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The High-Efficiency and Stable Hypercoagulation-Breast-Cancer Models for Study the Mechanism of Hematogenous Metastasis

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Abstract

Breast cancer is the commonest malignancy cancer in women. Accumulated research showed that more than 60% of patients with malignant tumor were accompanied by hypercoagulable state, which in turn cause cancer progression, followed by long distant metastasis, alert the worse tumor prognosis and even affect treatment strategy. In the clinical study, we showed that the MA (mm) were significantly higher in the patients with stage IV breast cancer than in stage I/II/ III patients(P=0.01), which indicated that platelet hyperfunction was positively associated with tumor metastasis in breast cancer patients. In order to explore the relational mechanisms, we were committed to construction of efficient models in vivo and in vitro. In vivo study, as tumor-bearing period (TBP) and postoperative incubation period (PIP) are two key factors for coagulation and metastasis, we established 4T1-luc breast-tumor-bearing mice model of 4 groups (1a= TBP25+PIP17, 1b= TBP25+PIP1, 1c= TBP20+PIP17, 1d= TBP20+PIP1) with different TBP and PIP. Through evaluation of the breast tumors, lung metastases, animal welfare, operational feasibility and TEG Assay, indicated that: Lung metastasis and hypercoagulability were successfully established in all tumor groups; The TBP and PIP were both played a decisive role in the occurrence of lung metastasis; The model hypercoagulation state was characterized not by coagulation factors, but by the hyperactivity of fibrin and platelet. Given animal welfare and operational feasibility, recommended 4Tl-luc cells be injected into the fourth intramammary gland fat pad with 1 x 106, and with TBP of 3 weeks and PIP of 2 weeks, then an ideal in vivo model can be established. In vitro study, we established an in vitro model (platelet-breast cancer co-action) by platelets (female BALB/c mice) and 4T1 cells co-incubation for 30 minutes (represent the early phase of platelet-breast cancer co-action in vivo) or 24 hours (represent the late phase of platelet-breast cancer co-action in vivo), then the co-incubation supernatants were obtained by differential centrifugation. Through evaluation of the physical characteristics and procoagulant activity of the supernatants, indicated that co-incubation of platelets and 4T1 cells could activate each other and promote the release of substance and procoagulant activity with prolonging the co-incubation time, and the reaction supernatants can be used for interest factors detection and bioactivity verification. In conclusion, we provided powerful hypercoagulation-breast-cancer model for research in this field in vivo and in vitro, which will also provide important insights into the intervention of hematological metastasis in breast cancer.

Keywords:

Breast Cancer;

Hypercoagulable State;

In Vivo Model;

In Vitro Model.

Introduction

Activation of the hemostatic system contributes significantly to the hypercoagulable state, thrombotic diathesis, metastasis and even death in these patients [1-6]. And the elevated of platelets function or counts were reported widely in numerous clinical cancer researches [7-8]. Furthermore, aberrant platelet activation and aggregation include disseminated intravascular coagulation, migratory thrombophlebitis and pulmonary embolism frequently discovered in cancer patients too [9]. However, reducing platelet count or inhibiting platelet function can effectively inhibit the number of experimental metastases [10-12]. In addition, clinical randomized controlled trials (RCTs) studies have revealed the anticancer effects of low-dose aspirin, and recommending it for primary prevention of cancer [13-17]. All of the above evidences suggest that platelets play a vital role of the hypercoagulable state formation and tumor metastasis.

Breast cancer is the commonest malignancy cancer in women and is considered to be associated with high risk of hypercoagulable state to make tumor progression [18]. The clinical data of our hospital showed that the degree of hypercoagulability worsens with cancer progression, and the hyperfunction of platelet is only appears in metastasis

breast cancer patients. Which prompted us to doubt whether the hyperfunction of platelet has a more directly relationship with metastasis than coagulation factors in breast cancer. Currently, the relevant mechanism researches indicated that the procoagulant microparticles (MPS), especially those expressing tissue factor (TF) or phospholipid (PS), which mainly come from tumor cells, platelets or both [19-22]. Contribute most to the hypercoagulability and metastasis of breast cancer.

Extracellular vesicles (EVs) which are classified into three groups, (a) exosomes, (b) microvesicles (MVs), and (c) apoptotic bodies (ABs), based on their sizes, origins, makers, shapes and derivation mechanism [23-25]. They act as carriers of nucleic acids, lipids and proteins and have been recognized as critical mediators of extracellular communications, which promote transformation, growth invasion, hypercoagulability and drug-resistance of cancer cells. PS exposure on MPS (belonging to MV) promote hypercoagulable state and metastasis have been reported largely in breast cancer. However, PS exposure on other EVs especially the small ones like exosomes, which whether contributes to hypercoagulability and progression in patients with breast cancer or not has not been investigated, so the interesting function of small EVs maybe a promising research direction.

For research in this field, construction of an in vivo model of tumor metastasis with hypercoagulability is the key. However, a high-efficiency and stable condition to establish the in vivo model suitable for study in this field has not been clearly elucidated. We took tumor-bearing period (TBP) and postoperative incubation period (PIP) as the two main intervention factors to build 4 groups, through the evaluation of the breast and lung metastases tumor, the characteristics of hypercoagulation state, animal welfare and operational feasibility, we got an excellent in vivo model which characterized not by coagulation factors, but by the hyperactivity of fibrin and platelet.

A lot of research indicated the dynamic crosstalk between tumors and platelet is charming which increasingly recognized as a key regulator of hypercoagulable state and malignant progression. For our research, we established an in vitro model (platelet-breast cancer co-action) by platelets and 4T1 cells co-incubation for 30 minutes (represent the early phase of platelet-breast cancer co-action in vivo) or 24 hours (represent the late phase of platelet-breast cancer co-action in vivo), then the co-incubation supernatants were obtained by differential centrifugation. Through the evaluation of the co-incubation supernatants in physical characteristics (concentration and size) and procoagulant activity (PS exposure), we confirmed co-incubation of platelets and 4T1 cells could activate each other and promote the release of substance and procoagulant activity with prolonging the co-incubation time. In addition, the reaction supernatant is rich in nucleic acids, lipids, proteins and numerous bioactive substances. You can use this model to detect anything you are interested in and set up intervention conditions.

This study provides a powerful research tool for the study of the correlation between hypercoagulable state and breast cancer progression through establishment of in vivo and in vitro research models, and novel antithrombotic treatments may arise from a better understanding of breast cancer associated hypercoagulable state.

Materials and methods

1. Clinical information of patients.

A retrospective analysis was performed on breast cancer patients who tested Thrombelastograph (TEG) assay in the Beijing Traditional Chinese Medicine Hospital Capital Medical University from July 2016 to January 2019. Clinical and test information were extracted via the institutional electronic data warehouse. We obtained pathological, clinical stage, combined disease, medical history, sex, age and the results of TEG assay included R (min), K (min), Angle (deg), MA (mm). Inclusion criteria: Breast cancer diagnosed by pathology; Patients have been tested for TEG assay. Exclusion criteria: Patients with unavailable clinical stage; Patients received anticoagulant therapy; Patients combined with coagulation-related diseases.

2. Cell culture

4T1/ 4T1-luc are a murine breast carcinoma cell line, which from Caliper (USA) were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FBS; Sigma-Aldrich, Oakville, ON) and 1% penicillin-streptomycin (Gibco; 10000 units/ml Penicillin, 10000 μg/ml Streptomycin) at 37˚C in a humidified atmosphere containing 5% CO2. The cells were passaged every two to three days to maintain exponential growth.

3. Animal

6-8-week-old female BALB/c mice used in this study were obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd, and were approved by China Laboratory Animal Welfare and Animal Experimental Ethical Committee. All mice were maintained in specific pathogen-free barrier facilities at the Beijing Institute of Traditional Chinese Medicine, and fed with a regular diet. They were carefully bred and would be humanely sacrificed by cervical dislocation at study endpoint.

4. Established of the 4T1-luc breast-tumor-bearing mice model / lung metastasis model

4T1-luc cells seeded at log phase were treated with 0.25% Trypsin-EDTA (Gibco) for two minutes and washed once with PBS prior to being passaged. Then the viability and number of 4T1-luc cells were measured by Trypan blue staining, and the cell concentration was adjusted to 2×10^7/mL with PBS. Finally, 50 ul cell suspension (1×10^6) were stead injected into the fourth intramammary gland fat pad of female BALB/c mice on each side at a uniform speed.

When the TBP of each group was reached to the design requirement, we removed the breast tumors as follows: Animals were fixed on the paraffin table in supine position after gas (oxygen: isoflurane = 98:2) anesthesia. After 70% alcohol disinfection, the skin was incised beside the tumors on the side of the body. Surgical scissors were used to carefully remove the tumor from the breast pad. During the operation, attention should be paid to the detachment of the blood vessel and the complete elimination of the tumor capsule according to the radical principle. The iatrogenic dissemination of the tumor cells was avoided according to the tumor-free principle. Attention also should be paid to sterilization of instruments and protection of surgical incision according to aseptic principle. Avoid animal death due to excessive bleeding or excessive anesthesia time and avoid secondary recurrence due to incomplete resection. Complete separation of main nutrient vessels and proximal ligation are important steps to reduce blood loss (fig 1). 5-0 non-absorbable surgical sutures to suture the skin, ligation of instruments, cutting of excess sutures, skin closure of incisions, daily observation.

