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Effects of Cryopreservation on Microparticles Concentration, Procoagulant Function, Size Distribution, and Morphology

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Statistical Analysis C
Data Interpretation D
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Background: Research on microparticles is rapidly evolving and has extended to the field of many diseases. It is unclear whether microparticles can be stored frozen. In this study, our goal was to verify whether cryopreservation had an effect on the properties of the microparticles.

Material/Methods: We obtained C57BL/6J mouse-derived microparticles by grinding and gradient centrifugation. The specimens were divided into 2 groups: without dimethyl sulfoxide and with dimethyl sulfoxide. The microparticles were then stored at 25°C, 4°C, –20°C, –80°C, and –196°C for 0.5 days, 1 day, 3 days, 5 days, and 7 days. We tested whether the concentration, coagulation function, diameter distribution, and morphology of the microparticles in the 2 groups changed compared to those of a fresh sample.

Results: We discovered that the concentrations of total microparticles, annexin V-positive microparticles, and brain-derived microparticles changed with freezing. The coagulation function, morphology, and size distribution of the microparticles were also affected by cryopreservation. Finally, there was no difference in the effects of cryopreservation on microparticles between the dimethyl sulfoxide group and the dimethyl sulfoxide-free group.

Conclusions: This study suggests that cryopreservation has diverse effects on microparticles within 1 week and that dimethyl sulfoxide has no protective effect on cryopreserved microparticles. Therefore, microparticles should be used fresh for future studies, and they should not be cryopreserved with or without dimethyl sulfoxide.

MeSH Keywords: **Annexin A5 • Cell-Derived Microparticles • Cryopreservation • Dimethyl Sulfoxide**

Abbreviations: **BDMPs** – brain-derived microparticles; **DMSO** – dimethyl sulfoxide; **MPs** – microparticles; **TBI** – traumatic brain injury

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Background

When a cell is activated or apoptotic, its plasma membrane protrudes outward in a bud-like manner and then forms microparticles (MPs) after membrane curvature and cytoskeleton detachment [1,2]. MPs, also known as microvesicles (MVs) or ectosomes, are a subset of extracellular vesicles [3]. Typically, the plasma membrane surface of MPs is rich in phosphatidylserine (PS), and their size is between 100 and 1000 nm. Annexin V can bind to PS on the outer surface of MPs. Therefore, the measurement of annexin V-positive vesicles using flow cytometry is a conventional method for detecting MPs [4]. Most cells can release MPs after activation, such as platelet-derived MPs (PMPs), erythrocyte-derived MPs (RMPs), leukocyte-derived MPs (LMPs), endothelial cell-derived MPs (EMPs), and tumor cell-derived MPs (TMPs) [5–7]. The parental cells can be identified by detecting the surface glycoproteins of MPs.

Increasing reports show that MPs plays an important role in many biological metabolic processes, such as signaling, intercellular communication, inflammatory responses, and clotting processes [8,9]. Thus far, MPs are considered to be an important intermediary for transcellular delivery systems and proinflammatory mediators within biological complex networks [10,11]. In addition, Ratajczak et al. showed that MPs can alter gene expression of target cells by transferring mRNAs and miRNAs [12]. More importantly, the concentration of MPs is mentioned in the literature on almost all thrombotic diseases occurring in the vascular bed [13–16]. MPs have strong procoagulant function, mainly because they are rich in tissue factor (TF) [17,18] and negatively charged PS [19]. The outer membrane of MPs expresses abundant PS and can be bound by annexin V [20], and thus, annexin V-positive MPs exhibit procoagulant activity [21]. Interestingly, in the recent study by Tian et al. [22], the procoagulant activity of brain-derived microparticles (BDMPs) was stronger than that of PMPs. More importantly, the involvement of BDMPs may be responsible for the occurrence of traumatic brain injury (TBI) coagulopathy. Therefore, the role of BDMPs is currently receiving increased attention [23].

However, before a large-scale study of MPs is carried out, whether MPs can be cryopreserved effectively must be determined. The purpose of this study was to determine whether cryopreservation had an effect on MPs within 1 week. In addition to detecting the total MPs concentration, we also detected annexin-positive MPs and BDMPs. This study will provide a reference for large-scale research on MPs.

Material and Methods

MPs production and collection

The brain tissue of C57BL/6J mice (8 weeks old, male) was taken out and kept at low temperature (containers for brain tissue were placed on ice) after euthanasia (sodium pentobarbital, 150 mg/kg intraperitoneal). The brain tissue of mice was ground for 5 minutes until a brain tissue homogenate was formed. First, the brain tissue homogenate was centrifuged at 1500 g for 20 minutes at 4°C; then, the supernatant was centrifuged at 13 000 g for 2 minutes at 4°C, and finally, the supernatant was centrifuged at 100 000 g for 60 minutes at 4°C. Then, the pellet was resuspended in sterile phosphate-buffered saline (PBS) (final concentration of 10^6/μL) [22]. All experimental procedures were approved by the Chinese Small Animal Protection Association.

Specimen storage and testing

The samples were divided into 2 groups. The samples in one group were stored without additives at 25°C, 4°C, –20°C, –80°C, and –196°C; dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) was added to the samples in the other group (original sample volume: DMSO volume=9: 1) before storage at 25°C, 4°C, –20°C, –80°C, and –196°C. The 2 groups of samples were stored for 0.5 days, 1 day, 3 days, 5 days, and 7 days.

Then, the frozen samples were removed from the freezer, and fresh particles were prepared for comparison. The MPs concentration was detected by flow cytometry; the function of the MPs was detected by a coagulation function meter; the particle size distribution of the MPs was detected by a NanoSight; and the morphology of the MPs was observed by electron microscopy. Each set of experiments was repeated 3 times (Supplementary Figure 1).

Flow cytometric detection of MPs

Prior to flow cytometric analysis, the samples were incubated with annexin V-PE (BD Biosciences, CA, USA), GFAP-FITC (eBioscience, CA, USA) and annexin V binding buffer for 30 minutes at room temperature in the dark. We used a flow cytometer (BD™ Biosciences, USA, LSRFortessa) to detect the MPs concentration. The data were collected and analyzed using FACSDiva software. MPs were defined as annexin V-positive vesicles that were less than 1.0 μm in diameter (Supplementary Figure 2C, 2D) [24]. BDMPs were defined as annexin V-positive and surface antigen GFAP-positive particles less than 1.0 μm in diameter (Supplementary Figure 2E, 2F) [22]. The MPs size gate was set with the help of microbeads that were 0.5, 0.9, and 3 μm in diameter (Biocytex, Marseille, France) (Supplementary Figure 2A). The total MPs concentration of each specimen

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was evaluated using fluorescent beads (Spherotec, CA, USA) (Supplementary Figure 2B). The following equation was used to calculate the total concentration of MPs: microparticles per uL=positive events/bead events×bead count per tube (lot–specific)/sample volume.

The concentrations of annexin V-positive MPs and BDMPs were determined on a dot plot, with GFAP-FITC on the horizontal axis and annexin V-PE on the vertical axis. The flow cytometer was set up to obtain samples at a low flow rate for 60 seconds to ensure consistent flow rates throughout the sample collection process.

