0	0	1	1	1	1	0	0	1	0	5
Si et al., 2007	0	0	1	1	1	0	0	0	1	0	4
Yin et al., 2008	0	0	1	1	1	0	0	0	1	0	4

Li et al., 2010	0	0	1	1	0	0	0	0	1	0	3
Peng et al., 2012	0	0	1	1	0	0	0	0	1	0	3
Guo et al., 2012	0	0	1	1	0	0	0	0	1	0	3
Xie et al., 1999	0	0	1	1	0	0	0	0	1	0	3
Chen et al., 2012	0	0	1	1	1	0	0	0	1	0	4
Zhang et al., 2014	0	0	1	1	1	1	0	0	1	0	5
Li et al., 2001	0	0	1	1	0	0	0	0	1	0	3

①: sample size estimation; ②: randomization application; ③: acute blood stasis (ABS) model in the theory of Qi stagnation and Cold condensation; ④: model verification; ⑤: anesthetic side-effects on the cardiovascular system; ⑥: experimental temperature control; ⑦: application of blinding method in results; ⑧: experimental animal protection principles; ⑨: publication through peer review; ⑩: conflicts of interest. (1 = positive; 0 = negative).

Whole blood viscosity changes

Whole blood viscosity meta-analysis included 15 studies, eight in the 0-24 h subgroup and seven in the 24-72 h subgroup. Studies were heterogeneous ($\chi^2 = 261.77$; $P < 0.05$; $I^2 = 95\%$). The random effects model and weighted mean difference (WMD) were used to assess differences in whole blood viscosity between the ABS experimental and control groups.

Low shear rate of whole blood viscosity changes

The low shear rate of whole blood viscosity increased in the ABS experimental group compared to the control group: showed an absolute difference between experimental and control rats: WMD = -7.74 mPa/s, (95% CI = -9.99 - 5.59; Z = 7.06; $P < 0.05$). The 0-24 h and 24-72 h subgroups of the model both increased compared to the control group: WMD = -11.45 mPa/s, (95% CI = -15.71 - 7.19; Z = 5.27; $P < 0.05$) and WMD = -3.05 mPa/s, (95% CI = -4.78 - 1.32; Z = 3.45; $P < 0.05$), respectively. A higher increase in whole blood viscosity was observed in the 0-24 h subgroup of the model.

Medium shear rate of whole blood viscosity changes

The ABS model experimental group showed an increase in medium shear rate of whole blood viscosity compared to the control group: WMD = -2.42 mPa/s, (95% CI = -3.1 - 1.73; Z = 6.94; $P < 0.05$). The ABS model showed significant increase in the 0-24 h and 24-72 h subgroups compared to the control group: WMD = -3.07 mPa/s, (95% CI, -3.98 - 2.15; Z=6.57, $P < 0.05$) and WMD = -1.7 mPa/s, (95% CI, -2.24 - 1.17; Z = 6.28; $P < 0.05$) respectively. The 0-24 h subgroup of the ABS model in showed the greatest degree of change in medium shear rate of whole blood viscosity.

High shear rate of whole blood viscosity changes

The ABS model experimental group showed an increase in high shear rate of whole blood viscosity compared to the control group: WMD = -1.76 mPa/s, (95% CI = -2.24 - 1.28; Z = 7.24; P < 0.05). The 0-24 h and 24-72 h subgroups of the ABS model both increased compared to the control group: WMD = -1.86 mPa/s, (95% CI = -2.34 - 1.37; Z = 7.52; P < 0.05) and WMD = -1.74 mPa/s, (95% CI = -2.57 - 0.9; Z = 4.07; P < 0.05) respectively.

Plasma viscosity changes

The plasma viscosity meta-analysis included 14 studies: 8 in the 0-24 h subgroup and 6 in the 24-72 h subgroup. Studies were heterogeneous ($\chi^2 = 144.7$; P < 0.05; $I^2 = 91\%$). The random effects model and WMD were used to assess differences in plasma viscosity between the ABS model and the control group.

The ABS model experimental group showed a clear increase in plasma viscosity compared to the control group: WMD = -0.39 mPa/s, (95% CI = -0.55 - 0.24; Z = 5.15; P < 0.05). Both the 0-24 h and 24-72 h subgroups of the experimental model showed increased plasma viscosity compared to the control group: WMD = -0.47 mPa/s, (95% CI = -0.71 - 0.23; Z = 3.87, P < 0.05) and WMD = -0.26 mPa/s, (95% CI = -0.4 - 0.11; Z = 3.47; P < 0.05) respectively.

