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ORIGINAL RESEARCH

Non-Clinical Safety Evaluation of Exosomes Derived from Human Umbilical Cord Mesenchymal Stem Cells in Cynomolgus Monkeys

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Purpose: In recent years, exosomes have been proved to be used to treat many diseases. However, due to the lack of uniform quality control standards for exosomes, the safety of exosomes is still a problem to be solved, especially now more and more exosomes are used in clinical trials, and its non-clinical safety evaluation is particularly important. However, there is no safety evaluation standard for exosomes at present. Therefore, this study will refer to the evaluation criteria of therapeutic biological products, adopt non-human primates to evaluate the non-clinical safety of human umbilical cord mesenchymal stem cell exosomes from the general pharmacology and immunotoxicity, aiming at establishing a safety evaluation system of exosomes and providing reference for the clinical application of exosomes in the future.

Methods: 3.85×10^{12} exosomes derived from human umbilical cord mesenchymal stem cells were injected into cynomolgus monkeys intravenously. The changes of general clinical conditions, hematology, immunoglobulin, Th1/Th2 cytokines, T lymphocytes and B lymphocytes, and immune organs were observed before and within 14 days after injection.

Results: The results showed that exosomes did not have obvious pathological effects on the general clinical conditions, blood, coagulation function, organ coefficient, immunoglobulin, Th1/Th2 cytokines, lymphocytes, major organs, and major immune organs (spleen, thymus, bone marrow) of cynomolgus monkeys. However, the number of granulocyte-macrophage colonies in exosomes group was significantly higher than that in control group.

Conclusion: To sum up, the general pharmacological results and immunotoxicity results showed that the injection of 3.85×10^{12} exosomes may have no obvious adverse reactions to cynomolgus monkeys. This dose of exosomes is relatively safe for treatment, which provides basis research for non-clinical safety evaluation of exosomes and provides reliable research basis for future clinical application of exosomes.

Keywords: exosomes, cynomolgus monkeys, safety, immunotoxicity

Introduction

Exosomes are tiny, membranous vesicles with a diameter of approximately 30–150 nm. Exosomes are surrounded by a lipid bilayer, secreted by most cells, and widely distributed in various body fluids, including serum, plasma, saliva, urine, cerebrospinal fluid, and emulsions. They are involved in physiological and pathological processes and the development of many diseases.[1,2]

Exosomes derived from human umbilical cord mesenchymal stem cells (HUCMSCs) have the potential to treat kidney, liver, ocular, spinal cord, and neurodegenerative diseases. However, with the increasing application of exosomes in disease

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treatment, safety issues need to be paid attention to. At present, there are few studies on the safety of exosomes. Some studies have suggested that the extracellular vesicles derived from mesenchymal stem cells have complex components and may contain potential therapeutic components, such as toxic proteins and harmful miRNAs, and there is a lack of large-scale and multi-center clinical trials to confirm the effectiveness and safety of extracellular vesicles.[3] Another study also shows that the safety of exosomes is still an unsolved problem, and further research in this field, such as clinical research or research using large animal models, still needs to prove the safety of exosomes in treatment, especially the safety of exosomes secreted by cancer cells.[4]

In addition, as the carrier or therapeutic segment of drug delivery, it is necessary to conduct preclinical tests before clinical application. First of all, exosomes have complex biological characteristics and functions, and their pharmacokinetics, action pathway, target and action mechanism in vivo are still unknown.[5] The researchers also pointed out that several key problems need to be solved in the successful transformation of exosomes into clinical use, such as production methods, quantification and characterization, pharmacokinetics and targeting, and safety evaluation.[6] Some researchers also said that it is very important to make a comprehensive analysis of exosomes (including protein, lipids and nucleic acids) and carry out animal toxicology research to predict any unforeseen safety problems.[7] Therefore, before clinical application, it is the key to ensure its safety and effectiveness to study the characteristics of exosomes through preclinical tests. In addition, preclinical trials can also optimize the administration scheme of exocrine drugs. By comparing the effects of different administration methods, doses and frequencies on the efficacy and safety of exocrine drugs, researchers can determine the best administration scheme and provide strong support for clinical trials.