MPs coagulation function test

All specimens were removed and placed at 37°C for rapid thawing. The concentration of each specimen was adjusted according to the flow cytometry results to generate a uniform concentration. The specimen, mouse plasma, and calcium ions were added to the coagulation function meter (TS6000, MD Pacific, China). The specimens were preheated at 37°C and then tested for prothrombin time (PT) values.

MPs size distribution detection

We used a NanoSight NS300 instrument (Malvern Instruments, Malvern, UK) to analyze the MPs size distribution. The air bubbles were removed from the instrument before testing, and the instrument was kept clean. The instrument was adjusted, and a 50-second video was taken with a 405-nm monochrome laser beam. The video was taken 3 times at a frame rate of 30 frames per second. Particle motion was analyzed by NTA software (version 3.2, NanoSight). We used the NTA system with a minimum detectable size of approximately 50 nm. The NTA setting parameters remained constant while the NTA tested each sample. The software was used to plot the particle size distribution of the MPs by analyzing the videos.

Morphological observation of MPs

The specimens were added to a carbon membrane/Formvar membrane-coated copper mesh and incubated for 5 minutes at 25°C. Phosphotungstic acid (pH 6.5) was added for 5 minutes to enhance the visibility of the membrane structure and then dehydrated with absolute ethanol. Digital images were acquired using a Hitachi HT-7700 transmission electron microscope (TEM, Japan).

Statistical analysis

The data are presented as the mean and standard deviation (Supplementary Tables 1–10). A general linear model was used to analyze the influencing factors of the data. The fixed effects were set as temperature, time, and the interaction between temperature and time. The subjects in each group were random effects; if the interaction between temperature and time was not statistically significant, the effect was removed and analyzed again. The influence of temperature, time and particle size on the data was analyzed using a general linear model. Multiple comparisons were performed using the least squares regression mean for pairwise comparisons. All statistical analyses were performed using SAS 9.4 statistical software; $P \leq 0.05$ was considered statistically significant.

Results

Flow cytometry

As shown in Figure 1, the horizontal axis represents the number of days of storage, the sample tested on day 0 was a fresh sample, and the vertical axis represents the MPs concentration ($\times 10^4$). The 5 curves represent the trends of the total MPs concentrations over 7 days of storage at 25°C, 4°C, –20°C, –80°C, and –196°C.

Total MPs concentration

From Supplementary Tables 1 and 2 and Table 1, we know that the total MPs concentration did not differ significantly according to temperature or the interaction of temperature and time. The total MPs concentration did differ significantly at different times ($P=0.0038 < 0.05$ in the DMSO-free group and $P=0.0033 < 0.05$ in the DMSO group).

Annexin V-positive MPs concentration

The interaction between temperature and time had no significant effect on the concentration of annexin V-positive MPs in the DMSO group, but there was a difference according to the temperature and time interaction ($P=0.0408$ in the DMSO-free group) (Supplementary Tables 3, 4 and Table 2). Concerning the concentration of annexin V-positive MPs, significant differences were observed at different temperatures in each group ($P=0.0004$ in the DMSO-free group and $P=0.0006$ in the DMSO group). There were significant differences over time in each group ($P=0.0002$ in the DMSO-free group and $P<0.0001$ in the DMSO group).

The concentration of BDMPs

From Supplementary Tables 5 and 6 and Table 3, we know that the concentration of BDMPs did not differ significantly in terms of the temperature and time interaction. There was a significant difference across temperatures in the DMSO-free group ($P=0.0388 < 0.05$). There was no significant difference across

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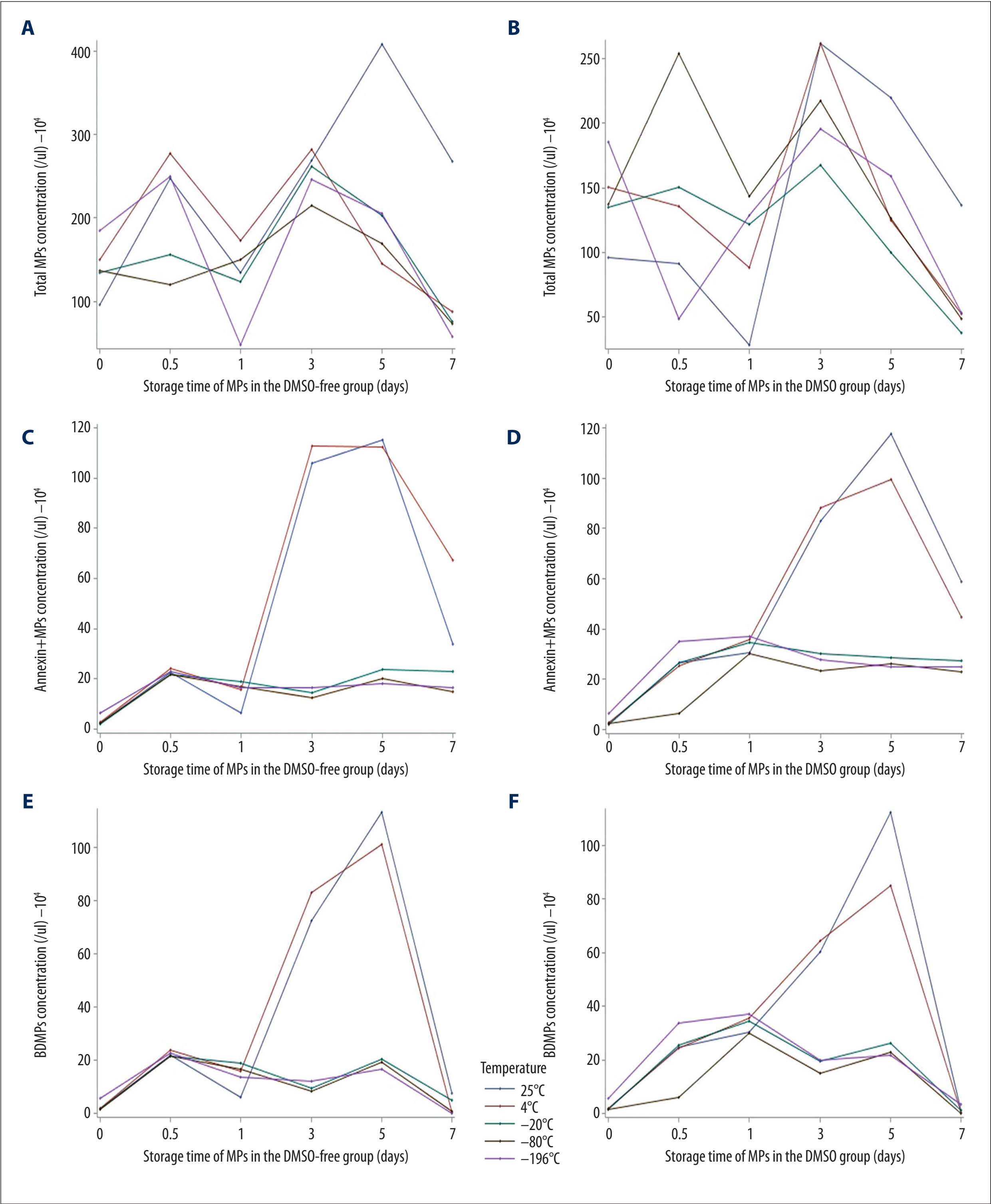

Figure 1. The figure presents the changes in MPs concentrations over 7 days of storage at different temperatures. (**A**) The change in the total MPs concentration over 7 days in the DMSO-free group. (**B**) The change in the total MPs concentration over 7 days in the DMSO group. (**C**) The change in the annexin V-positive MPs concentration over 7 days in the DMSO-free group. (**D**) The change in the annexin V-positive MPs concentration over 7 days in the DMSOs group. (**E**) The change in the BDMPs concentration over 7 days in the DMSO-free group. (**F**) The change in the BDMPs concentration over 7 days in the DMSO group.