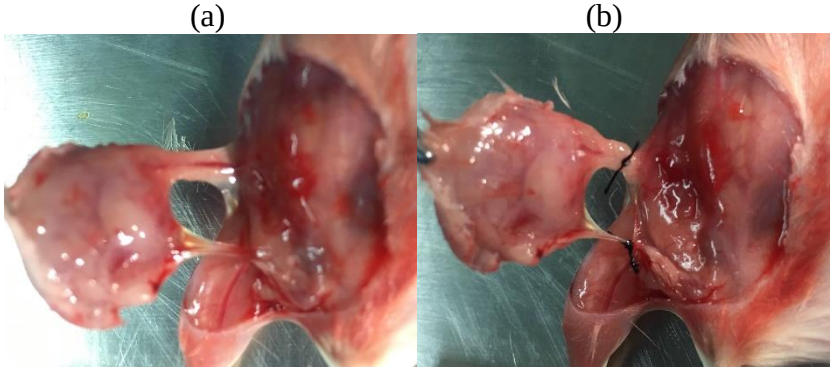


Fig. 1 Complete separation of the main nutrient vessels and proximal ligation: (a). Complete separation of the main nutrient vessels: Peeled off the main nutrient vessels along the blood vessel as carefully and rapidly as possible at the 5-6 o'clock position and the 10-11 o'clock position of the breast tumor. (b) Proximal ligation of the main nutrient vessels: After complete separation of the main nutrient vessels, they were ligated by instrument knotting at the proximal end of them, then severed sharply.

5. In vivo experimental design

In order to study the effect of TBP and PIP on lung metastasis in mice, different experimental groups were set up in this study. According to the TBP, the mice were divided into group 1 (27 days ± 2 days, n=17), and group 2 (20 days ± 2 days, n=14). According to the PIP (15 days ± 2 days/ 1 day), the group 1 and the group 2 were further divided into 4 subgroups: group 1a (with TBP 25 days and PIP 17 days, n=8), group 1b (with TBP 29 days and PIP 1 days, n=9), group 2a (with TBP 22 days and PIP 13 days, n=9), group 2b (with TBP 18 days and PIP 1 days, n=5), and set up a blank control group (n=11). After modeling, the mice were observed for general activity, nutritional status, and tumor formation rate.

6. Evaluation of the in vivo model

6.1 Evaluation of the breast tumors

To investigate and verify the ability of tumorigenesis at breast. The breast tumors were measured using a caliper once every 3 days from the 10th day after model established. The tumor volume was calculated using the formula: tumor volume (mm3) = $0.5\times a\times b^2$, where a represents the shortest diameter and b represents the longest diameter of the tumor. For pathological confirmation, mice were euthanized to collection of breast tumors according to the study design. All tissues were weighed and took photos for recording, then embedded in paraffin using standard procedures, sectioned, and stained with hematoxylin and eosin (H & E).

6.2 Evaluation of the lung metastases

Lung and extrapulmonary metastasis was assessed by optical in vivo imaging (IVIS-Lumina Ⅲ) before the experiment was terminated according to the study design. The specific operation was as follows: Each mouse was injected with 200 ul fluorescein substrates in the subcutaneous space, isoflurane was inhaled and anesthetized for 3 minutes, then placed into a living fluorescence imaging system to record lung metastases and extrapulmonary

metastases. Finally, using Living Image 4.5 software for analysis.

After recorded photon number of lung and extrapulmonary metastases. The mice were humanely sacrificed by cervical dislocation to collection their lung and extrapulmonary metastases. All tissues were weighed and took photos for recording, then embedded in paraffin using standard procedures, sectioned, and stained with hematoxylin and eosin (H & E).

6.3 Evaluation of the animal welfare/ operational feasibility

Based on the principle of animal welfare, we compare the following indicators between experiment groups: 1. Total time (anesthesia duration) spent on complete removal of breast tumors, the shorter the time, the less harmful the animal is; 2. The length and the stitches of the breast surgical incision, the shorter the incision, the less harmful the animal is; 3. The trend of animal weight change, the milder the trend, the less harmful the animal is.

6.4 Routine blood tests

For hypercoagulation state analysis, 20μL mice whole blood was collected into the prepared tubes with special diluent, then routine blood tests were immediately performed using a Sysmex XT-2000i automated hematology analyzer (Sysmex Corp., Hyogo, Japan) for the platelets (PLT) counts.

6.5 TEG Assay

For hypercoagulation state analysis, 500μL mice whole blood was collected into the prepared anti - coagulation tubes (sodium citrate 3.8%). Then the TEG test which measures clot formation, clot strength, and clot degradation in whole blood by adding kaolin and calcium chloride (final concentration, 10.5mMol/L) were carry out for enable a quantitative analysis of blood coagulation factor and platelet function. The specific operation is as follows: After the instrument is calibrated, 20 ul of calcium chloride is pushed to the bottom of the test cup, 500 ul of anticoagulant is thoroughly mixed with 20 ul of kaolin, 360 ul of the stimulated blood is pushed to the TEG test cup, and the TEG analyzer is started to measure the blood. The entire process of clot formation and degradation.

The parameters of the TEG are explained as follows: R(min) reflects the prothrombin start-up time, which reflects the function of coagulation factor, and the prolongation of R (min) indicates that the activity of coagulation factor is decreased or absent, and conversely, the activity of coagulation factor is hyperactive; K (min) and Angle (deg) reflect the rate of blood clot formation, which reflects fibrin function. K-time prolongation or Angle (deg) reduction suggests that fibrin function is reduced, and vice versa. MA (mm) reflects the absolute intensity of fibrin clot which reflecting platelet function, a decrease in the MA (mm) indicates a decrease in platelet function, and conversely indicates an increase in platelet function.

7. Preparation of mice platelets

Blood samples were obtained from female BALB/c mice. Washed platelet suspensions were prepared from blood in Tyrode's solution as previously described ($1*10^8$ /ml) [26].

8. Established of the in vitro model

We established the in vitro model to study the crosstalk between platelets and breast tumor cells in cancer progression. As we all known, the life-span of platelets in vivo is 7-10 days, and in vitro, the survival time is greatly shortened. So, we cultured platelets and 4T1 cells in vitro for 24 hours to represent the late phase of platelet-breast cancer co-action in vivo, and 30 minutes to represent the early phase of platelet-breast cancer co-action in vivo. The specific operation is as follows: 4T1 cells seeded at log phase were treated with 0.25% Trypsin-EDTA for two minutes and washed once with PBS prior to being passaged. Then the viability and number of 4T1 cells were measured by Trypan blue staining, and the cell concentration was adjusted to 1×10^6/mL with RPMI-1640 (10% FBS and 1% penicillin-streptomycin), Washed platelet suspensions(1ml) and 4T1 cells suspensions(1ml) co-incubation for 30 minutes or 24 hours at 37˚C in a humidified atmosphere containing 5% CO2. Then the co-incubation supernatants were centrifuged twice (first at 1400×g for 20 minutes and then at 13000×g for 2 min) at 4˚C to complete removal all of the 4T1 cells and platelets [27]. Platelets/4T1 cells incubation lonely for 24 hours/30 minutes all as control, and the supernatants stored at −80°C until assayed.

9. Identification of the co-incubation supernatants

To evaluation of the co-incubation supernatants in physical characteristics, we used a nano-flow cytometer (N30 Nanoflow Analyzer, NanoFCM Inc., Xiamen, China) to quantify the co-incubation supernatants and control supernatants by their concentration, size. As phosphatidylserine (PS) is an anionic phospholipid generally distributed in the cell membrane. But when the cells are activated or upon apoptosis, PS will externalize to the outer membrane to enhance the procoagulant activity, A large number of research reports circulating MPs and exposed PS from different cells contribute to the procoagulant activity in patients with cancer [27-28]. To evaluation of the co-incubation supernatants in procoagulant activity, we used a nano-flow cytometer to quantify the co-incubation supernatants and control supernatants by their PS exposure.

10. Statistical analysis

Data was analyzed using SPSS 16.0 (IBM, Armonk, NY, USA) or GraphPad Prism 5 (GraphPad, CA, USA). Groups were compared by using Mann-Whitney test or Kruskal-Wallis test. p-value<0.05 was considered statistically significant.

Results

1. Clinical results

Between July 2016 and January 2019, 81 subjects who had undergone TEG testing in our hospital were identified. Based on the criteria for inclusion and exclusion, 76 women subjects were eligible for analysis (Fig 2).

