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Original Article

Annexin-V positive microparticles correlate with systemic inflammation response in patients with traumatic extremities fracture

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Abstract: Aims: Physiological inflammation response is one of the critical initial factor for fracture healing. Microparticles are emerging as an important participant and regulator in inflammation response and tissue regeneration. In this study, we mainly investigated the influence of traumatic fracture on circulating MPs and the relationship between microparticles and inflammation response level. Methods and results: In this study we enrolled 18 young traumatic extremities fracture patients and 15 control subjects without recent fracture. The circulating MPs was labeled by Annexin-V and quantified by flow cytometry. To analyze the systemic inflammation level, the levels of serum inflammation factors, including IL-1β, IL-6 and C-reactive protein were measured by ELISA. The results showed that Annexin-V positive MPs level was significantly higher in the patients group than that of the healthy control group (4008 ± 442.2 vs 9333 ± 976.1, $P < 0.001$). Circulating MPs level was correlated with C-reactive protein (CRP) concentration instead of white blood cells counts. Further investigation found the elevation of MPs was independence of the coagulation status. Conclusion: In this study, we found that circulating MPs was elevated in traumatic fracture patients for the first time. Our result also found that the level of circulating MPs was correlated with CRP concentration, suggesting MPs may be an indicator for systemic inflammation response.

Keywords: Bone fracture, microparticles, inflammation, tissue regeneration

Introduction

Microparticles (MPs) are 0.1 to 1 μm germinative membrane vesicles released from parent cells. Circulating MPs are generated from different type of heamocytes, including platelet, erythrocyte and leukocyte. Platelets are the primary source of MPs in the circulation of healthy individuals, although other cells including erythrocyte and leukocyte also release MPs. Recent years, MPs are gradually recognized as intracellular messenger for their function of carrying nucleic acids, proteins, and other antigens from parental cells to target cells. They have been proposed to play roles in numerous processes, including coagulation, inflammation, immune response, cell activation and cancer [1-3]. Platelets MPs were considered play an import role in inflammation and tissue regeneration [4, 5].

Of note, inflammation response is one of the initial factor for bone healing [6]. Platelet-derived growth factor, transforming growth factor-beta (TGF-β), and interleukin (IL) 1 and 6 and prostaglandins are among the key group of inflammatory chemokines that initiate repair processes through their effects on the marrow, periosteum, and hematoma. Microparticles, and platelet membranes can stimulate the mitogenic activity of bone cell, thereby contributing to the regeneration of mineralized tissue [5]. A recent study showed that Osteoblast-derived microparticles may shed from osteoblasts contain RANKL protein and can transfer it to osteoclast precursors, thus may influence bone modeling and remodeling [7].

The inflammatory response after trauma has been extensively studied and SIRS, CARS and MARS have been indicated to exists in a variety

Table 1. Clinical Characteristics for different groups

	Control (n = 15)	Facture group (n = 18)
Male/Female (n)	9/6	11/7
Age (Mean ± SE, y)	25.86 ± 0.53	29.56 ± 2.36
BMI (Mean ± SE)	24.88 ± 1.48	23.44 ± 0.93
Blood Pressure (Mean, mmHg)	123.00/74.00	128.00/75.00
Glucose (Mean ± SE, mmol/L)	5.80 ± 0.50	6.10 ± 0.40
ALT (Mean ± SE, U/L)	19.80 ± 1.43	20.20 ± 1.50
CR (Mean ± SE, mmmol/L)	71.20 ± 5.12	69.40 ± 4.64

BMI: body mass index; ALT: alanine aminotransferase; CR: creatinine.

of settings of patients following trauma [8, 9]. However, the impact of trauma on microparticles is poorly understood. In this study we compared the circulation MPs profiles between traumatic bone fracture patients and healthy volunteers, and investigated the relationship between circulation MPs and inflammation factors and coagulation status.

Materials and methods

Study design and participant protection

This is a single-center retrospective study comparing the circulation MPs profiles between bone fracture patients and healthy volunteers. The study protocol was approved by Peking University Third Hospital Medical Science Research Ethics Committee. Informed consent was obtained from all participants.