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Table 1. Statistical analysis of the influence of time, temperature, time×temperature factors on the total MPs concentration.

Dependent	Group	Source	P
Total MPs concentration	DMSO-free group	Temperature	0.1481
		Time	0.0038
		Temperature×time	0.6116
	DMSO group	Temperature	0.8597
		Time	0.0033
		Temperature×time	0.6692

MPs – micropartles; DMSO – dimethyl sulfoxide.

Table 2. Statistical analysis of the effect of time, temperature and time×temperature factors on the concentration of Annexin V positive MPs.

Dependent	Group	Source	P
Concentration of Annexin V positive MPs	DMSO-free group	Temperature	0.0004
		Time	0.0002
		Temperature×time	0.0408
	DMSO group	Temperature	0.0006
		Time	<.0001
		Temperature×time	0.0990

MPs – micropartles; DMSO – dimethyl sulfoxide.

Table 3. Statistical analysis of the effects of temperature, time and time×temperature on the concentration of BDMPs.

Dependent	Group	Source	P
Concentration of BDMPs	DMSO-free group	Temperature	0.0388
		Time	0.0011
		Temperature×time	0.2646
	DMSO group	Temperature	0.0662
		Time	0.0001
		Temperature×time	0.4123

BDMPs – brain-drived micropartles; DMSO – dimethyl sulfoxide.

temperatures in the DMSO group. There were significant differences between the groups at different times (P=0.0011 in the DMSO-free group and P=0.0001 in the DMSO group).

Functional assays

The trend of the MPs PT value over 7 days is shown (Figure 2). The horizontal axis represents the number of days of storage. The sample tested on day 0 was a fresh sample, and the vertical axis represents the PT value(s) of the MPs. The 5 curves represent the trends of the MPs PT values over 7 days of storage at 25°C, 4°C, –20°C, –80°C, and –196°C.

From Supplementary Tables 7 and 8 and Table 4, we know that the temperature and time interaction had no significant effect on the MPs procoagulant time. There was no statistically significant difference across storage times in the 2 groups. There was a statistically significant difference across temperatures in both groups (P<0.0001 in the DMSO-free group and P=0.0055 <0.05 in the DMSO group).

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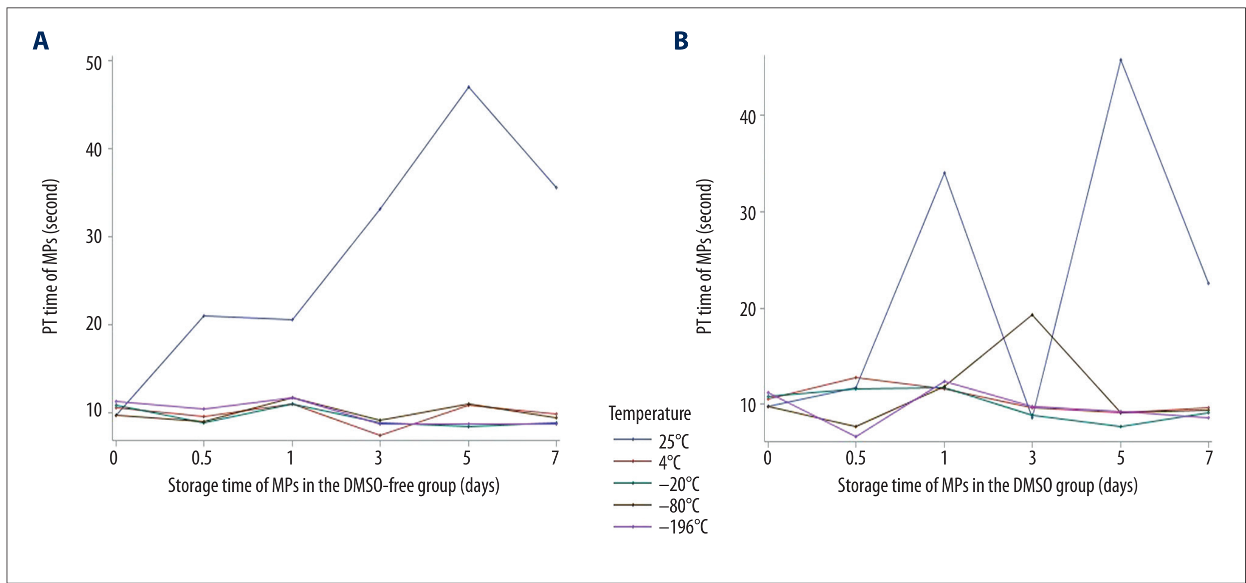

Figure 2. The figure presents the changes in the MPs PT values over 7 days at different temperatures (Figure 2). (**A**) The change in the MPs PT value over 7 days in the DMSO-free group. (**B**) The change in the MPs PT value over 7 days in the DMSO group.

Table 4. Statistical analysis of the influence of time, temperature and time×temperature factors on the procoagulant time of MPs.

Dependent	Group	Source	P
PT value	DMSO-free group	Temperature	<.0001
		Time	0.4008
		Temperature×time	0.1268
	DMSO group	Temperature	0.0055
		Time	0.4324
		Temperature×time	0.1961

PT – prothrombin time; MPs – micropartles; DMSO – dimethyl sulfoxide.

Nanoparticle tracking analysis

From Figure 3, we know that at the same temperature, the change trends of MPs with different particle sizes differed between the DMSO-free group and the DMSO group over 7 days. At the same particle size, the changing trend of MPs stored at different temperatures differed between the DMSO-free group and the DMSO group over 7 days. After storage at different temperatures for 7 days, the 0 to 250 nm size accounted for the greatest number of MPs, followed by the 250 to 500 nm size, and the 500 to 1000 nm size accounted for the smallest number of MPs.

From Supplementary Tables 9 and 10 and Table 5, we found that at the same storage temperature and storage time, there were significant differences in the particle size distributions of each group ($P<0.0001$). At the same storage temperature and MPs size, there were significant differences across different storage times in each group ($P<0.0001$).

Transmission electron microscopy

As shown by the photos of fresh specimens, the number of MPs was large, and the MPs were separated from each other and presented a spherical or horseshoe shape (the black arrow in Figure 4). The background of the field of view was also very clean, with no impurities or fragments of MPs.

In the DMSO-free group, the number of MPs stored at 4°C, –20°C, –80°C, and –196°C decreased with increasing storage time, and the MPs shape changed from the normal spherical or horseshoe shape to an unusual or irregular shape. After one day of storage at 25°C, the specimen began to grow bacteria, and on the 5th and 7th days, MPs could not be seen in the field of view. The growth of bacteria may be the cause of this phenomenon.