Some researchers believe that exosomes have strong biocompatibility but low immunogenicity.[8,9] To date, experimental data using animal models has shown that exosomes from different sources are safe. The safety of exosome administration has been studied through hemolysis tests, vascular and muscle stimulation tests, hematological indices, and pathological tests, and results have shown that exosomes are well tolerated in animal models.[10] In another study, the toxicological characteristics and safety of adipose tissue-derived exosomes were evaluated using skin sensitization, acute oral toxicity, and photosensitivity tests. The results showed that adipose tissue-derived exosomes were safe as a local treatment, and no adverse reactions occurred in toxicological tests.[11] However, these studies mainly focused on the general toxicological effects of exosomes on the body, whereas few studies have investigated whether exosomes produce immunotoxicity after entering the body.

The immune system plays a key role in maintaining the integrity of organisms. In addition to protecting against pathogens, it participates in the prevention, development, and defense against cancer.[12] The adverse effect of exogenous substances on the immune system, including immunosuppression and immunoenhancement,[13] may lead to damage to normal cells and tissue, known as immunotoxicity. Abnormal immune system reactions play an important role in many diseases and may be the direct cause of a disease or aggravate its progression and the associated damage caused. Over the past few decades, a large number of in vivo and in vitro studies have demonstrated the immunotoxic potential of various nanoparticles.[14] While exosomes are also a type of nanoparticle, their immunotoxic potential remains unclear. Therefore, further studies are needed, especially since exosomes are now an important choice in the treatment of many diseases, to verify their safety and immunotoxicity.

At present, most studies investigating exosome safety have focused on small animals, and research on nonhuman primates is lacking. During drug development, conducting non-clinical safety evaluations is a key part for human risk assessments and clinical development support, and the appropriate selection of animals for toxicity testing is important.[15] Non-human primate experimental animals are one of the most important experimental animal species in the field of life science research. Monkeys are the closest genetic species to humans, and experimental monkeys have become an indispensable resource for life science and biomedical research and development. Monkeys, particularly cynomolgus monkeys, are considered the only relevant species for use in biopharmaceutical safety assessments.[16] Their use in verifying the safety of exosomes in non-human primates will assist in promoting the clinical application of exosomes.

However, there is no internationally recognized guide to define exosomes and their clinical applications, and there is no standard for evaluating the safety of exosomes. At present, there is still controversy about exosomes. Some people think it is a medical product, while others think it is a biological product similar to maternal cells.[17] In a study, researchers thought that exosomes might be a ready-made biological product.[18] Therefore, we refer to the non-clinical safety evaluation standard of therapeutic biological products to evaluate the safety of exosomes. In this study, we used

cynomolgus monkeys to evaluate the safety of exosomes derived from HUCMSCs according to the requirements of non-clinical pharmacological and toxicological research of therapeutic biological products and to provide research basis for clinical study of exosomes in the future.

Materials and Methods

Extraction, Identification and Quality Study of Exosomes

HUCMSCs-derived exosomes were provided by Professor Wang Yue's research group from the Research Center of Translational Medicine, Naval Medical University, Shanghai, China. Exosomes were identified using Nanoparticle Tracking Analysis (NTA) (ZetaView, Particle Metrix, Germany) to measure their concentration and particle size, and a transmission electron microscope (TEM) (HT-7700, Hitachi High-tech Company, Japan) was used to observe their shape and size. Western blotting was performed to detect the surface marker proteins CD9 (Proteintech, 20,597-1-AP), Tsg101 (Proteintech, 28,283-1-AP), and Calnexin (Proteintech, 10,427-2-AP) in the exosomes.

The exosomes were sequenced in the early stage by Professor Wang Yue's research group. We used the dataset GSE159814 and protein levels which provided by professor Wang Yue to analyze the characteristics of exosomes.

Animal Treatment

Six male cynomolgus monkeys were provided by Guangxi Xiongshen Primate Experimental Animal Breeding and Development Co., Ltd. (SCXK Gui 2021–0004). They were conventional animals aged between 4 and 10 years and weighed 4.73 ± 1.39 kg. The monkeys were raised at Thinxon Biomedical Co., Ltd. (SYXK Gui 2020–0008). The animal room environment was controlled at a temperature of 18–28 °C, relative humidity of 40%–70%, and 12 h of light and 12 h of darkness. Except for the fasting time, they were fed once in the morning and once in the afternoon, with an average of approximately 80 g of feed per animal per feeding, an additional 60 g of fruit per day, and free access to water. The monkey feed was provided by Beijing Keao Xieli Feed Co., Ltd., and the drinking water met the national urban living drinking water hygiene standards.

Six cynomolgus monkeys were randomly divided into control and exosome groups, with three animals in each group. Each cynomolgus monkey in the exosome group was injected with 3.85×10^{12} particles in 10 mL 1X phosphate-buffered saline (PBS). In the control group, 1X PBS (10 mL) was intravenously injected into each cynomolgus monkey. The injection speed was set to 2 mL/min. The day of injection was recorded as day 0, and after 14 days of observation, the cynomolgus monkeys were euthanized.