Study population

In this study, 20 healthy control volunteers and 30 patients aged between 15 to 44 years were recruited. Healthy subjects were excluded if they were active smokers or had hypertension, hyperlipidemia, tumor or diabetes. Bone fracture patients were recruited between September 2014 and January 2015. Diagnosis of traumatic fracture was confirmed by X-ray and a recently history of trauma. Age, sex, height, weight, total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), triglycerides (TG), blood pressure, heart rate, and smoking status (never, past, current) were assessed on the date of recruitment for all subjects. Body mass index (BMI) was calculated using height and weight measurements. Inclusion criteria for the patients group included youth (younger than 65 years old), traumatic extremities fracture. Exclusion criteria included aging (older than 65 years old), chronic hypertension, heavy smoking (more than 1 pack per day) hyperlipidemia diabetes mellitus, antiphospholipid syndrome, tumor and autoimmune disease. Finally, 15 healthy volunteers and 18 patients were enrolled.

Measurement of microparticles

All samples were obtained, prepared, and stored as follows: 5 mL of venous blood were withdrawn using a 21 G needle and collected in citrated tubes after discarding the first 1 mL of blood. All blood sample were obtained with in 1 weeks after trauma. Within 2 hours of collection, blood was centrifuged at 2500 g for 15 minutes at room temperature to prepare platelet-poor plasma (PPP). The PPP was then collected, aliquoted, and stored at 80°C.

Reagents

FITC-Annexin V (Catalog No. 556570) was purchased from Boster (Wuhan, China). Calibrator beads, 0.3 lm (Catalog No. LC-3) were purchased from Sigma. Calibrator beads, 1 lm (Catalog No. BCP-10-5) and 3 lm (Catalog No. BCP-30-5) were purchased from Spherotech. The antibodies were double-filtered before labeling with a 0.1 lm low protein-binding filter (Millipore, Cat#SLVV033RS). Aliquots of 25 μL of each sample were stained, after which 2.5 μL of 3.0 lm beads (equivalent to 25000 beads) was added to each tube as reference counting beads. Annexin Buffer (10 mmol/L Hepes, pH 7.4, 140 mmol/L NaCl, and 2.5 mmol/L $CaCl_2$) was added to each tube to make the total volume 250 μL. The Annexin Buffer was double-filtered by a 0.22 lm filter followed by a 0.1 lm filter.

Microparticles analysis by flow cytometry

Microparticles analysis by flow cytometry MPs were measured using a Beckman Coulter GalliosTM flow cytometer (Beckman Coulter, Inc. Brea, CA, USA). The MP-gate determination was based on the use of fluorescent calibrated beads (Megamix beads; BioCytex, Marseille, France), comprising 0.5 lm, 0.9 lm, and 3 lm fluorescent beads [10]. Standardization of MP count requires mastering this limit located at 0.5 lm for an optimal compromise between MP analysis and background exclusion. Set FL1