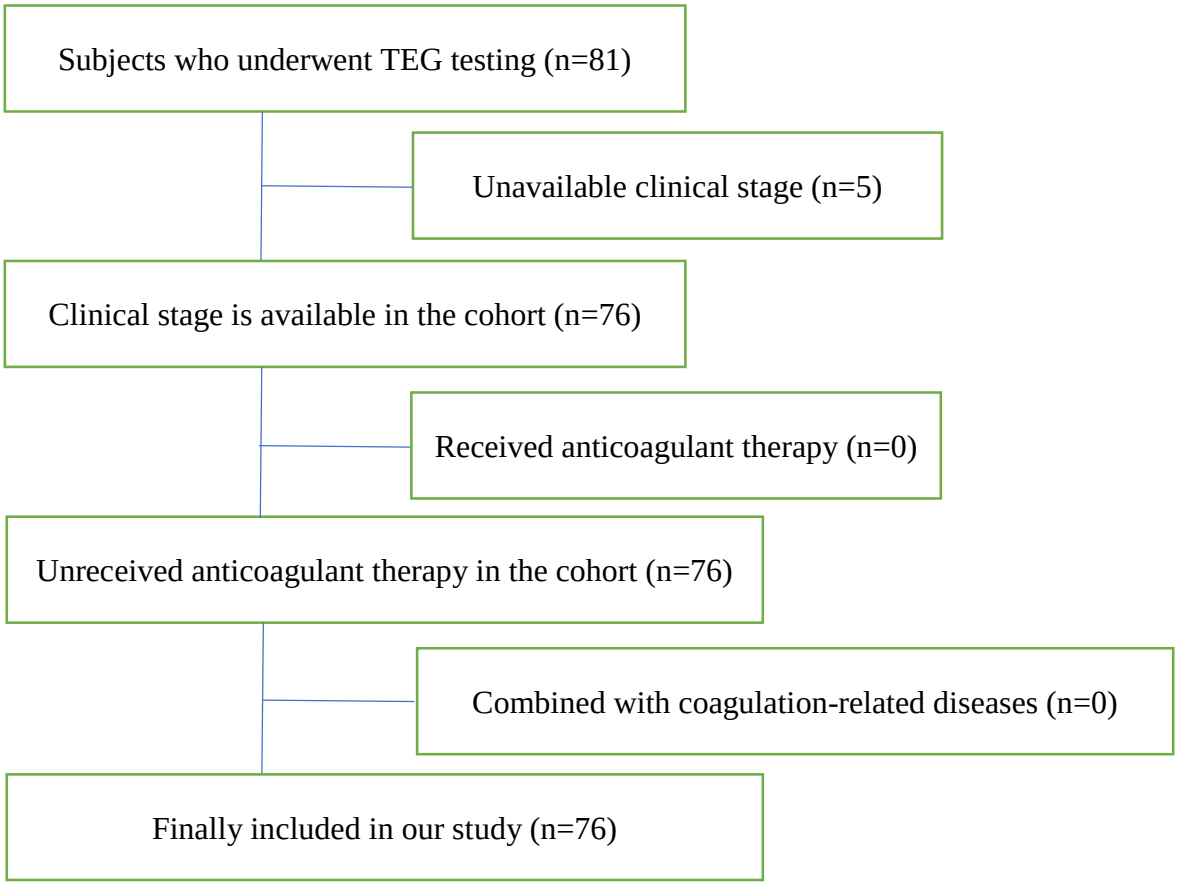


Fig. 2 Study flow chart showing step-wise exclusion algorithm based on eligibility

Among the eligible subjects (age from 34 to 88), the characteristic of clinical stage is: stage Ⅰ is 12, stage Ⅱ is 16, stage Ⅲ is 12, stage Ⅳ is 36; M0 is 40, M1 is 36. The results of TEG assay showed that the R (min) among stage Ⅰ, stage Ⅱ, stage Ⅲ with stage Ⅳ is no difference, and the MA (mm) among stage Ⅰ, stage Ⅱ, stage Ⅲ with stage Ⅳ is no difference. In order to explore the difference of coagulation state between metastasis and no metastasis subjects, we take M0 compare to M1 with the results of TEG assay. Which showed that the R (min) between M0 and M1 is no difference (P＞0.05), but the MA (mm) between M0 and M1 is difference (P=0.01). In other words, the hyperfunction of coagulation factor is appears in all clinical stages, but the hyperfunction of platelet is only appears in metastasis subjects. We doubt that maybe the hyperfunction of platelet has a more directly relationship with metastasis than coagulation factor in breast cancer (Fig 3).

Table 1. Comparison of the coagulation status between the two groups by TEG assay: The R (min) between M0 and M1 is no difference (P＞0.05), but the MA (mm) between M0 and M1 is difference (P=0.01).

	M0	M1	x^2	p value
R (min)			1.11	>0.05
<5	10	13		
≥5	30	23		
MA (mm)				=0.01

<70	40	30
≥70	0	6

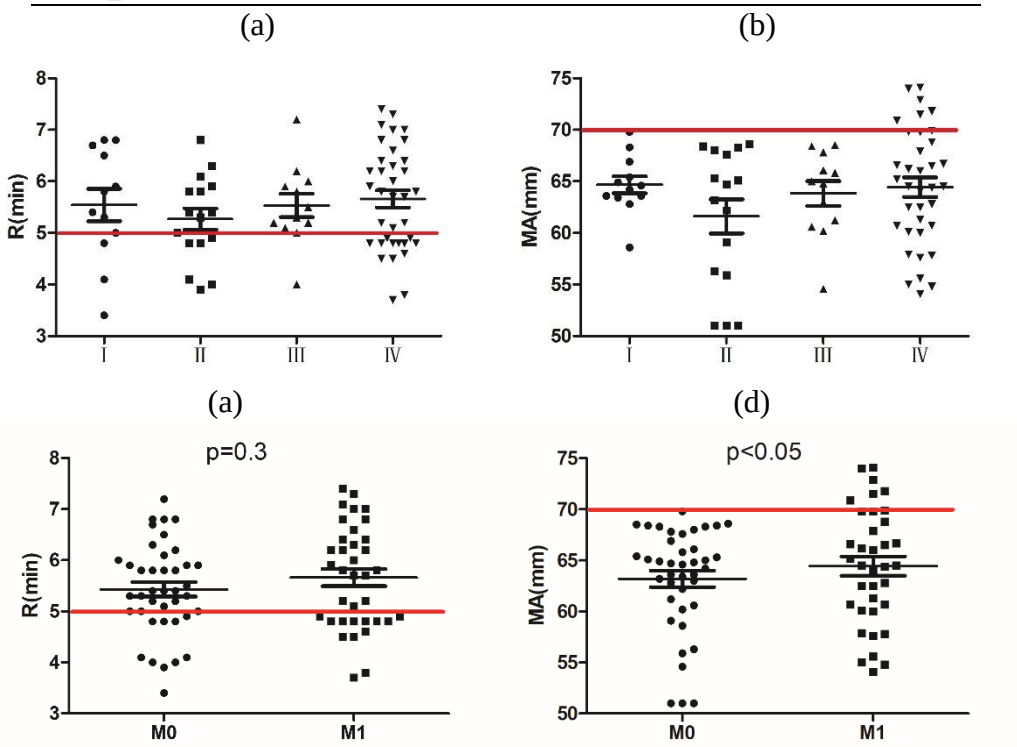


Fig. 3 Comparison of TEG assay among different clinical stages. (a) R(min): There is no difference of the R(min) among stage Ⅰ, stage Ⅱ, stage Ⅲand stage Ⅳ; (b) MA (mm): There is no difference of the MA (mm) among stage Ⅰ, stage Ⅱ, stage Ⅲand stage Ⅳ; (c) R(min): There is no difference of the R(min) between M0 and M1, P=0.3 ; (d) MA (mm): There is a statistical difference between M0 and M1, P＜0.05.
*The red line represents the normal value, the normal value of R is no less than 5.0(min), the normal value of MA is less than 70(mm).

2. The characteristics of breast tumor among four groups

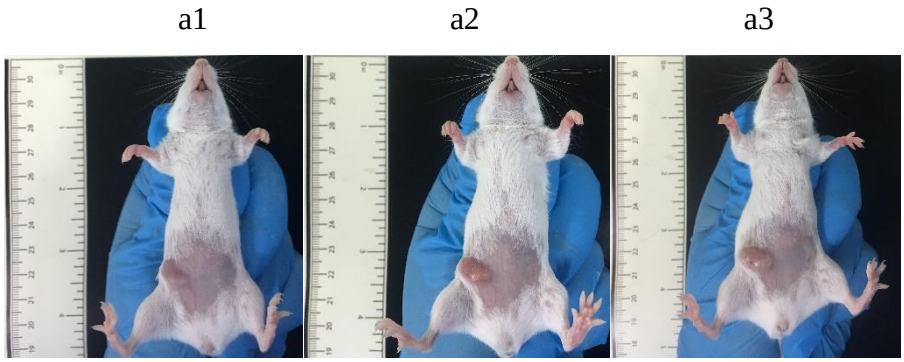
2.1 Comparison the volume of breast tumor