Platelet aggregation rate changes

The platelet aggregation rate meta-analysis included 10 studies, 4 in the 0-24 h subgroup and 6 in the 24-72 h subgroup. Studies were heterogeneous ($\chi^2 = 62.95$; P < 0.05; $I^2 = 86\%$). The random effects model and WMD were used to assess differences in the platelet aggregation rate between the ABS model and the control group.

The ABS experimental group showed an increase in the platelet aggregation rate compared to the control group: WMD = -13.66%, (95% CI = -17.55 - 9.78; Z = 6.89; P < 0.05). The 0-24 h and 24-72 h subgroups of the ABS model both increased compared to the control group: WMD = -14.45%, (95% CI = -22.34 - 6.57; Z = 3.59; P < 0.05) and WMD = -12.8%, (95% CI = 14.61 - 10.98; Z = 3.47; P < 0.05), respectively.

Erythrocyte aggregation index changes

Erythrocyte aggregation index changes meta-analysis included 7 studies. Studies were heterogeneous ($\chi^2 = 39.54$; P < 0.05; $I^2 = 85\%$). The random effects model and WMD were used to assess differences in erythrocyte aggregation rate between the ABS model and the control group. The ABS experimental group showed a clear increase in erythrocyte aggregation index compared to the control group: WMD = -0.84, (95% CI = -1.16 - 0.53; Z = 5.26; P < 0.05).

Fibrinogen concentration changes

The meta-analysis of fibrinogen concentration changes included 7 studies. Studies were heterogeneous ($\chi^2 = 30.68$; P < 0.05; $I^2 = 77\%$). The random effects model and WMD were used to assess differences in fibrinogen concentration between the ABS model and the control group. The ABS experimental group showed a clear increase in fibrinogen concentration compared to the control group: WMD = -1.22 g/L, (95% CI = -1.67 - 0.76; Z = 5.28; P < 0.05).