In the DMSO group, after storage at 4°C, –20°C, –80°C, and –196°C, similar to the DMSO-free group, the number of MPs

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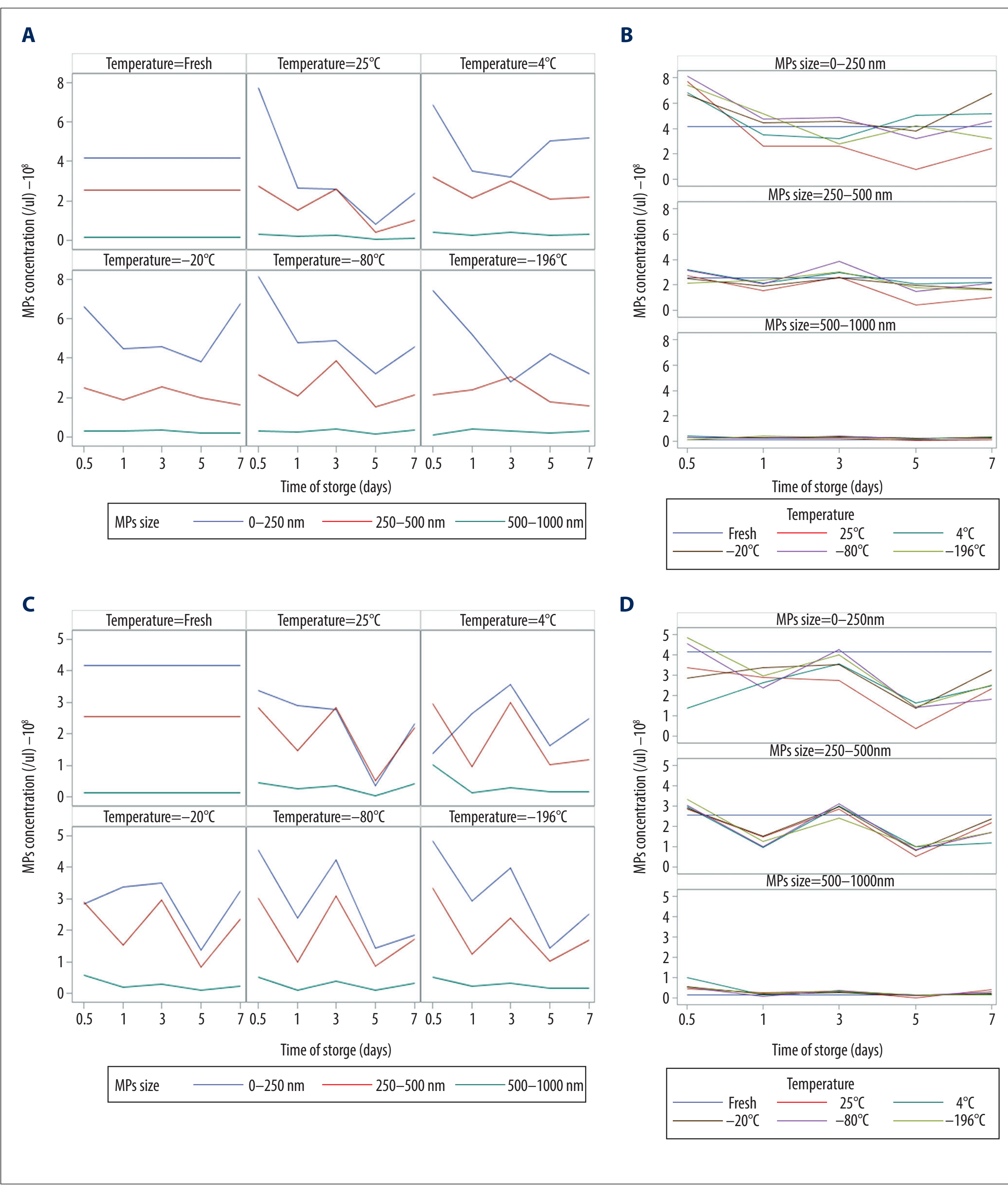

Figure 3. The change trends of MPs with different particle sizes. (**A**) The change trends of the concentrations of 0 to 250 nm, 250 to 500 nm, and 500 to 1000 nm MPs at the same temperature over 7 days in the DMSO-free group. (**C**) The change trends of the concentrations of 0 to 250 nm, 250 to 500 nm, and 500 to 1000 nm MPs at the same temperature over 7 days in the DMSO group. (**B**) The change trends of the concentration of MPs of the same size at different temperatures over 7 days in the DMSO-free group. (**D**) The change trends of the concentration of MPs of the same size at different temperatures over 7 days in the DMSO group.

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Table 5. The effect of different temperatures on MPs 0–250 nm, 250–500 nm, 500–1000 nm (×10⁸) in the DMSO-free group and the DMSO group within 7 days.

Variable	Constant	P
Time	Temperature, size	<0.0001
Temperature	Time, size	<0.0001
Size	Time, temperature	<0.0001

MPs – micropartles; DMSO – dimethyl sulfoxide.

decreased as the storage time increased, and the MPs morphology also became unusual or irregularly shaped. Unlike the DMSO-free group stored at 25°C, the DMSO group stored at 25°C for 7 days showed no bacterial growth or reproduction. This effect is related to the toxicity of DMSO, which is not suitable for the growth and reproduction of bacteria. In conclusion, compared to the DMSO-free group, the DMSO group not only showed no protective effect on MPs cryopreservation but also showed changes in MPs morphology, suggesting that the added DMSO may also cause damage to the MPs.