Behavioral Score

During the observation period, the cynomolgus monkeys were observed once every morning and evening to determine their mental state, behavioral activities, respiratory status, nervous system function, and whether they had died. Behavioral videos of cynomolgus monkeys were recorded at baseline, day 1, day 7 and day 14; they were scored by two staff members. Behavioral scoring was carried out according to a refined and modified Kito's improved rating scale,[19] where the behavioral performance of the monkeys was observed while they moved freely and without intervention in the behavior cage and when their body and face were touched.

General Clinical Observation

During the observation period, the blood pressure, pulse, heart rate, and respiration of the cynomolgus monkeys were measured daily. The animals were fixed in a monkey chair, and after standing for 10 min, an animal multiparameter monitor (vet-12v, Sinnor Instruments Inc.) was connected to measure blood pressure, pulse, heart rate, and respiration, and the values were read and recorded. An electronic thermometer was used to measure the inner thigh body temperature of each animal, in a region where there was comparatively less fur. The weight and electrocardiogram results of the cynomolgus monkeys were recorded at baseline, day 1, day 7, and day 14. The electrocardiogram examination method proceeded as follows: cynomolgus monkeys were fasted for more than 16 h and then atropine sulfate (0.1mL/kg, IM) and

Zoletil 50 (0.04mL/kg, IM) were used to induce anesthesia. Subsequently, a digital twelve-channel electrocardiograph (ECG-1210, Shenzhen Biocare Bio-Medical Equipment Co., Ltd.) was employed to record heart activity.

Routine Examination of Blood and Cerebrospinal Fluid

After fasting for 16 h, the cynomolgus monkeys were anesthetized, and blood samples were collected from the superficial veins of the limbs to examine routine blood, blood biochemistry, and coagulation function. The routine blood tests included white blood cell count (WBC), red blood cell count (RBC), hematocrit (HCT), hemoglobin (HGB), platelet count (PLT), platelet hematocrit (PCT), lymphocyte count (LYM#), % of lymphocytes (LYM%), mean amount of red blood cell hemoglobin (MCH), mean hemoglobin concentration of red blood cells (MCHC), mean red blood cell volume (MCV), mean platelet volume (MPV), monocyte count (MON#), % of monocytes (MON%), red blood cell volume distribution width (RDW), and platelet volume distribution width (PDW). Blood biochemistry tests included glucose (GLU), urea (UREA), creatinine (CREA), cholesterol (CHOL), triglycerides (TG), total bilirubin (TBIL), total protein (TP), lactate dehydrogenase (LDH), creatine kinase (CK), and glutaminase/alanine aminotransferase (AST/ALT) levels. Cerebrospinal fluid was collected under aseptic conditions through the subarachnoid space using a disposable puncture needle to conduct routine biochemical evaluation. Cerebrospinal fluid biochemistry included glucose (GLU), chlorine (CL), and total protein in the cerebrospinal fluid (CSFTP), while the coagulation function included plasma prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), prothrombin activity (PTA) and fibrinogen (FIB). All items were collected at baseline, day 1, day 7, and day 14.

Immunoglobulin and Th1/Th2 Detection

Blood samples were collected from the superficial veins of the limbs at baseline, day 1, day 7, and day 14. After the samples were maintained at room temperature for approximately 1 h, they were centrifuged at 4 °C for 15 min at 3000 rpm, and the supernatant was extracted for later use. The IFN-γ (Laibio, JL11054), IL-6 (Laibio, JL21801), IL-10 (Laibio, JL21797), IgA (Elabscience, E-EL-MK0397c), IgG (Mlbio, ml023342V), and IgM (Laibio, JL13817) contents in serum were detected using ELISA. Before the experiment, the kit was placed at room temperature for 20–30 min and detection were conducted in accordance with the manufacturer's instructions. Each sample was provided with two wells in 96-well plate, and the OD value at 450 nm was detected using a microplate reader (BioTek, USA).