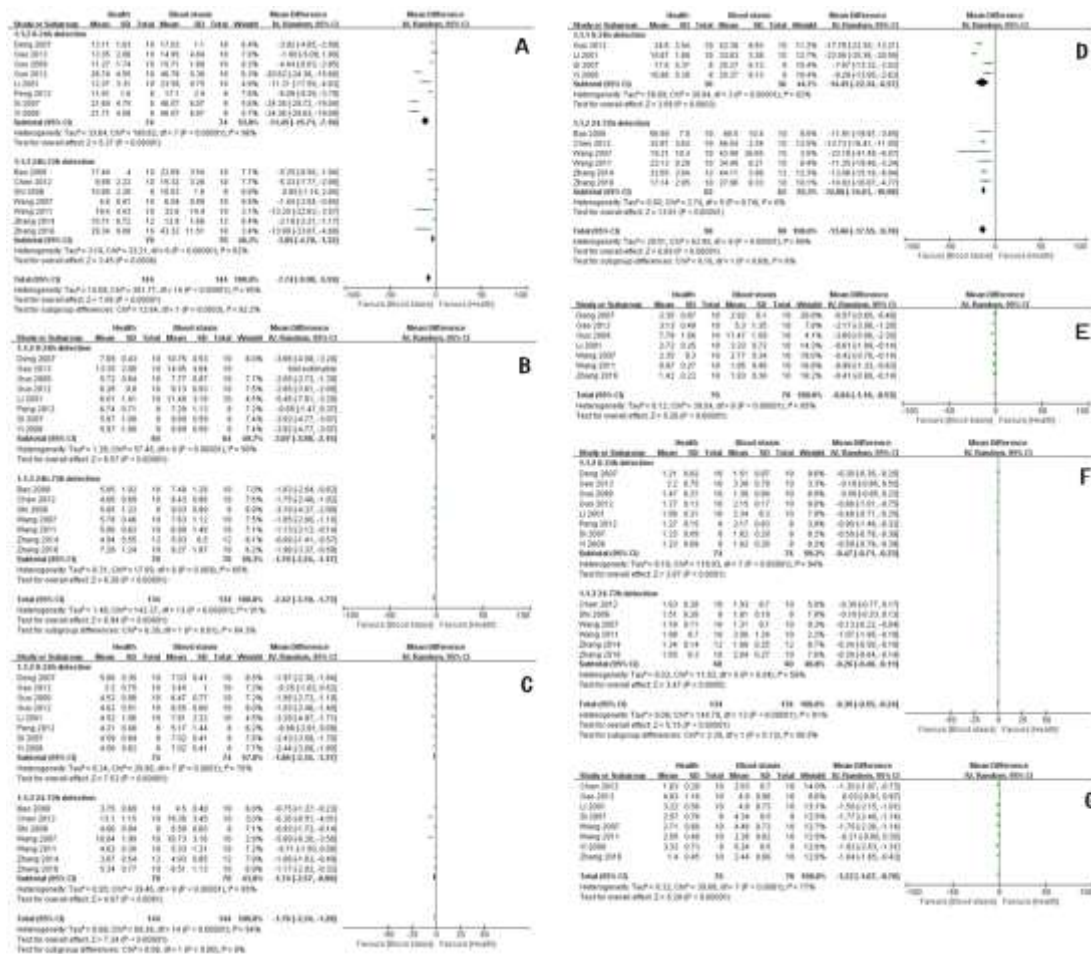


Figure 2: Forrest plot of hemorheology indexes. Mean, standard deviation, sample size, weighted mean difference (WMD), and 95% confidence interval (CI) were calculated from included studies. A: whole blood viscosity (low shear rate); B: whole blood viscosity (medium shear rate); C: whole blood viscosity (high shear rate); D: platelet aggregation rate; E: RBC aggregation index; F: plasma viscosity; G: fibrinogen concentration.

Risk of bias in included studies

Egger's linear regression test was applied to analysis of the publication bias in the meta-analysis of whole blood viscosity, plasma viscosity, platelet aggregation rate, erythrocyte aggregation rate, and fibrinogen concentration. Egger's publication bias plot of whole blood viscosity (low shear rate) revealed a significant publication bias (P = 0.002). Egger's publication bias plots of whole blood viscosity (medium shear rate), whole blood viscosity (high shear rate), plasma viscosity, platelet aggregation rate, erythrocyte aggregation index, and fibrinogen concentration showed negative publication bias (P = 0.819, 0.391, 0.402, 0.221, 0.131, and 0.471, respectively).

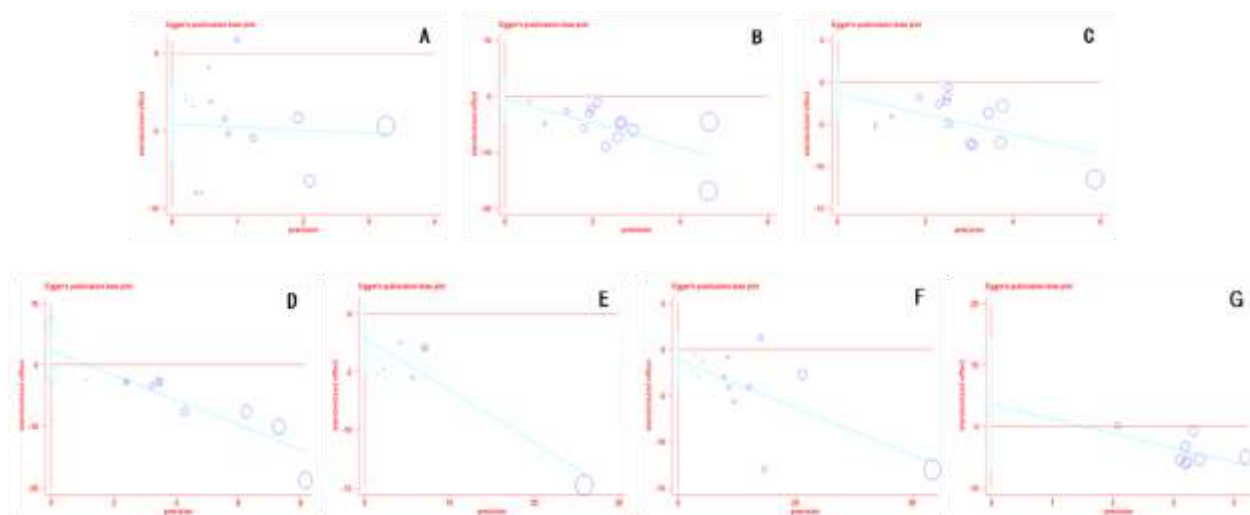


Figure 3: Funnel plot of publication bias (Egger's test). A: whole blood viscosity (low shear rate); B: whole blood viscosity (medium shear rate); C: whole blood viscosity (high shear rate); D: platelet aggregation rate; E: RBC aggregation index; F: plasma viscosity; G: fibrinogen concentration.