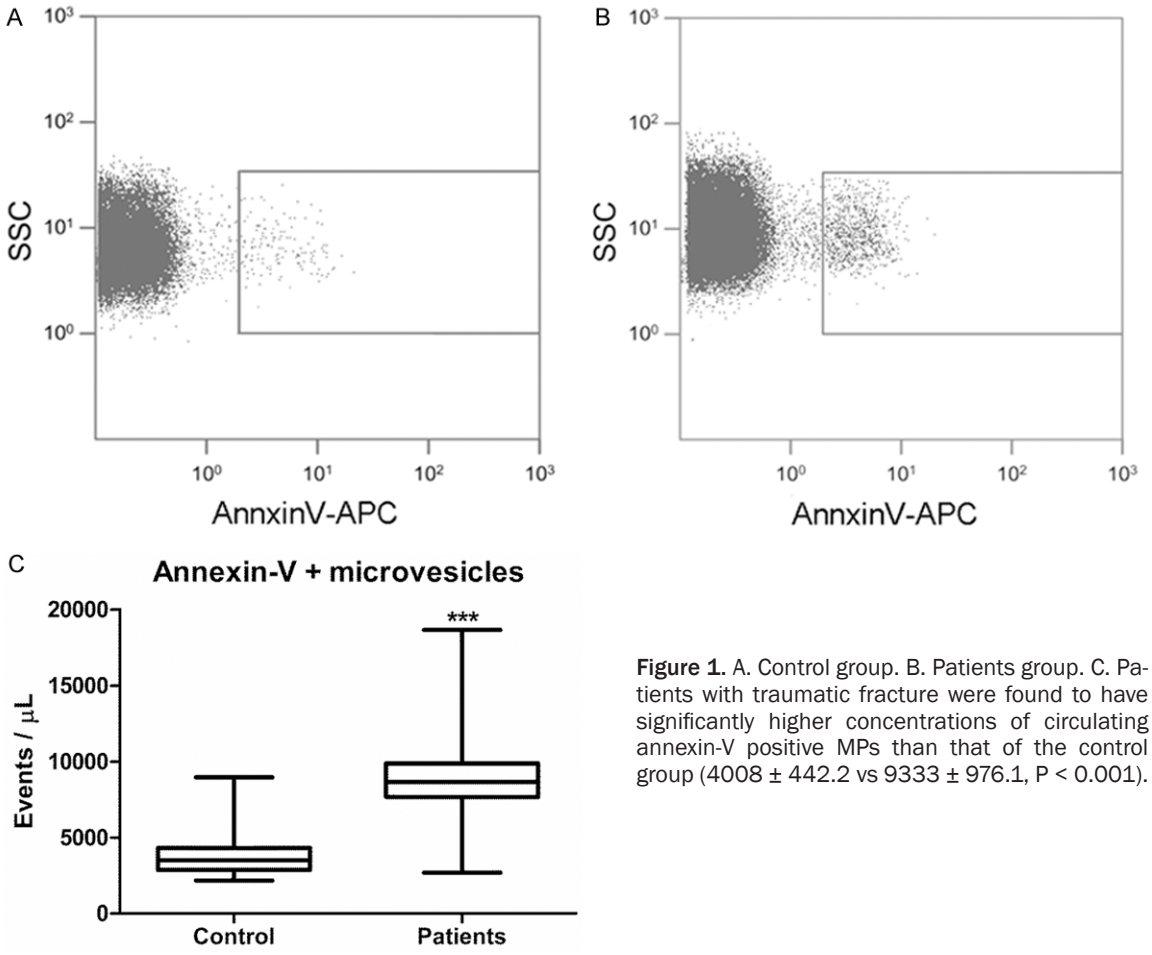

Figure 1. A. Control group. B. Patients group. C. Patients with traumatic fracture were found to have significantly higher concentrations of circulating annexin-V positive MPs than that of the control group (4008 ± 442.2 vs 9333 ± 976.1, P < 0.001).

PMT and SS PMT to locate beads cloud separately.

Adjust FS PMT to let 0.5 lm bead percentage in F Log x Count histogram close to 50%. The MP analysis region is defined as follows: The lower side is defined by the threshold allowing acquisition of events of at least 0.5 lm, and the upper side is the end of the 0.9 lm bead cloud. On the SS Log x FS Log cytogram, an MP autogate with maximum sensitivity around 0.9 lm was created.

Characterization of MP subsets was performed by Annexin V and fluorescence label staining of characteristic ligands [11]. Background reactivities to isotypic were subtracted. 50 µL of serum sample was added to a mixture of four fluorescence stains (4 µL each) and 4 µL heparin. The mixture was incubated at room temperature for 15 min, and 200 µL loading buffer (KeyGen Biotech Company, China) was added to get the final analyses for flow cytometry detection.

Statistical analysis

Continuous data, with the exception of MP data, were expressed as mean ± SD if normally distributed. Normality of continuous variables was assessed using tests for skewness and kurtosis. Means between 2 groups were compared using the 2-tailed, unpaired Student's t test. Medians between 2 groups were compared using the Wilcoxon rank-sum test. Qualitative data are presented as number of subjects with percentages. Frequencies between or among groups were compared using Fisher's exact test. Multivariable linear regression of log-transformed MP concentrations was used to assess the association between each MP concentration and inflammation factors. All models were adjusted for age, sex, BMI, systolic blood pressure (SBP), LDL, HDL, and triglyceride (TG) level, and smoking status (included in the model as former or never smokers since active smoking was an exclusion criterion for the healthy comparators) regardless of their statistical association with MP concentration

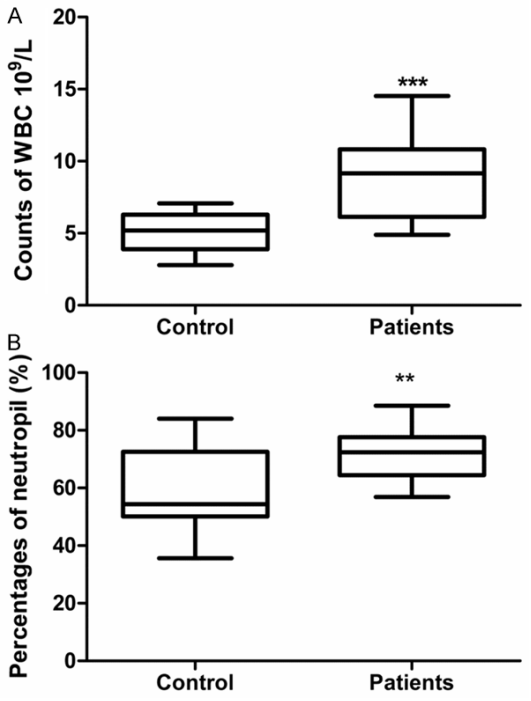

Figure 2. White blood cell (WBC) counts (A), nuetropil percentage (B) were also elevated in the patients group, comparing with those of the control group ($P < 0.001$, $P < 0.01$ respectively).