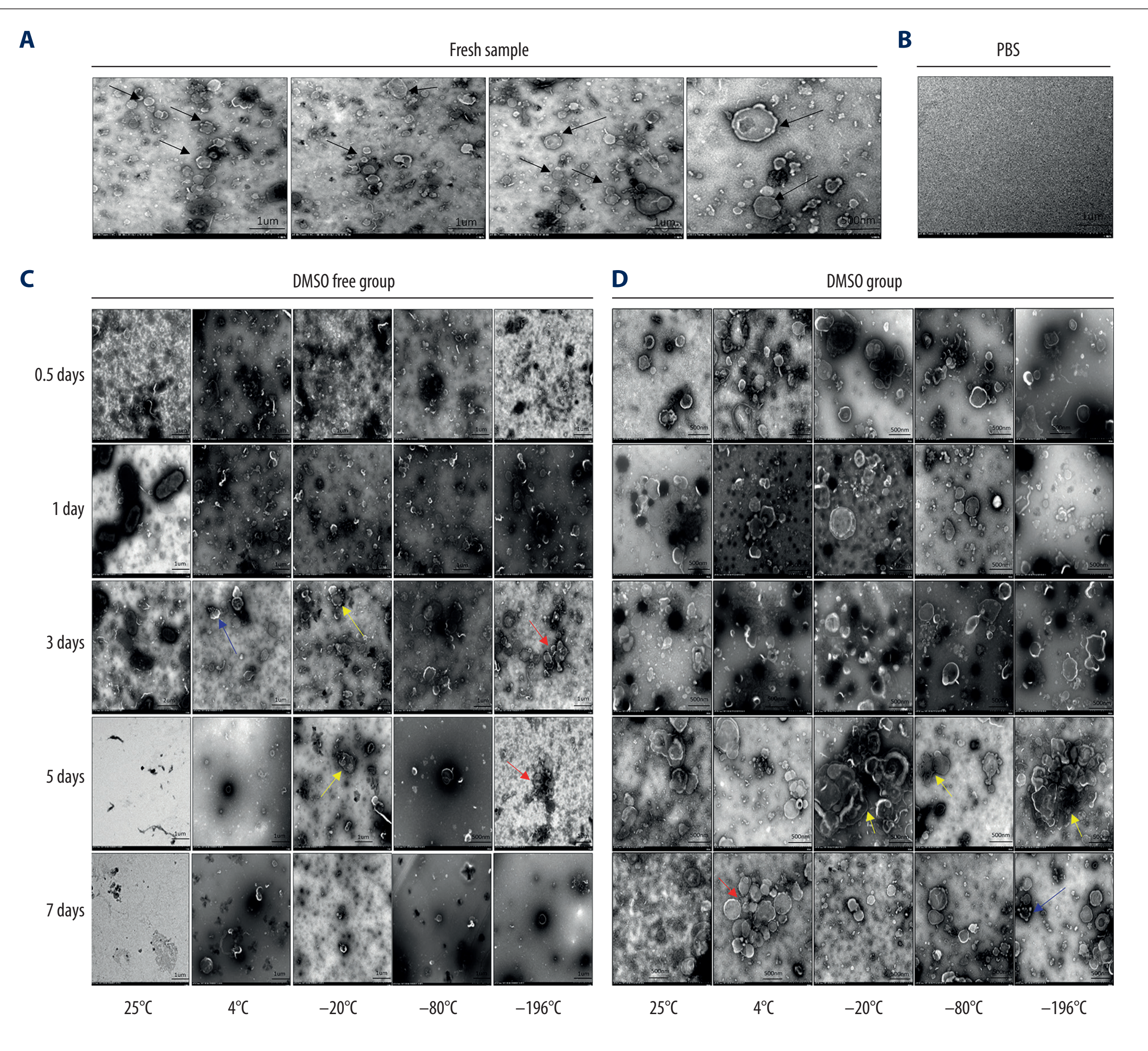

Figure 4. Electron micrograph of MPs stored over 7 days of cryopreservation (magnified 4000 to 10 000 times). (**A**) Electron micrograph of fresh MPs. (**B**) Electron micrograph of the diluent PBS. (**C**) Electron micrograph of MPs stored for 7 days of cryopreservation in the DMSO-free group. (**D**) Electron micrograph of MPs stored for 7 days of cryopreservation in the DMSO group. The photos show MPs stored at 25°C, 4°C, −20°C, −80°C, and −196°C, from left to right. The photos show MPs stored for 0.5 days, 1 day, 3 days, 5 days, and 7 days from top to bottom.

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We found that cryopreservation may cause damage to the morphology of MPs, as cryopreservation may result in MPs agglomeration (the red arrow in Figure 4), lysis (the green arrow in Figure 4) or fusion (the yellow arrow in Figure 4).

Discussion

Research on MPs is attracting more and more research teams. However, before performing large-scale discovery work, there is an obstacle that must be overcome; that is, can MPs be cryopreserved? The results of different studies differ on this topic. Jy et al. suggested rapid freezing of platelet-free plasma in liquid nitrogen followed by storage at –80°C to reduce the formation of ice crystals [25]. However, Lacroix et al. found no significant difference between these 2 methods in a comparison between rapid freezing in liquid nitrogen and direct storage at –80°C [26] although it is unclear whether there are differences in the fine structure or cytoplasmic content of MPs. Shah et al. observed a decrease in the number of CD144+ EMPs in samples stored at 4°C and –80°C [27]. However, van Ierssel et al. proposed that when samples are stored at 4°C and –80°C, cryopreservation has different effects on different phenotypic subsets of MPs [28]. Our research showed that the concentrations of total MPs, annexin V-positive MPs, and BDMPs change with freezing. Although the identification by flow cytometry may be inaccurate for MPs less than 0.5 μm, we improved the specificity and sensitivity of flow cytometry by using the 3 basic features of MPs (size, annexin V positivity, and cell-specific markers) in this study. In summary, although flow cytometry has a certain detection threshold, it is still the most common method for detecting MPs.

MPs have strong procoagulant activity. Some studies have reported an increase in the number of MPs after cryopreservation [29]. This change may be the cause of the increased platelet clotting activity after freezing [30]. PMPs expressing phosphatidylserine can participate in the coagulation pathway by binding to FXa and thrombin to produce a thrombin complex, resulting in an obvious increase in the blood coagulation activity of cryopreserved apheresis platelet concentrates [30,31]. However, there is controversy regarding the coagulation function of MPs themselves after cryopreservation. Lacroix et al. found that the procoagulant activity of MPs did not change after 12 months of storage at –80°C [26]. However, our results show that cryopreservation affects the coagulation function of the MPs.

DMSO is a widely used reagent in cell biology. It can dehydrate cells during cryopreservation, thereby preventing the formation of ice crystals in the intracellular environment [32]. Our results showed that compared to the fresh group, the total MPs concentration, the concentration of annexin V-positive MPs, the concentration of BDMPs, the procoagulant function of MPs, the MPs size distribution, and the morphology of the MPs were significantly changed in the DMSO group; however, there was no difference between the DMSO group and the DMSO-free group. Therefore, we conclude that DMSO has no protective effect on cryopreserved MPs. Notably, there are currently no reports of reagents that can protect frozen MPs well.

Studies by Matijevic et al. have shown that MPs may be degraded into smaller vesicles and may aggregate or fuse into larger vesicles after 5 days of refrigeration [33]. Our research confirms this finding. We found that cryopreservation may cause damage to the morphology of the MPs, such as MPs agglomeration, lysis or fusion. Compared to fresh MPs, there are many impurities and MPs fragments in the field of view after cryopreservation. Morphological changes may cause a change in MPs concentration. We observed changes in the morphology of MPs by electron microscopy; however, this change did not rule out the effects of dehydration and fixation on sample morphology. Therefore, cryo-EM that maintains the activity and functional status of biological samples will be considered in the future.

Specifically, we used a method involving the grinding of the mouse brain to obtain a large number of MPs (including annexin V-positive MPs and BDMPs) [22]. This is one of the advantages of this experiment. In addition, this experiment focused on the effects of 4 different storage temperatures on MPs, including MPs concentration, function, and particle size distribution, and MPs morphology. This work will provide a reference for the storage of MPs in the future. However, this study has several limitations, reflected mainly in the following 3 aspects: 1) this study lacks data on the separate storage of blood cell-derived MPs. The effect of cryopreservation on blood cell-derived MPs may be different from that on other MPs. 2) This study lacks data on MPs proteomics after cryopreservation, and the protein changes in the MPs after cryopreservation are unclear. Protein changes during cryopreservation may be a key cause of fluctuations in MPs concentration and procoagulant activity. Therefore, proteomics of MPs during cryopreservation will become a hot spot in future research. 3) Further research is needed on the specific mechanism by which freezing affects MPs changes.