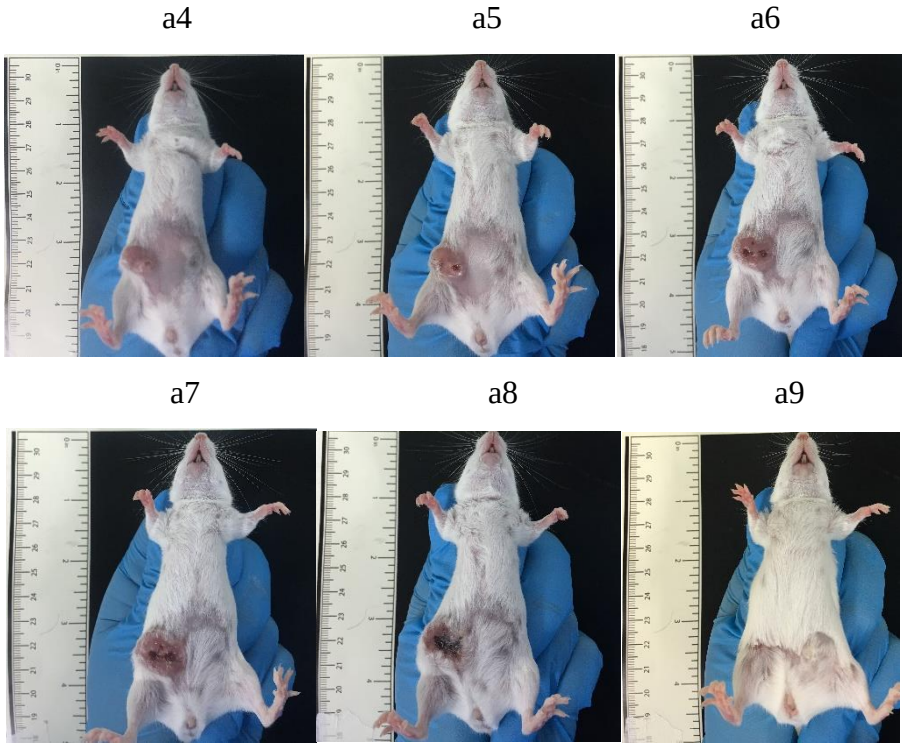
The volume of breast tumors was measured every 3 days from the 8th day after in vivo model established, the growth trend was consistent among the four groups, and the growing pictures were also collected (Fig 4 a).

2.2 Comparison the weight of breast tumor

Compliance with the termination time of the in vivo experimental design, tumor weight recorded immediately after resection (Fig 4 b). Comparison the weight of breast tumor among the groups: The breast weights of experimental group 1a, 2a increased significantly compared with group 2b, the differences were statistically significant, and the P value was 0.0006, 0.0041, respectively; The breast weights of experimental group 1b, increased significantly compared with group 1a, 2a and 2b, the differences were statistically significant, and the P value was 0.0006, 0.0021 and 0.0021, respectively.

(a)





(b)

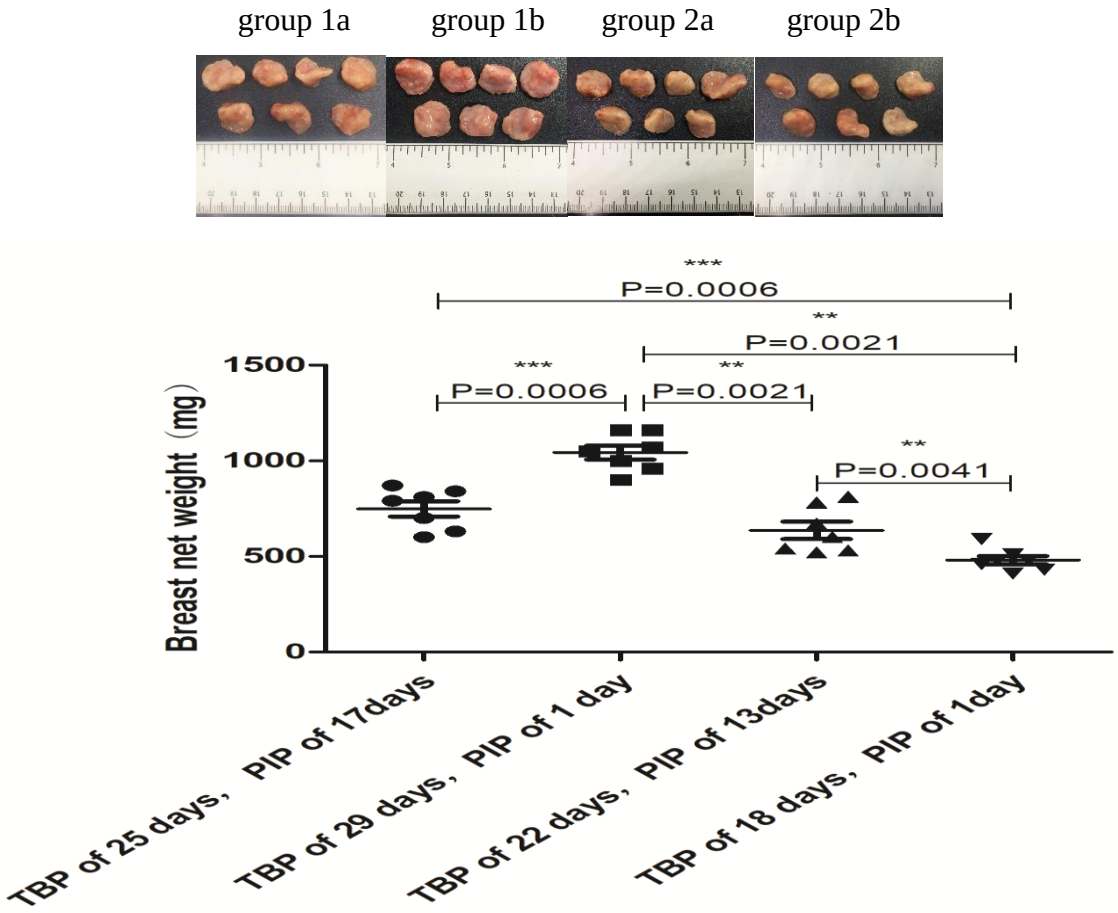


Fig. 4 The growth trend of breast tumors: (a). The picture collection of breast tumors from the 8th day after in vivo model established. a1=8th day, a2=11th day, a3=14th day, a4=17th day, a5=20th day, a6=23th day, a7=26th day, a8=33th day, a9=7th day after the breast tumor resection. (b). Comparison the weight of breast tumor among the groups:

The breast weights of experimental group 1a, 2a increased significantly compared with group 2b, the differences were statistically significant, and the P value was 0.0006, 0.0041, respectively; The breast weights of experimental group 1b, increased significantly compared with group 1a, 2a and 2b, the differences were statistically significant, and the P value was 0.0006, 0.0021 and 0.0021, respectively.

3. The characteristics of lung and extrapulmonary metastases among four groups

3.1 Comparison of the lung weight

The results of lung weight showed that: 1. Comparison between the experimental groups and the blank group: The lung weights of experimental group 1a, 1b, 2a and 2b increased significantly compared with the blank group, the differences were statistically significant, and the P value was 0.0024, 0.0002, 0.0003 and 0.0025, respectively (Fig. 5 a). 2. Comparison between the experimental groups: Compared with experimental group 1b, 2a and 2b, the lung weight of experimental group 1a increased significantly, and the P value was 0.0181, 0.0123 and 0.0295, respectively. Which means the lung metastasis of the experimental group 1a was more obvious than that of the other experimental groups (Fig. 5 a).

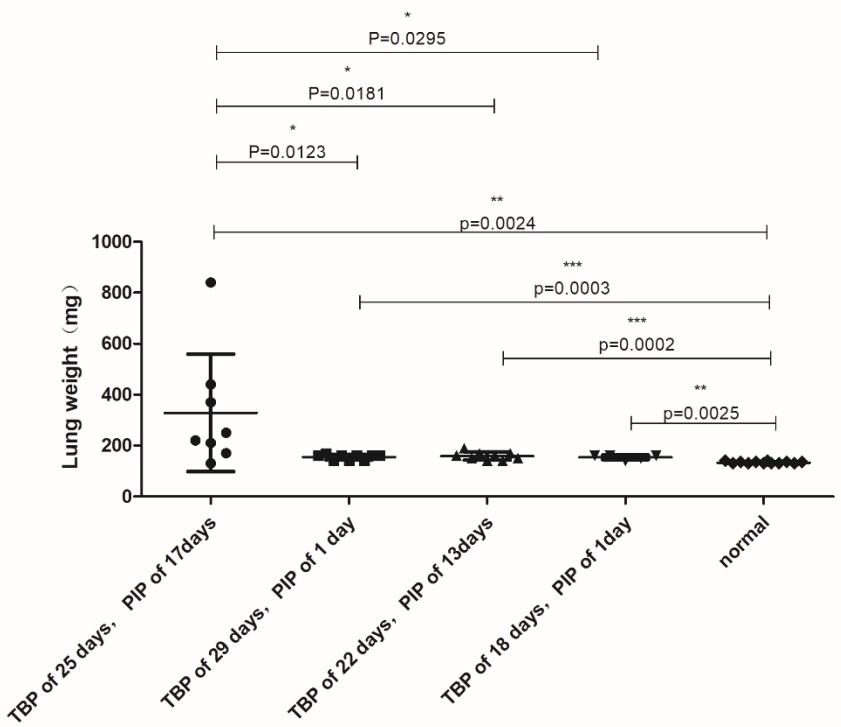
3.2 Comparison photon number of lung metastasis

The result of optical in vivo imaging analysis showed that the photon number of experiment 1a group > experiment 2a group > experiment 1b group > experiment 2b group. The general view of lung specimens showed that the lung metastases in group 1a were mostly fused, and the single lung metastases were larger, but there was no obvious lung metastases in group 2b (Fig 5 b).