as these covariates have previously been shown to have significant associations with MP levels. All analysis were carried by GraphPad Prism5 software. The two groups were compared using Student's t test. Differences with $P < 0.05$ were considered statistically significant.

Result

Subject characteristics

In our study, 18 traumatic extremities fracture patients and 15 control subjects without recent fracture were included in the study. The fracture group was slightly older (29.56 ± 2.36 versus 25.86 ± 0.53). The differences of other characteristics between the two groups were out of significant (**Table 1**).

Microparticle and systemic inflammation response levels in traumatic fracture patients and healthy control volunteers

Patients with traumatic fracture were found to have significantly higher concentrations of circulating annexin-V positive MPs than that of the control group (4008 ± 442.2 vs 9333 ± 976.1, $P < 0.001$, **Figure 1**). White blood cell (WBC) counts (**Figure 2A**), neutrophil percentage (**Figure 2B**) and CRP (**Figure 3**) were also elevated in the patients group, comparing with those of the control group ($P < 0.001$, $P < 0.01$, $P < 0.05$ respectively).

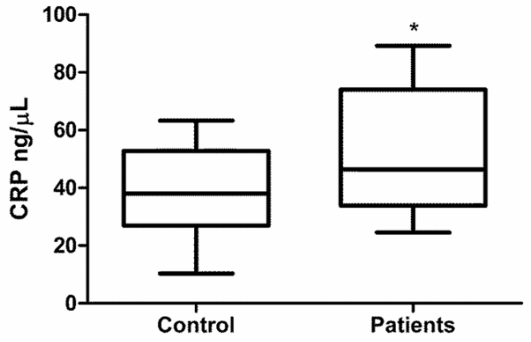

Figure 3. CRP were also elevated in the patients group, comparing with those of the control group ($P < 0.05$).

Correlation between MPs and inflammation factors in patients with traumatic fracture and healthy control volunteer

In fracture group, the circulating MPs levels were slightly correlated with the CRP levels ($r^2 = 0.266$, **Figure 4A**). However, there was no such correlation in the control group ($r^2 = 0.0103$, **Figure 4B**). Of note, no correlation was found between circulating MPs counts and WBC as well as neutrophil percentage (data not shown).

Coagulation status in in traumatic fracture patients and healthy control volunteers

Platelet counts (PLT), activated partial thromboplastin time (APTT), prothrombin time (PT), and international normalized ratio (INR) were examined in both fracture and healthy group. There was no differences between the two groups (**Figure 5**). No correlation was found between MPs levels and these factors (data not shown), suggesting the elevation of MPs was independent of coagulation status.

Discussion

Microparticles (MPs) were first time described as an unwanted contamination of an experimental preparation of platelets and eventually called "platelets dust" [12]. Advanced method-