Conclusions

This study confirms that 1) the concentrations of total MPs, annexin V-positive MPs, and BDMPs changed with freezing; 2) the coagulation function, morphology and size distribution of MPs were also affected by cryopreservation; and 3) there was no difference in the effect of cryopreservation on MPs between the DMSO group and the DMSO-free group. Our research

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shows that cryopreservation affects the concentration, function, size distribution, and morphology of MPs. Therefore, we believe that MPs may not be suitable for cryopreservation. If MPs are required for clinical or scientific research, fresh MPs are an ideal choice.

Acknowledgments

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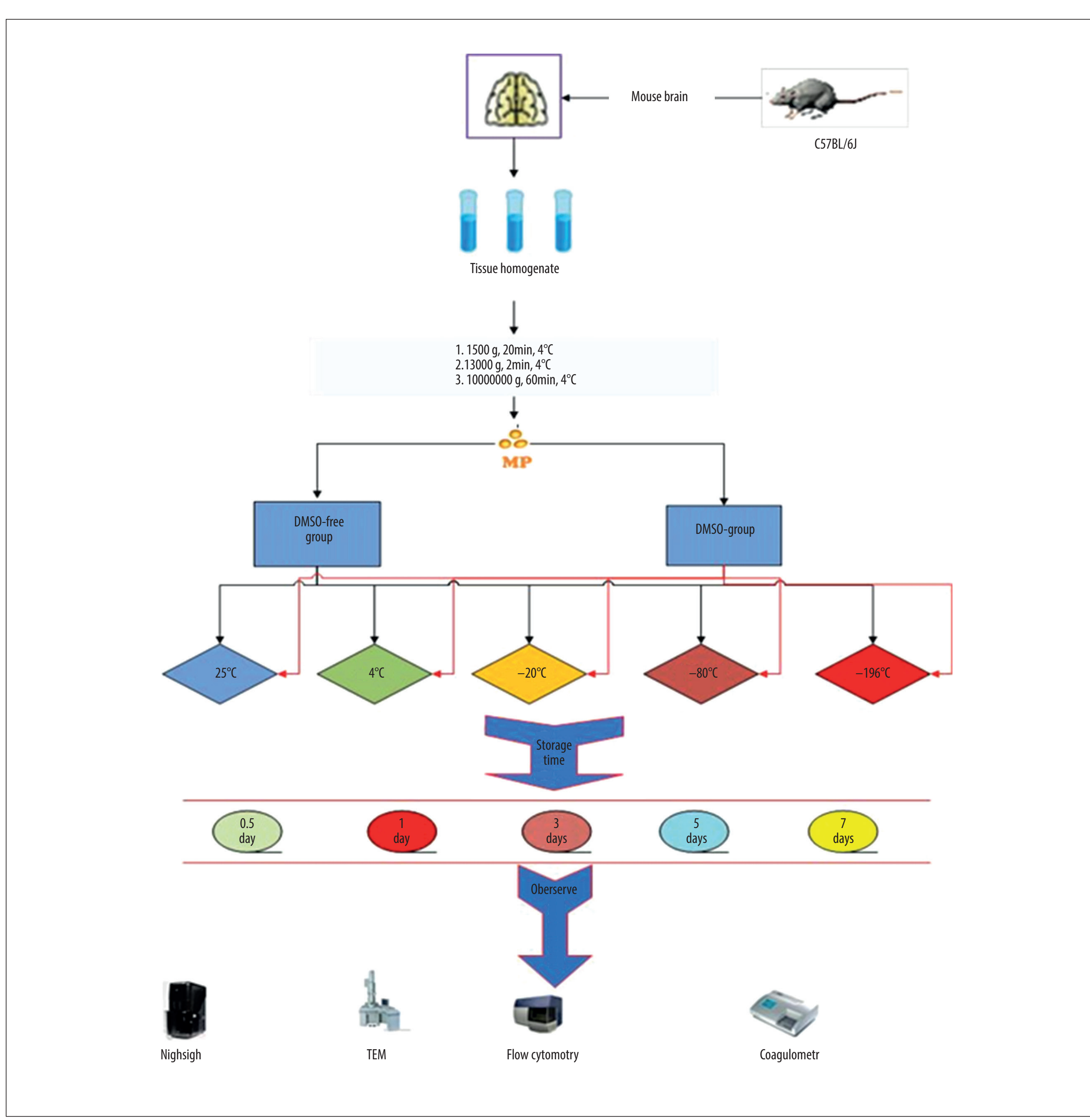
Supplementary Data



Supplementary Figure 1. Cryopreservation protocol and experimental method. We obtained C57BL/6J mouse-derived MPs by grinding and gradient centrifugation. The specimens were divided into 2 groups: without DMSO and with DMSO. The MPs were then stored at 25°C, 4°C, –20°C, –80°C, and –196°C for 0.5 days, 1 day, 3 days, 5 days, and 7 days. We then tested whether the concentration of MPs, the coagulation function of the MPs, the diameter distribution, and the morphology of the MPs in the two groups changed compared to those of the fresh sample.

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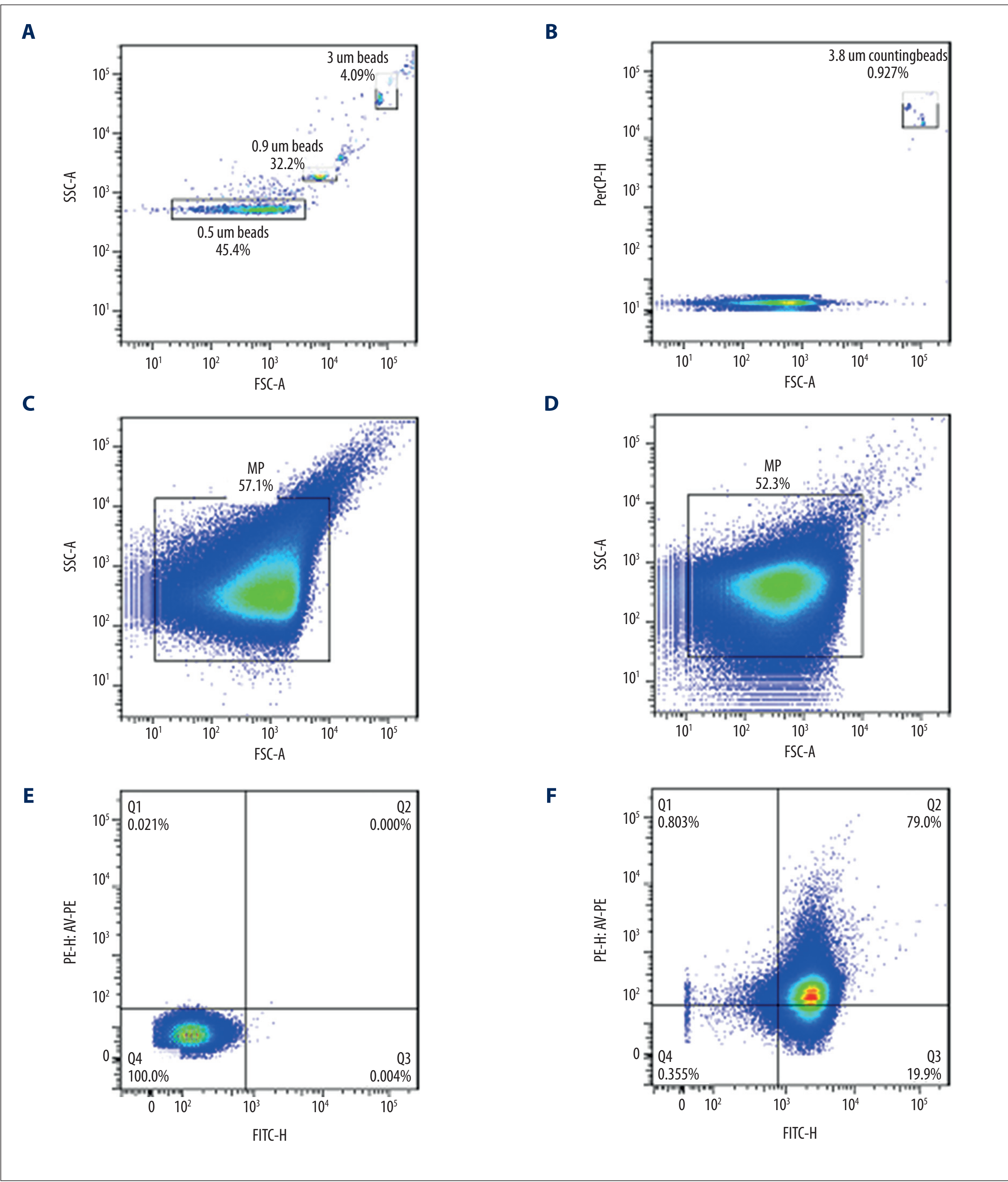