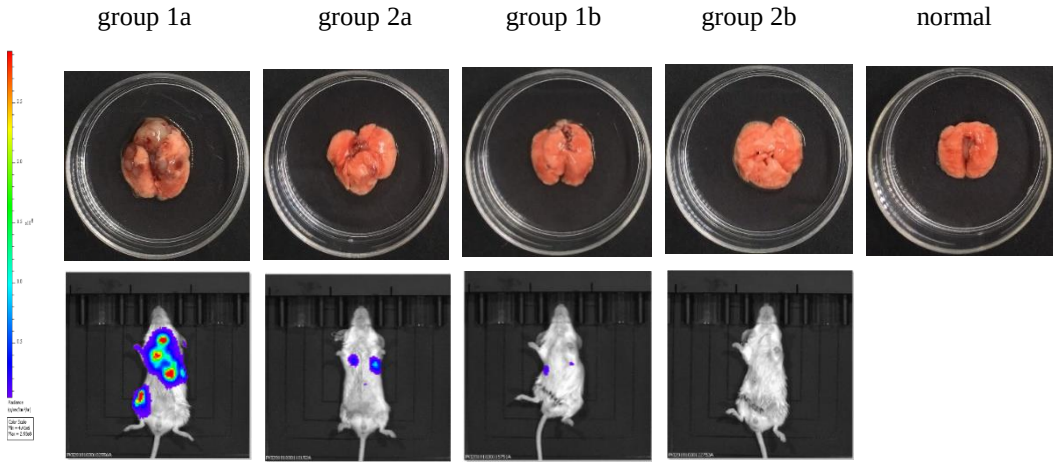
4. The characteristics of extrapulmonary metastases

To our surprise, the group 1a, besides lung metastasis, extrapulmonary metastases such as chest wall, back subcutaneous, axillary lymph nodes, inguinal lymph nodes and pericardium were also found (Fig 5 c).

(a)



(b)



(c)

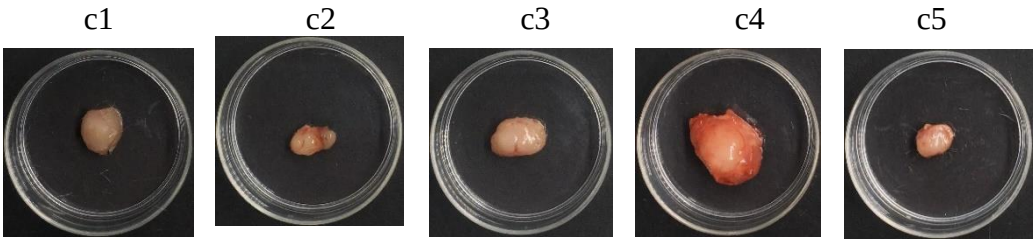


Fig. 5 The characteristics of lung and extrapulmonary metastases among four groups: (a). Comparison of the lung weight: The lung weights of experimental group 1a, 1b, 2a and 2b increased significantly compared with the blank group, the differences were statistically significant, and the P value was 0.0024, 0.0002, 0.0003 and 0.0025, respectively; The lung weights of experimental group 1a increased significantly compared with group 1b, 2a, 2b, the differences were statistically significant, and the P value was 0.0181, 0.0123, 0.0295. (b). Comparison photon number of lung metastasis: The photon number of group1a > group 2a> group 1b> group 2b, and the photos of lung specimens are displayed simultaneously. (c). The extrapulmonary metastases of group 1a: c1= chest wall metastases, c2=back subcutaneous metastases, c3=axillary lymph nodes metastases, c4=inguinal lymph nodes metastases, c5=pericardium metastases.

5. The characteristics of hypercoagulation state

TEG results showed that: 1. Comparison between the experimental groups and the blank group: The K(min) of experimental group 1a, 1b, 2a and 2b had significantly shorter than the blank group, and the P values were 0.0087, 0.0009, 0.0060 and 0.0012, respectively (Fig.6 a). Suggesting the fibrin function is hyperactive in all the experimental groups; The Angle (deg) of experimental group 1a, 1b, 2a and 2b had significantly increased than the blank group, and the P values were 0.0104, 0.0083, 0.0018 and 0.0022, respectively (Fig.6 b). Suggesting the fibrin function is hyperactive in all the experimental groups; The MA (mm) of experimental group 1a, 1b, 2a and 2b had significantly increased than the blank group, and the P values were 0.0022, 0.0001, 0.0018 and 0.0046 respectively (Fig.6 c). Suggesting the platelet function is hyperactive in all the experimental groups. However, the R(min) had no significant difference between the experimental groups and the blank group (Fig.6 d). Suggesting there was no significant abnormality in coagulation factor function in all of the experimental groups. 2. Comparison between the experimental groups: The only statistically significant was the MA (mm) of the experimental group 2a increased than group 1b, and P value was 0.0274 (Fig.6 c). Suggesting prolonged tumor-bearing time and post-operative stress may cause hyper platelet function in group 1b.

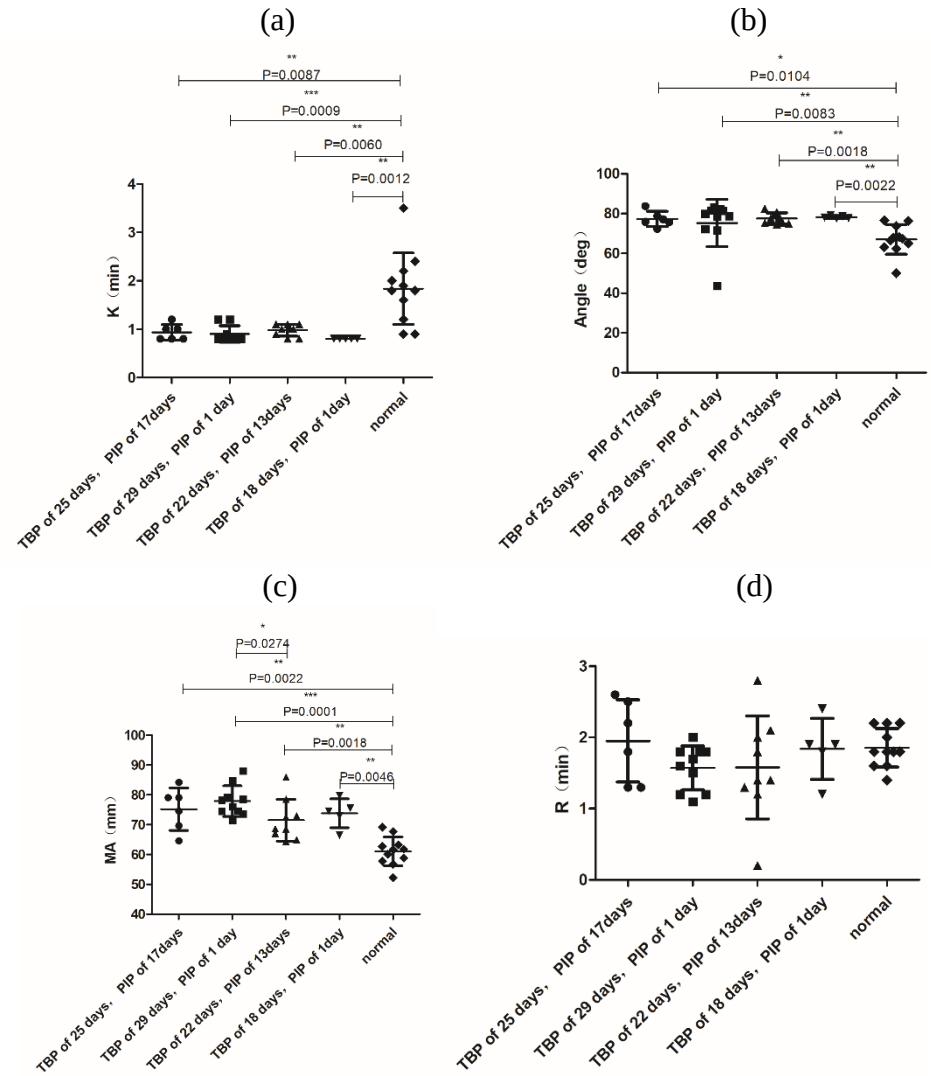


Fig. 6 The characteristics of hypercoagulation state: a. Comparison between the experimental groups and the blank group: The K(min) of experimental group 1a, 1b, 2a and 2b had significantly shorter than the blank group, and the P values were 0.0087, 0.0009, 0.0060 and 0.0012, respectively; b. The Angle (deg) of experimental group 1a, 1b, 2a and 2b had significantly increased than the blank group, and the P values were 0.0104, 0.0083, 0.0018 and 0.0022, respectively; c. The MA (mm) of experimental group 1a, 1b, 2a and 2b had significantly increased than the blank group, and the P values were 0.0022, 0.0001, 0.0018 and 0.0046 respectively, The MA (mm) of the group 2a increased than group 1b, the differences were statistically significant, and P value was 0.0274.d. the R(min) had no significant difference between the experimental groups and the blank group.