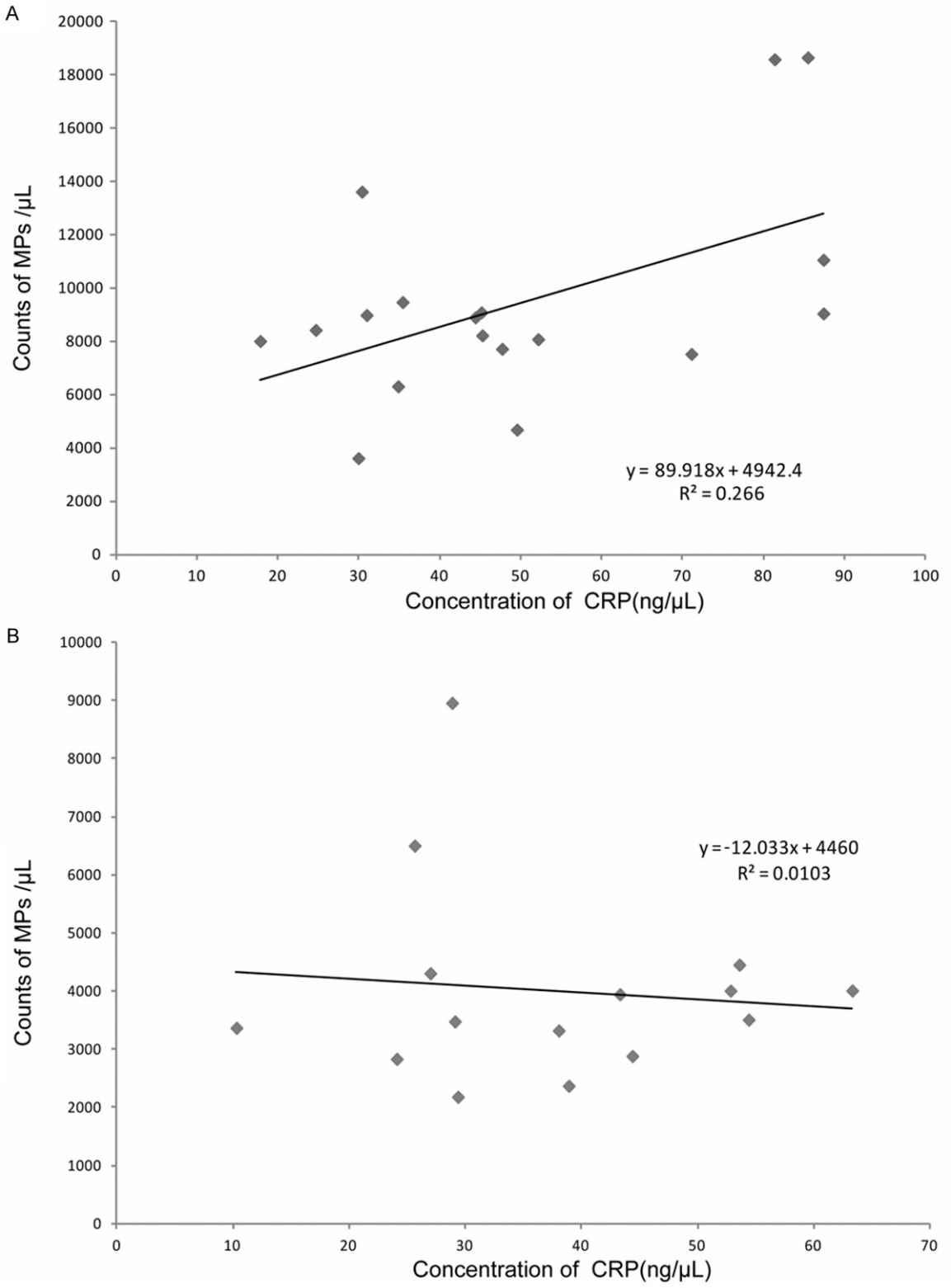

Figure 4. In fracture group, the circulating MPs levels were slightly correlated with the CRP levels (r^2 = 0.266, A). No such correlation was found in the control group (r^2 = 0.0103, B).

ologies such as fluorescence activated cell sorting (FACS) enabled their detailed characterization during the last decade. The relatively novel term MPs includes exosomes and