Supplementary Figure 2. Flow cytometric detection of MPs/BDMPs. Detection of MPs by flow cytometry (**A**) The microbead (0.5, 0.9, and 3 µm) size gate was set. (**B**) The fluorescent bead (3.8 µm) size gate was set. (**C**) The MPs size gate was set with the help of microbeads that were 0.5, 0.9, and 3 mm in diameter in samples unstained with antibodies. (**D**) The MPs size gate was set with the help of microbeads in samples stained with antibodies. (**E**) Application of flow cytometric detection for unstained MPs. (**F**) Application of flow cytometric detection for stained MPs.

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Supplementary Table 1. Concentration of total MPs in the DMSO-free group (×10^4).

Temperature	25°C (N=3)	4°C (N=3)	–20°C (N=3)	–80°C (N=3)	–196°C (N=3)
0 day	95.97±7.30	150.24±19.00	135.04±19.40	137.42±11.16	185.45±82.87
0.5 days	247.87±84.52	277.43±132.25	155.89±98.98	120.37±37.10	249.12±125.45
1 day	134.79±115.25	173.38±151.54	123.93±120.77	150.84±145.74	48.28±62.27
3 days	269.29±155.38	281.44±139.24	261.93±69.83	215.51±115.00	246.22±92.94
5 days	407.81±330.80	145.80±113.43	202.97±186.77	169.11±138.64	205.76±193.13
7 days	267.14±36.13	87.60±54.20	76.41±84.41	73.55±66.92	58.17±44.16

Mean and standard deviation of the total MPs concentration (×10^4) in the DMSO-free group.

Supplementary Table 2. Concentration of total MPs in the DMSO group (×10^4).

Temperature	25°C (N=3)	4°C (N=3)	–20°C (N=3)	–80°C (N=3)	–196°C (N=3)
0 day	95.97±7.30	150.24±19.00	135.04±19.40	137.42±11.16	185.45±82.87
0.5 days	91.57±90.55	135.96±162.07	150.15±178.45	254.13±89.16	48.35±7.25
1 day	28.30±26.27	88.14±65.40	121.64±110.24	143.47±154.96	128.34±119.89
3 days	261.63±145.87	261.71±166.38	167.91±75.08	217.75±112.29	195.84±121.90
5 days	220.05±120.32	124.97±81.42	100.03±76.51	126.14±83.76	159.07±158.22
7 days	136.62±70.18	52.68±33.92	38.11±44.35	48.55±41.76	53.12±43.26

Mean and standard deviation of the total MPs concentration (×10^4) in the DMSO group.

Supplementary Table 3. Results of Annexin V positive MPs concentration in DMSO-free group (×10^4).

Temperature	25°C (N=3)	4°C (N=3)	–20°C (N=3)	–80°C (N=3)	–196°C (N=3)
0 day	2.45±0.07	2.69±0.05	2.19±0.10	2.40±0.06	6.45±6.89
0.5 days	22.48±5.30	24.31±6.14	21.76±7.58	21.96±7.76	23.07±8.50
1 day	6.38±8.03	15.94±13.10	18.91±14.00	16.95±13.34	16.45±13.16
3 days	106.02±102.63	112.56±112.05	14.63±10.73	12.56±6.70	16.49±10.47
5 days	115.12±99.53	112.54±66.21	23.91±9.37	20.12±14.48	18.00±13.59
7 days	33.85±15.21	67.25±44.02	23.14±7.44	14.76±1.08	16.36±2.68

Mean and standard deviation of the annexinV-positive MPs concentration (×10^4) in the DMSO-free group.

Supplementary Table 4. Results of Annexin V positive MPs concentration in DMSO group (×10^4).

Temperature	25°C (N=3)	4°C (N=3)	–20°C (N=3)	–80°C (N=3)	–196°C (N=3)
0 day	2.45±0.07	2.69±0.05	2.19±0.10	2.40±0.06	6.45±6.89
0.5 days	26.51±17.79	25.35±18.52	26.64±18.32	6.40±3.13	35.23±0.39
1 day	30.56±3.90	35.70±4.69	34.61±0.70	30.32±15.25	37.16±3.23
3 days	83.12±76.53	88.26±81.38	30.14±15.13	23.30±14.03	28.02±6.83
5 days	117.47±90.26	99.67±38.02	28.77±13.00	26.14±13.34	25.11±12.12
7 days	58.80±9.83	44.82±14.73	27.27±4.02	22.87±4.66	24.88±4.49

Mean and standard deviation of the concentration of annexinV-positive MPs (×10^4) in the DMSO group.

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Supplementary Table 5. Results of BDMPs concentration in the DMSO-free group (×10^4).

Temperature	25°C (N=3)	4°C (N=3)	–20°C (N=3)	–80°C (N=3)	–196°C (N=3)
0 day	2.09±0.04	1.76±0.10	1.67±0.06	1.72±0.04	5.81±7.15
0.5 days	21.98±4.90	23.94±6.07	21.49±7.65	21.63±7.63	22.67±8.23
1 day	6.17±8.15	15.79±13.10	18.72±14.05	16.74±13.32	13.65±13.12
3 days	72.26±105.42	82.79±117.93	9.42±7.32	8.20±8.00	12.25±12.58
5 days	113.00±99.09	100.83±79.43	20.44±9.66	19.11±15.26	16.47±13.88
7 days	7.51±12.63	0.25±0.36	5.01±8.64	0.85±1.45	0.21±0.33

Mean and standard deviation of the BDMPs concentration (×10^4) in the DMSO-free group.

Supplementary Table 6. Results of BDMPs concentration in the DMSO group (×10^4).