Analysis of Lymphocyte Phenotype in Peripheral Blood, Spleen, and Thymus

Blood was collected from the superficial veins of the limbs at baseline, day 1, day 7, and day 14. The fresh spleen and thymus of cynomolgus monkeys were collected to prepare a single-cell suspension on day 14. The phenotypes of T- and B-lymphocytes present in the peripheral blood, spleen, and thymus were detected using flow cytometry (CytoFLEX S, Beckman Coulter). The antibodies used for flow cytometry were anti-CD45 (BD Biosciences, 558,411), anti-CD3 (BD Biosciences, 557,749), anti-CD4 (BD Biosciences, 550,628), anti-CD8 (BD Biosciences, 561,421), and anti-CD20 (BioLegend, 302,306).

Lymphocyte Proliferation Assay

At day 14, sterile spleen was obtained as previously described, and a single-cell suspension was prepared in 1640 complete medium. The cell concentration was adjusted to 2×10^6 cells/mL, 1 mL of cell suspension was inoculated into each well of a 24-well plate, and 75 μL ConA (100 μg/mL) (Sigma, C2010) and LPS (400 μg/mL) (Solarbio, L8880) were added to the designated wells, while the control wells received the same volume of 1X PBS. The plates were then incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 68 h. 100 μL of Cell Counting Kit-8 solution (Uelandy, C6005M) was then added to each well, and the OD at 450 nm was detected after incubating for 3 h.

Detection of Granulocyte-Macrophage Colony Forming Unit

On day 14, the bone marrow from the hip bone was collected to prepare a single-cell suspension, and a certain volume of erythrocyte lysate was added for 5 min. The cell density was then adjusted to 4×10^5 cells/mL, then out the next experiment according to the protocols of MethoCult medium (MethoCult™ H4034, StemCell Technologies,

Vancouver, BC, Canada). Finally, two covered 35 mm Petri dishes with the cell suspension and one uncovered Petri dish with 3 mL sterile water were placed into a 150 mm covered Petri dish, which was placed in an incubator at 37°C, with 5% CO_2 and 95% humidity, for 14 days. On day 14, CFU-granulocyte-macrophage (CFU-GM) and burst-forming unit erythroid (BFU-E) cell colonies were counted under a microscope.

Bone Marrow Smear Examination

Following bone marrow extraction from the hip bone as previously described, a drop was placed on a glass slide and smeared. After Wright's staining, bone marrow cells were observed under an oil mirror microscope.

HE Staining

Following the observation period, the subjects were euthanized, and the main organs (heart, liver, spleen, lung, kidney, brain, testis, and spinal cord) were HE stained.

Immunohistochemical and TUNEL Staining

Following euthanasia, the harvested spleens were embedded in paraffin and cut into paraffin sections, followed by incubation with anti-CD4 (Novus, NBP2-52,663, 1:100) and anti-CD8 (GeneTex, GTX74773, 1:50) antibodies. The images were then observed under a microscope, and photographs were taken.

Primary and secondary antibodies were added to the embedded, sectioned spleen and incubated. DAPI was used to counterstain the nucleus, and the sections were finally observed and imaged using a fluorescent microscope (MF43-N, Guangzhou Mingmei Photoelectricity Technology Co., Ltd.).

Statistical Analysis

SPSS 26.0 software (IBM Corp., Armonk, NY, USA) was used to conduct statistical analyses. GraphPad Prism 8 (Graphpad software, USA) was used to draw the graphs. Data are expressed in mean ± SD. Normally distributed data were analyzed using an independent sample *t*-test, and the one-way analysis of variance (ANOVA) was used to compare the results among the three groups. Statistical significance was defined as $P < 0.05$.

Results

Characteristics and Content Determination of Exosomes

NTA showed that the exosomes had a mean particle size of 100.3 nm, which was consistent with a previously reported size range, and the particle concentration was 8.9E+11 particles/mL (Figure 1A). Western blotting showed positive CD9 and Tsg101 expression, which were the typical exosome protein markers, but negative Calnexin expression (Figure 1B). The typical exosome cup-shaped structure was observed by TEM (Figure 1C).

Through the results of miRNA sequencing and proteomics sequencing of exosomes, we found that there are 546 kinds of miRNA mainly expressed in our exosomes (Figure 1D), among which miR-21-5p, miR-24-3p and miR-22-3p on the top 3 (Figure 1E). In addition, 3179 proteins were detected in exosomes, and the top 1% proteins were expressed as shown in Figure 1F. Among them, the three highest contents are Albumin (ALB), Apolipoprotein B (APOB) and Immunoglobulin heavy constant mu (IGHM).

Behavioral Score Results

During the observation period, all monkeys were breathing regularly and moving normally. They also appeared to be in a good mental state, and there were no obvious abnormal reactions or deaths. There was no evidence of redness, swelling, or rupture at the site of administration. The nerve function scores of both groups of cynomolgus monkeys were zero, indicating no nerve function damage (Table 1).