5. Animal welfare/ Feasibility analysis of operation

Increased size of breast tumors caused by prolonged TBP significantly prolonged the operation time, the length and stitches of the incision, which all brought animal trauma undoubtedly.

5.1 The weight records of mice

Mice weight records (Once a week) showed that the weight of the mice increased smoothly, there were no statistically differences among all the groups, and the tumors did not cause them obvious pain.

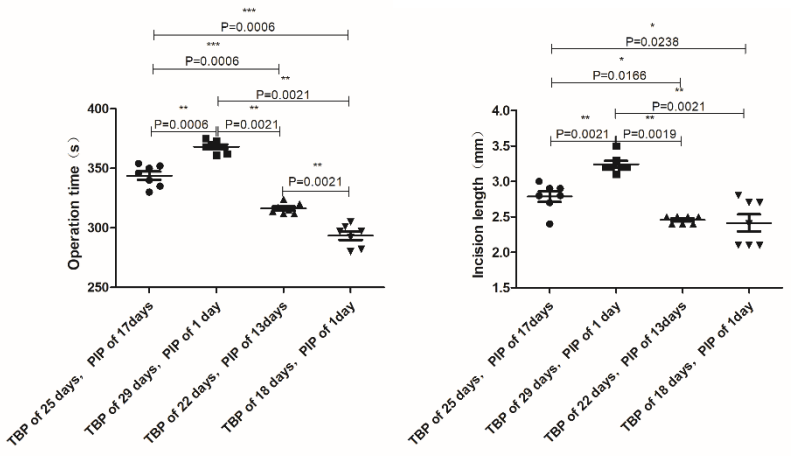
5.2 Comparison of the operation time

The operation time of experimental group 1b had significantly longer than group 1a, 2a and 2b, and the P values were 0.0103, 0.0014 and 0.0021, respectively. The operation time of experimental group 1a had significantly longer than group 2a and 2b, and the P values were 0.0006, 0.0006, respectively. The operation time of experimental group 2a had significantly longer than group 2b, and the P values was 0.0021. (Fig.7 a).

5.3 Comparison of the incision length and stitches

Because the size of breast tumors varies with the time of tumor-bearing, so the lengths and stitches of incisions were different. The incision length of experimental group 1b had significantly longer than group 1a, 2a and 2b, and the P values were 0.0021, 0.0019, and 0.0021, respectively. The incision length of experimental group 1a had significantly longer than group 2a and 2b, and the P values were 0.0166, 0.0238, respectively. But there was no obvious statistical difference between group 2a and 2b. (Fig.7 b). The incision stitches of experimental group 1a had significantly longer than group 2a and 2b, and the P values were 0.0093, 0.0020, respectively. The incision stitches of experimental group 1b had significantly longer than group 1a, 2a and 2b, and the P values were 0.0103, 0.0014 and 0.0014, respectively. And the incision stitches of experimental group 2a had significantly longer than group 2b, and the P values was 0.0075. c= group 1a, d= group 2a, e= group 2b. (d). The pictures of the incisions were collected after operation. (Fig.7 c).

(a) (b)



（c）

group 1a group 1b group 2a group 2b

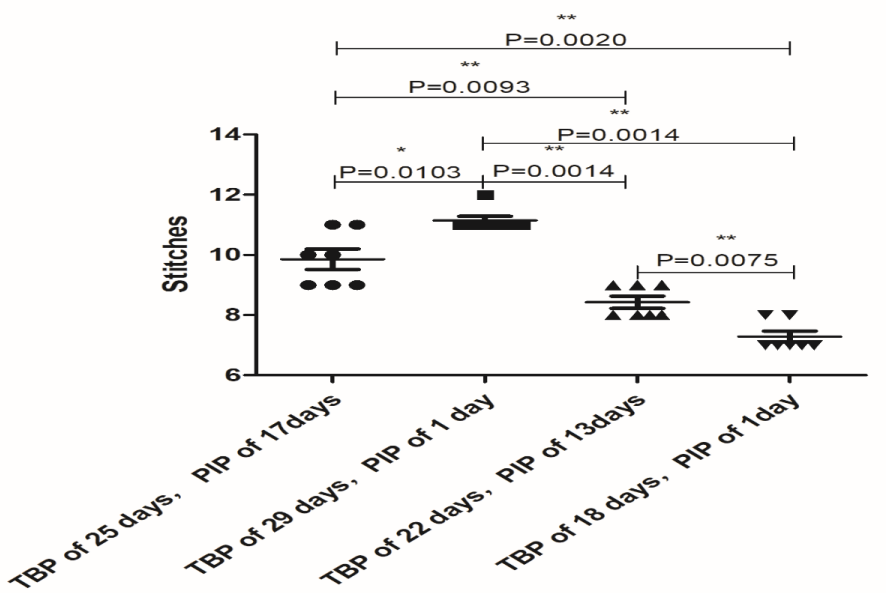


Fig. 7 Animal welfare/ Feasibility analysis of operation: (a). Comparison of the operation time: The operation time of experimental group 1b had significantly longer than group 1a, 2a and 2b, and the P values were 0.0103, 0.0014 and 0.0021, respectively. The operation time of experimental group 1a had significantly longer than group 2a and 2b, and the P values were 0.0006, 0.0006, respectively. The operation time of experimental group 2a had significantly longer than group 2b, and the P values was 0.0021. (b). Comparison of the incision length: The incision length of experimental group 1b had significantly longer than group 1a, 2a and 2b, and the P values were 0.0021, 0.0019, and 0.0021, respectively. The incision length of experimental group 1a had significantly longer than group 2a and 2b, and the P values were 0.0166, 0.0238, respectively. But there was no obvious statistical difference between group 2a and 2b. (c). Comparison of the incision stitches: The incision stitches of experimental group 1a had significantly longer than group 2a and 2b, and the P values were 0.0093, 0.0020, respectively. The incision stitches of experimental group 1b had significantly longer than group 1a, 2a and 2b, and the P values were 0.0103, 0.0014 and 0.0014, respectively. And the incision stitches of experimental group 2a had significantly longer than group 2b, and the P values was 0.0075. c= group 1a, d= group 2a, e= group 2b. (d). The pictures of the incisions were collected after operation.
*Increased size of breast tumors caused by prolonged TBP significantly prolonged the operation time, the length and stitches of the incision, which all brought animal trauma undoubtedly.

6. The characteristics of supernatants

Co-incubation of platelets and 4T1 cells could activate each other and promote the release of substance and procoagulant activity with prolonging the co-incubation time.

6.1 The physical characteristics of supernatants

The concentration and size characteristics of supernatants were listed as follows (Table 2), (Fig.8 a), (Fig.8 b): 1. The concentration of supernatants increased with the incubation time, 4T1 (24h) > 4T1 (30min), PLT (24h) > PLT (30min), 4T1+PLT(24h) >4T1+PLT (30min); 2. Co-incubation promoted release, and the efficiency increased with prolonging the co-incubation time, 4T1+PLT (30min) > 4T1 (30min) + PLT (30min), 4T1+PLT (24h) > 4T1 (24h) + PLT (24h); 3. There were no obvious size difference among the supernatants.

Table 2. The physical characteristics of supernatants: The concentration of supernatants increased with the incubation time, 4T1 (24h) > 4T1 (30min), PLT (24h) > PLT (30min), 4T1+PLT (24h) >4T1+PLT (30min); Co-incubation promoted release, and the efficiency increased with prolonging the co-incubation time, 4T1+PLT (30min) > 4T1 (30min) + PLT (30min), 4T1+PLT (24h) > 4T1 (24h) + PLT (24h);

	Sample	Size(nm)	Concentration
1	4T1 (30min)	61.14 ± 16.45	5.45×10^9
2	4T1 (24h)	57.63 ± 16.91	9.47×10^9
3	PLT (30min)	64.26 ± 27.31	2.39×10^{10}
4	PLT (24h)	57.35 ± 19.73	3.28×10^{10}
5	4T1+PLT (30min)	61.79 ± 24.34	2.97×10^{10}
6	4T1+PLT (24h)	57.37 ± 19.55	5.73×10^{10}

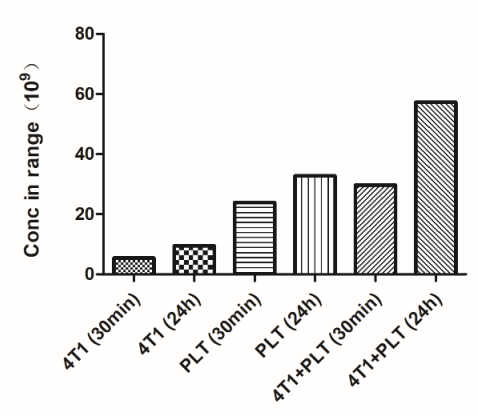
6.2. The procoagulant activity of supernatants

The small EVs with PS-exposed in the 24-hour co-incubation supernatant were significantly more than 24-hour platelet supernatant (Table 3) (Fig.8 c).