Discussion

Blood stasis was characterized by microcirculation disorder and hemorheology changes. The hemorheology index has been regarded as a criterion to assess blood stasis in recent studies (Chen et al., 1990). The hemorheology index, including whole blood viscosity, plasma viscosity, platelet aggregation rate, erythrocyte aggregation rate, and fibrinogen concentration, increased during blood stasis (Jiang, 1990). We systematically reviewed the publication of rat ABS models and meta-analyzed the changes of the hemorheology indexes.

Literature retrieval results found that 34 different methods have been used to establish rat blood stasis models. Physical methods included alpine cold, anoxia, fatigue, positive acceleration, and radiation injury. Biochemical methods used drugs including endotoxins, epinephrine, and dextran. The majority of studies used a combination of two or three methods to establish a rat blood stasis model, such as subcutaneous injection of epinephrine combined with an ice bath, or a high-fat diet combined with swimming. The TCM features of cold coagulation, Qi stagnation, heat toxin, blood deficiency, and Qi deficiency in the rat blood stasis model corresponding to different modeling methods explained TCM blood stasis theory. Subcutaneous injection of epinephrine combined with an ice bath was the most frequently used modeling method based on TCM theory of Qi stagnation and Cold condensation, accounting for one third of rat blood stasis studies, but only about 17% of those studies assessed rat ABS model using pathology-based methods; the rest compared the hemorheology index between the control group and the experimental group. There was a lack of unified hemorheology index evaluation criteria. The methodological quality of included studies indicated a low-quality study. Inadequate experimental design and incomplete experimental quality control were the highest contributors to the low study quality.

Integrated conclusions of the meta-analysis of included studies was reached as follows: studies of the low shear rate of whole blood viscosity included a significant publication bias, and the results were not reliable. The medium shear rate of whole blood viscosity, high shear rate of whole blood viscosity, plasma viscosity, platelet aggregation rate, erythrocyte aggregation index, and fibrinogen concentration all increased compared to the control group. The hemorheology results above were extracted and analyzed from studies in which the ABS model had been tested using pathology methods. We conclude that the observed increases compared to the control group could serve as criteria for successful establishment of a rat ABS model, independent of pathological methods. Among those hemorheology

indexes, the medium shear rate of whole blood viscosity and platelet aggregation rate were more sensitive. Both methods were also more sensitive at 0-24 hours sampling than at 24-72 hours, which suggests a better sampling timepoint in therapeutic studies. In general, the unified hemorheology index could be useful for assessing the rat acute blood stasis model; its advantages over pathology methods include convenient detection, variety, quantitative analysis, and accurate evaluation.

Conclusions

Rat blood stasis model studies have contributed a great deal to the understanding of blood stasis, a TCM pathology condition. Different rat blood stasis models have illustrated a modern medicine pathological mechanism from a variety of aspects, which have helped the global spread and development of the TCM blood stasis theory. However, systematic reviews of rat blood stasis studies revealed deficiencies in experimental design and quality control, which require integrated improvements. Meta-analysis of the included studies indicated that the unified hemorheology index of whole blood viscosity (medium and high shear rate), platelet aggregation rate, erythrocyte aggregation rate, and fibrinogen concentration could serve as criteria for the successful establishment of a rat ABS model independent of pathological methods.

Limitations

We included studies of rat ABS models induced by subcutaneous injection of epinephrine combined with an ice bath; the conclusion of meta-analysis determined that this model might not be suitable for all the types of rat ABS models, but provides suggestions for the assessment of rat ABS models. The sample sizes of included studies were similar; there is a lack of large sample analyses of rat ABS models. Our review only focused on the assessment of rat ABS models. Many studies examining drug therapy for rat ABS models, which should be investigated in future studies.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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