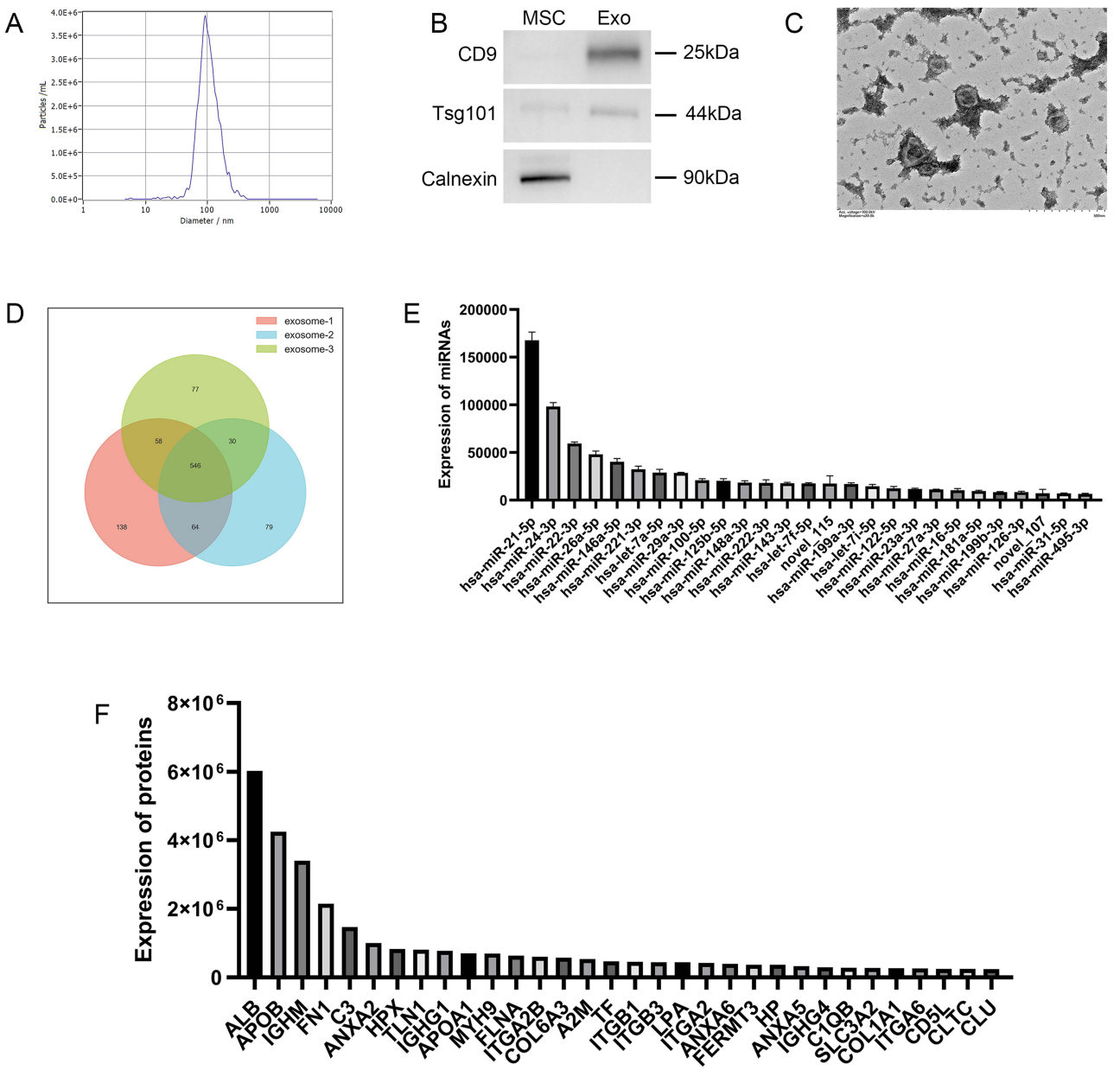

Figure 1 Characteristics and content determination of exosomes.
Notes: (**A**) Size distributions of exosomes derived from HUCMSCs by NTA. (**B**) Identification of exosome marker proteins. (**C**) Representative images of exosomes by TEM. Scale bar = 500 nm. (**D**) Venn diagram for three samples in GSE159814. (**E**) The expression of miRNA in the overlapping part accounts for the top 5%. (**F**) The expression of proteins in exosomes on the top 1%.