Table 3. The procoagulant activity of supernatants: PS-exposed microparticles in the 24-hour co-incubation supernatant were significantly more than 24-hour platelet supernatant, co-incubation significantly increased the procoagulant activity.

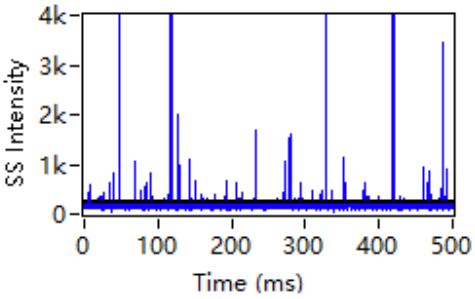
	Sample	Size(nm)	Concentration
1	PLT (24h)	82.36±28.29	5.84×10^9
2	4T1+PLT (24h)	76.91±29.26	9.86×10^9

（a）

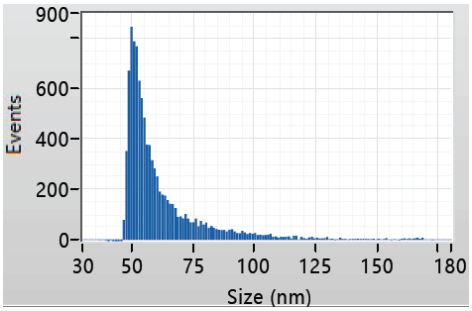


(b)

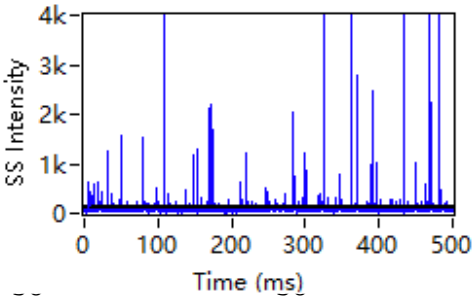
b1



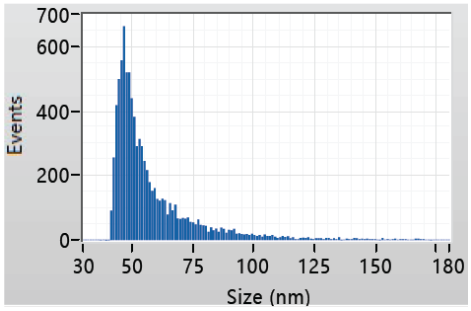
b2



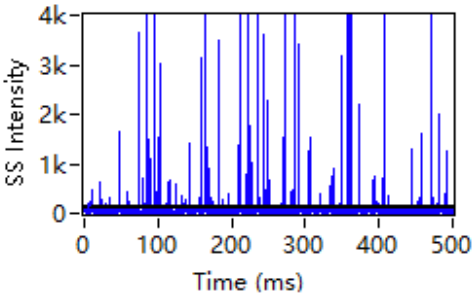
b3



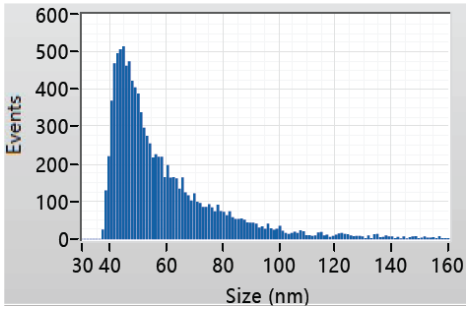
b4

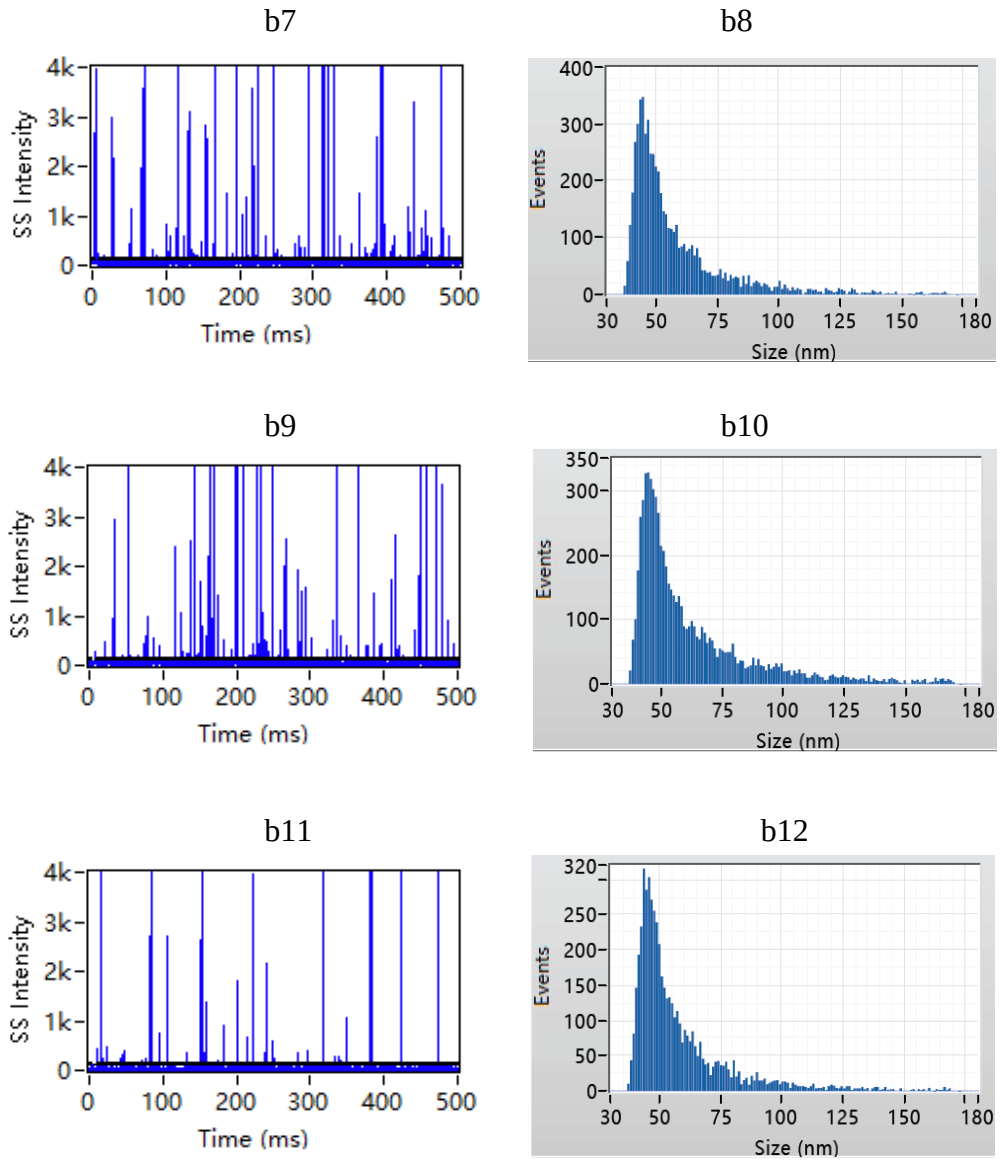


b5

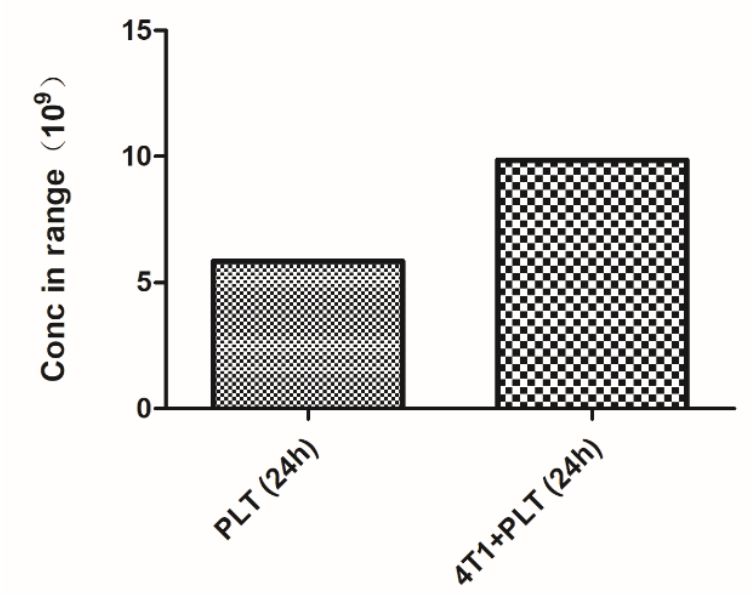


b6




b7
b8
b9
b10
b11
b12
SS Intensity
Time (ms)
Events
Size (nm)

(c)


Conc in range（10⁹）
PLT (24h)
4T1+PLT (24h)

(d)