Temperature	25°C (N=3)	4°C (N=3)	–20°C (N=3)	–80°C (N=3)	–196°C (N=3)
0 day	2.09±0.04	1.76±0.10	1.67±0.06	1.72±0.04	5.81±7.15
0.5 days	24.81±16.55	24.17±17.57	25.49±17.47	6.19±3.01	33.68±0.41
1 day	30.38±3.92	35.51±4.71	34.42±0.76	30.13±15.31	37.00±3.30
3 days	60.35±84.39	64.35±90.10	19.47±13.83	15.15±15.54	19.80±11.65
5 days	112.19±91.19	85.00±55.14	26.24±15.43	22.96±15.05	21.70±13.96
7 days	1.31±1.69	0.31±0.49	1.06±1.80	0.03±0.01	3.33±5.72

Mean and standard deviation of the BDMPs concentration in the DMSO group (×10^4).

Supplementary Table 7. Results of procoagulant time of MPs in the DMSO-free group (s).

Temperature	25°C (N=3)	4°C (N=3)	–20°C (N=3)	–80°C (N=3)	–196°C (N=3)
0 day	9.83±0.12	10.60±0.72	10.90±0.69	9.83±0.06	11.27±0.49
0.5 days	21.10±23.13	9.57±1.39	8.87±2.49	9.10±2.30	10.53±0.95
1 day	20.63±13.42	11.00±0.53	11.00±0.17	11.70±0.50	11.70±0.26
3 days	33.23±23.72	7.47±2.65	8.93±0.91	9.20±0.56	8.70±1.39
5 days	47.07±14.99	10.87±2.80	8.47±0.91	11.07±3.45	8.83±2.36
7 days	35.57±25.63	9.87±3.23	8.97±4.15	9.53±3.84	8.80±3.52

Mean and standard deviation of the procoagulant time value in the DMSO-free group.

Supplementary Table 8. Results of the procoagulant time of MPs in the DMSO group (s).

Temperature	25°C (N=3)	4°C (N=3)	–20°C (N=3)	–80°C (N=3)	–196°C (N=3)
0 day	9.83±0.12	10.60±0.72	10.90±0.69	9.83±0.06	11.27±0.49
0.5 days	11.70±3.33	12.83±1.36	11.67±3.01	7.73±0.76	6.67±0.31
1 day	34.00±35.34	11.63±0.42	11.73±0.78	11.83±0.85	12.43±1.10
3 days	8.60±3.20	9.70±1.31	8.87±1.81	19.30±15.44	9.87±1.50
5 days	45.70±43.43	9.13±1.48	7.67±2.54	9.17±1.50	9.27±0.72
7 days	22.60±16.80	9.63±2.93	9.20±1.84	9.40±3.31	8.63±2.91

Mean and standard deviation of the procoagulant time value in the DMSO group.

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Supplementary Table 9. Mean and standard deviation statistics of 0–250 nm, 250–500 nm, and 500-1000 nm distributions of MPs in the DMSO-free group (×10^8).

Temperature	Time (days)	Size distribution		
		0–250 nm	250–500 nm	500–1000 nm
Fresh	–	4.16±0.28	2.55±0.24	0.14±0.03
25°C	0.5	7.74±0.43	2.76±0.21	0.32±0.15
	1	2.64±0.33	1.54±0.11	0.23±0.02
	3	2.60±0.16	2.61±0.34	0.27±0.05
	5	0.80±0.12	0.4±0.04	0.06±0.02
	7	2.42±0.23	1.02±0.06	0.12±0.03
4°C	0.5	6.86±2.01	3.19±0.26	0.39±0.09
	1	3.50±0.31	2.11±0.14	0.27±0.08
	3	3.23±0.37	3.00±0.28	0.39±0.13
	5	5.06±0.21	2.11±0.29	0.23±0.12
	7	5.20±0.61	2.20±0.09	0.31±0.05
-20°C	0.5	6.63±0.38	2.50±0.03	0.32±0.09
	1	4.48±1.01	1.88±0.37	0.31±0.19
	3	4.59±0.36	2.57±0.19	0.35±0.11
	5	3.82±0.48	1.97±0.25	0.22±0.07
	7	6.78±1.97	1.65±0.43	0.22±0.07
-80°C	0.5	8.14±0.11	3.14±0.16	0.31±0.05
	1	4.76±1.07	2.11±0.54	0.25±0.02
	3	4.89±0.38	3.87±0.29	0.43±0.05
	5	3.20±0.19	1.51±0.27	0.16±0.08
	7	4.58±0.54	2.13±0.19	0.37±0.11
-196°C	0.5	7.45±0.28	2.16±0.48	0.12±0.08
	1	5.17±0.65	2.37±0.23	0.39±0.07
	3	2.82±0.76	3.06±0.34	0.33±0.07
	5	4.23±0.25	1.78±0.02	0.19±0.04
	7	3.20±0.65	1.60±0.07	0.33±0.07

Mean and standard deviation of the distribution of 0–250 nm, 250–500 nm, and 500–1000 nm MPs in the DMSO-free group (×10^8).

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Supplementary Table 10. Mean and standard deviation statistics of the distribution of MPs 0–250 nm, 250–500 nm, 500–1000 nm (×10^{8}) in the DMSO group.

Temperature	Time (days)	Size distribution		
		0–250 nm	250–500 nm	500–1000 nm
Fresh	–	4.16±0.28	2.55±0.24	0.14±0.03
25°C	0.5	3.36±0.41	2.84±0.17	0.45±0.15
	1	2.89±0.18	1.48±0.19	0.26±0.08
	3	2.76±0.13	2.84±0.32	0.35±0.14
	5	0.36±0.06	0.52±0.10	0.02±0.02
	7	2.32±0.17	2.19±0.09	0.40±0.16
4°C	0.5	1.36±0.20	2.97±0.44	1.01±0.15
	1	2.65±0.30	0.97±0.04	0.14±0.04
	3	3.56±0.37	3.01±0.21	0.30±0.10
	5	1.62±0.05	1.02±0.12	0.16±0.06
	7	2.48±0.66	1.20±0.04	0.17±0.05
-20°C	0.5	2.84±0.54	2.90±0.14	0.57±0.19
	1	3.36±0.47	1.52±0.14	0.20±0.05
	3	3.52±0.20	2.96±0.13	0.28±0.05
	5	1.36±0.18	0.83±0.13	0.11±0.04
	7	3.26±0.31	2.36±0.46	0.24±0.03
-80°C	0.5	4.56±0.47	3.02±0.35	0.50±0.06
	1	2.39±0.40	0.99±0.31	0.09±0.02
	3	4.24±1.01	3.09±0.25	0.38±0.13
	5	1.42±0.07	0.85±0.10	0.10±0.06
	7	1.83±0.15	1.71±0.38	0.31±0.08
-196°C	0.5	4.84±1.36	3.33±0.67	0.51±0.02
	1	2.95±0.48	1.25±0.38	0.23±0.07
	3	3.99±0.30	2.40±0.20	0.32±0.13
	5	1.45±0.17	1.02±0.16	0.15±0.04
	7	2.53±0.03	1.70±0.17	0.15±0.06

Mean and standard deviation of the distribution of 0–250 nm, 250–500 nm, and 500–1000 nm MPs (×10^{8}) in the DMSO group.

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