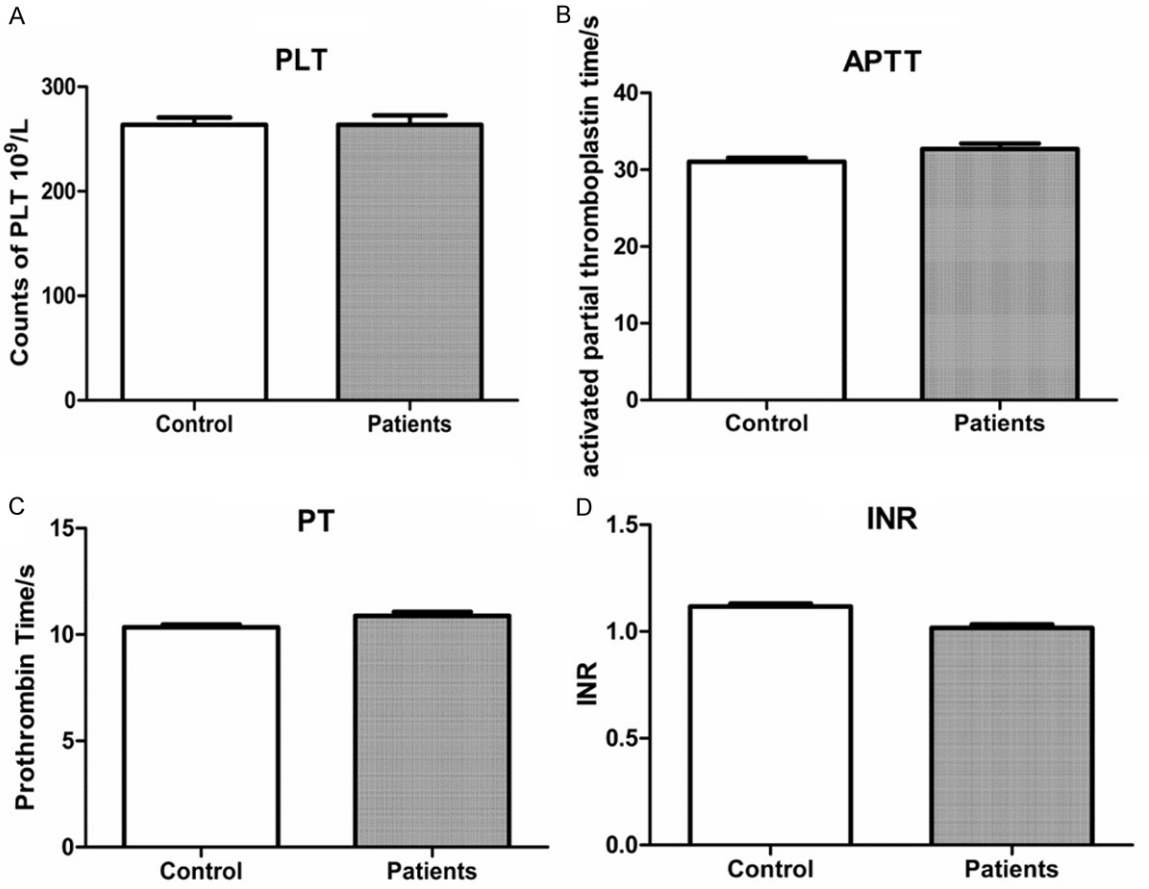

Figure 5. Platelet counts (PLT), activated partial thromboplastin time (APTT), prothrombin time (PT), and international normalized ratio (INR) were examined in both fracture and healthy group. There was no differences between the two group.

activation- or apoptosis-induced microparticles/microvesicles (MPs/MVs). MPs/MVs are 100-1000 nm in diameter, sometimes referred to exosomes and representing a novel route of horizontal communication between cells within the living organism through various body fluids. In contrast, exosomes are smaller in size, < 100 nm, and opposite to MPs they are formed and stored within the cell before their release.

Recent studies indicated that microvesicles may play an emerging role in tissue regeneration [13]. The SDF-1/CXCR4 pathway is crucial in the migration of MSCs to bone fractures [14]. It's very interesting that microvesicles could transfer CXCR4 co-receptor to CXCR4-null cells [15]. Although this finding was observed on T-cells, it's also reasonable to assume microvesicles may affect the sensitivity/responsiveness of CXCR4+ cells to an SDF-1 gradient [16], and play some subtle role in the recruitment of MSF after bone fracture.

Furthermore, it has been hypothesized that the early physiological responses to a bone fracture, namely, hypoxia and inflammation, induce gene expression pathways and promote cell proliferation and migration into the fracture site to promote healing [17]. Inflammatory factors including IL-1β were found capable stimulating proliferation of osteoblasts and production of mineralized bone matrix [18]. Other study has indicated aseptic inflammation may negatively affects the osteoinductivity of BMP-2 [19]. Meanwhile, microvesicles were considered play an important role in the inflammation response. Platelet-derived microvesicles, which is the main compound of circulating microvesicles, were found able to mediate inflammation by regulating IL-1β [20].

However, the relationship between bone fracture induced inflammation response and microvesicles has not been investigated. In this study, we investigated the relationship between

microvesicles and inflammation factors for the first time. Our result indicated that microvesicles were elevated after bone fracture compared with healthy volunteer. Our study also found that annexin-V positive microvesicles were positively correlated with CRP instead of total WBC nor neutrophil. Previous studies also indicated that circulating MPs level was correlated with the coagulation status [21]. Our studies also suggested that the elevation of MPs was independent of the coagulation status in fracture patients.

From our results, we could conjecture that systemic inflammation response may be the main cause of MPs' elevation. And MPs may be serve as biomarker for the inflammation response after trauma. However, being a single center study and limited by the subjects number, the result in our research needs more further studies.

Disclosure of conflict of interest

None.

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