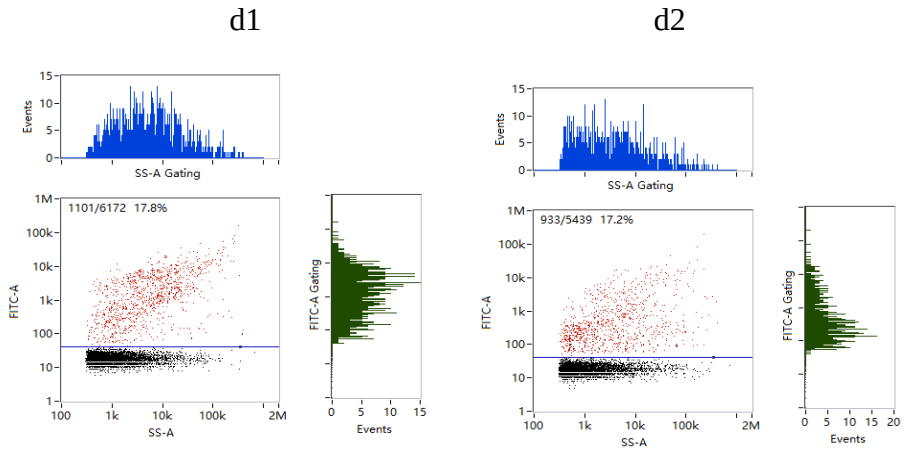


Fig.8 (1). The physical characteristics of supernatants: (a). The concentration of supernatants increased with the incubation time, 4T1 (24h) > 4T1 (30min), PLT (24h) > PLT (30min), 4T1+PLT (24h) >4T1+PLT (30min); Co-incubation promoted release, and the efficiency increased with prolonging the co-incubation time, 4T1+PLT (30min) > 4T1 (30min) + PLT (30min), 4T1+PLT (24h) > 4T1 (24h) + PLT (24h); (b). b1=the concentration of 4T1 (30min) supernatant, b2= the microparticle size of 4T1 (30min) supernatant, b3=the concentration of 4T1 (24h) supernatant, b4= the microparticle size of 4T1 (24h) supernatant, b5=the concentration of PLT (30min) supernatant, b6= the microparticle size of PLT (30min) supernatant, b7=the concentration of PLT (24h) supernatant, b8= the microparticle size of PLT (24h) supernatant, b9=the concentration of 4T1+PLT (30min) supernatant, b10= the microparticle size of 4T1+PLT (30min) supernatant, b11=the concentration of 4T1+PLT (24h) supernatant, b12= the microparticle size of 4T1+PLT (24h) supernatant. (2). The procoagulant activity of supernatants: (c). PS-exposed microparticles in the 24-hour co-incubation supernatant were significantly more than 24-hour platelet supernatant; (d). d1. PS-exposed microparticles of 24-hour platelet supernatant; d2. PS-exposed microparticles of 24-hour co-incubation supernatant. * Co-incubation of platelets and 4T1 cells could activate each other and promote the release of substance and procoagulant activity with prolonging the co-incubation time.

Discussion

Breast cancer is the commonest malignancy cancer in women, and about 3%-8% of patients are with distant metastasis at the time of initial diagnosis [29]. Even 30% of the early patients who underwent radical resection and standardized treatment would still have recurrence and metastasis [30], even lead to death. Accumulated studys showed that more than 60% of patients with malignant tumor were accompanied by hypercoagulable state, which in turn cause cancer progression, followed by long distant metastasis, alert the worse tumor prognosis and even affect treatment strategy. In the clinical study, we showed that the MA (mm) were significantly higher in the patients with stage IV breast cancer than in stage I/II/ III patients(P=0.01), which indicated that platelet hyperfunction was positively associated with tumor metastasis in breast cancer patients. The interesting clinical phenomenon has inspired us to explore the mechanisms related to platelet hyperactivity and breast cancer metastasis.

Given construction of models in vivo and in vitro are the key and the basis of study in this field [31], which both play a decisive role in mimic blood hypercoagulability and the biological characteristics of hematogenous metastasis with clinical efficiently. We were committed to the construction of efficient models in vivo and in vitro.

For selected an excellent in vivo model, we established four experimental groups by limiting the TBP and PIP. Through evaluation of the lung metastasis, we found: 1. The TBP and PIP were both played a decisive role in the occurrence of lung metastasis. For the decisive role of TBP, all of the group 1a, 1b and 2a achieved lung metastasis successfully, but there was no obvious lung metastasis in the group 2b. Given the influence of TBP on metastasis is mainly decided by the follows: When the TBP is too short, the tumor has not yet occurred invasion and metastasis, which directly affects the success of metastasis; When the TBP is too long, the tumor neovascularization increases and the rent increases, the tumor adheres to the surrounding tissue, and is prone to local necrosis, which brings difficulties to radical resection of the tumor, and also easy to cause death by excessive bleeding and prolonged anaesthesia during resection, even leads to incomplete tumor resection to cause recurrence. Combined with the characteristics of our in vivo models, it is indicated that 3 weeks is the baseline of TBP to achieve lung metastasis. 2. For the decisive role of PIP, we found the lung metastasis of the group 1a was more obvious than that of the group 1b, and the group 1a even achieved extrapulmonary metastases. But the fact was TBP of group 1a was longer than that of group 1b, which indicated if the TBP was over 3 weeks, continued extension of it does not increase the incidence of lung metastasis. On the contrary,

the PIP of group 1a was longer than that of group 1b, which indicated when TBP was over 3 weeks prolonged PIP (about 2 weeks) can increase the incidence of lung metastasis. In conclusion, with TBP of 3 weeks and PIP of 2 weeks is the optimum modeling conditions for achieve lung metastasis.

Through evaluation of the hypercoagulation state, we found: 1. Compared with the blank group, all of the experimental groups showed hypercoagulable state, and the remarkable characteristic is the hyperfunction of fibrin and platelet, but the function of coagulation factors were normal. It is further suggested that our in vivo model is particularly suitable for the study of the hematological mechanism of hypercoagulation with hyperactivity of fibrin and platelet; 2. The difference between the group 1b and the blank group was more obvious than that of the other experimental groups. Because the prolonged TBP is the most significant feature of the group 1b, which indicated that the TBP had a great influence on the hyperfunction of platelet; 3. The group 2b showed hypercoagulability before the obvious lung metastasis, indicating that the hypercoagulable state of our model is an early event of metastasis, which can effectively mimic the biology process of hypercoagulable state promoting metastasis.

By compared the operation time, the incision length and stitches among the experimental groups, animal welfare and feasibility analysis of operation were evaluated. We found: Increased size of breast tumors caused by prolonged TBP significantly prolonged the operation time, the length and stitches of the incision, which all brought animal trauma undoubtedly. Therefore, we should shorten the TBP as much as possible on the premise that the model can be successfully established. However, for surgical techniques, we emphasize that complete separation of main nutrient vessels and proximal ligation are important steps to reduce blood loss, which decreased animal trauma obviously.

In conclusion, our study recommended that 4Tl-luc cells be injected into the fourth intramammary gland fat pad with 1 x 10^6, and with TBP of 3 weeks and PIP of 2 weeks, then an ideal in vivo model can be established.

For further promote the study of in vitro mechanism, we established an in vitro model (platelet-breast cancer co-action) by platelets (female BALB/c mice) and 4T1 cells co-incubation for 30 minutes (represent the early phase of platelet-breast cancer co-action in vivo) or 24 hours (represent the late phase of platelet-breast cancer co-action in vivo), then the co-incubation supernatants were obtained by differential centrifugation. Through compared the physical characteristics of supernatants, we found: 1. The concentration of supernatants increased with the incubation time, 4T1 (24h) > 4T1 (30min), PLT (24h) > PLT (30min), 4T1+PLT(24h) >4T1+PLT (30min); 2. Co-incubation promoted release, and the efficiency increased with prolonging the co-incubation time, 4T1+PLT (30min) > 4T1 (30min) + PLT (30min), 4T1+PLT (24h) > 4T1 (24h) + PLT (24h); 3. There were no obvious size difference among the supernatants. The results of physical characterization showed that co-incubation of platelets and 4T1 cells could activate each other and promote substance release with prolonging the co-incubation time, and the reaction supernatants can be used for interest factors detection and bioactivity verification.

From the study of our in vivo model, we known the TBP had a great influence on the hyperfunction of platelet, which means in vitro model, the reaction supernatants by 4T1 and platelet co-incubation for 24 hours (represent the late phase of platelet-breast cancer co-action in vivo) should have a more obvious procoagulant activity, and through compared the PS-exposed, we found the small EVs in the 24-hour co-incubation supernatant were significantly more than 24-hour platelet supernatant, which means co-incubation significantly increased the procoagulant activity with prolonging the co-incubation time.

In conclusion, through the clinical study, we found platelet hyperfunction was positively associated with tumor metastasis in breast cancer patients. And through established models in vivo and in vitro, we have helped to provide powerful research tools for understanding the underlying mechanism related to platelet hyperactivity and breast cancer metastasis. Maybe these models will also provide important insights into the intervention of hematological metastasis in breast cancer.

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