General Clinical Features and Pathological Changes

There was no significant difference between the body weight of two groups (Figure 2A, $P > 0.05$). However, at day 14, there was a significant difference in body temperature between the two groups ($P = 0.016$, $P < 0.05$), but this remained within a normal body temperature range (Figure 2B). Besides, there were no significant differences between the pulse rate and respiratory rate (Figure 2C and D, $P > 0.05$). There were significant differences between the systolic blood pressure ($P = 0.044$, $P < 0.05$) of the two groups on day 9 and diastolic blood pressure ($P = 0.026$, $P < 0.05$) on day 14 (Figure 2E and F). However, there were no significant differences between the two indexes at rest. The ECG results showed no significant differences between the heart rate, P-R interval, QRS duration, and Q-T interval of the two groups (Figure 2G, $P > 0.05$).

Table 1 Neurological Function Scores of Cynomolgus Monkeys

Group	Observation Time	Total Score	Consciousness (0–28 Scores)	Musculoskeletal Coordination (0–18 Scores)	Sensory System (0–22 Scores)	Motion System (0–32 Scores)
Control	baseline	0	0	0	0	0
	day 1	0	0	0	0	0
	day 7	0	0	0	0	0
	day 14	0	0	0	0	0
Exosome	baseline	0	0	0	0	0
	day 1	0	0	0	0	0
	day 7	0	0	0	0	0
	day 14	0	0	0	0	0

Following euthanasia, the main organs were weighed and organ coefficients were calculated. The data showed that there were no significant differences between the organ coefficients of the two groups' main organs (Figure 2H). In addition, HE staining revealed no obvious pathological abnormalities or inflammatory cell infiltration in either group (Figure 2I).

Effects of Exosomes on Blood and Cerebrospinal Fluid

The routine blood test results showed that significant differences were found at baseline in BAS% ($P = 0.024$, $P < 0.05$), # BASO ($P = 0.016$, $P < 0.05$), and MON% ($P = 0.037$, $P < 0.05$) (Figure 3A). However, there was no significant difference between the two groups on day 1 and day 7 (Figure 3B and C); and there was a significant difference between the # BASO ($P = 0.035$, $P < 0.05$), MON% ($P = 0.020$, $P < 0.05$), and MON# ($P = 0.002$, $P < 0.05$) of the two groups on day 14 (Figure 3D).

Blood biochemical analysis showed that the LDH levels of the exosomes group were significantly higher than those of the control group at baseline (Figure 3E, $P = 0.038$, $P < 0.05$). However, there was no significant difference between the index at day 1 and day 7 (Figure 3F and G), but LDH levels ($P = 0.002$, $P < 0.01$) and GLU ($P = 0.018$, $P < 0.05$) were significantly different from those of the control group on day 14 (Figure 3H).

There was no significant difference between the cerebrospinal fluid biochemistry of the two groups at different time points (Figure 4A-D). The coagulation function results showed that there were no significant differences between the prothrombin time (PT), prothrombin time (TT), prothrombin activity (PTA), and fibrinogen (FIB) of the two groups at different time points (Figure 4E and F), but the activated partial thromboplastin time (APTT) of the exosome group was significantly higher than that of the control group (Figure 4G and H) at day 7 ($P = 0.026$, $P < 0.05$) and day 14 ($P = 0.006$, $P < 0.01$).

The ELISA results showed that there were no significant differences in the serum levels of IgA (Figure 5A), IgG (Figure 5B) between two groups. There was a significant difference between the IgM levels at baseline ($P = 0.041$, $P < 0.05$) and on day 1 ($P = 0.021$, $P < 0.05$); however, this difference was no longer apparent on day 7 and day 14 (Figure 5C). For Th1/Th2 cytokines, there was no significant different in INF-γ (Figure 5D), IL-6 (Figure 5E), and IL-10 (Figure 5F) between the two groups.

Effects of Exosomes on Lymphocyte Phenotypes in Peripheral Blood, Spleen, and Thymus

There were no significant differences between the percentages of B- and T-lymphocytes in the peripheral blood of the two groups (Figure 6A and B), and there were no significant differences between the $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, or the $CD4^+/CD8^+$ ratio of the two groups (Figure 6C-E). In addition, the phenotypic results of T- and B-lymphocytes in the spleen (Figure 6F) and thymus (Figure 6G) showed no significant differences between $CD3^+$, $CD4^+$, $CD8^+$ T lymphocytes, $CD4^+/CD8^+$ and $CD20^+$B lymphocyte levels of the two groups.

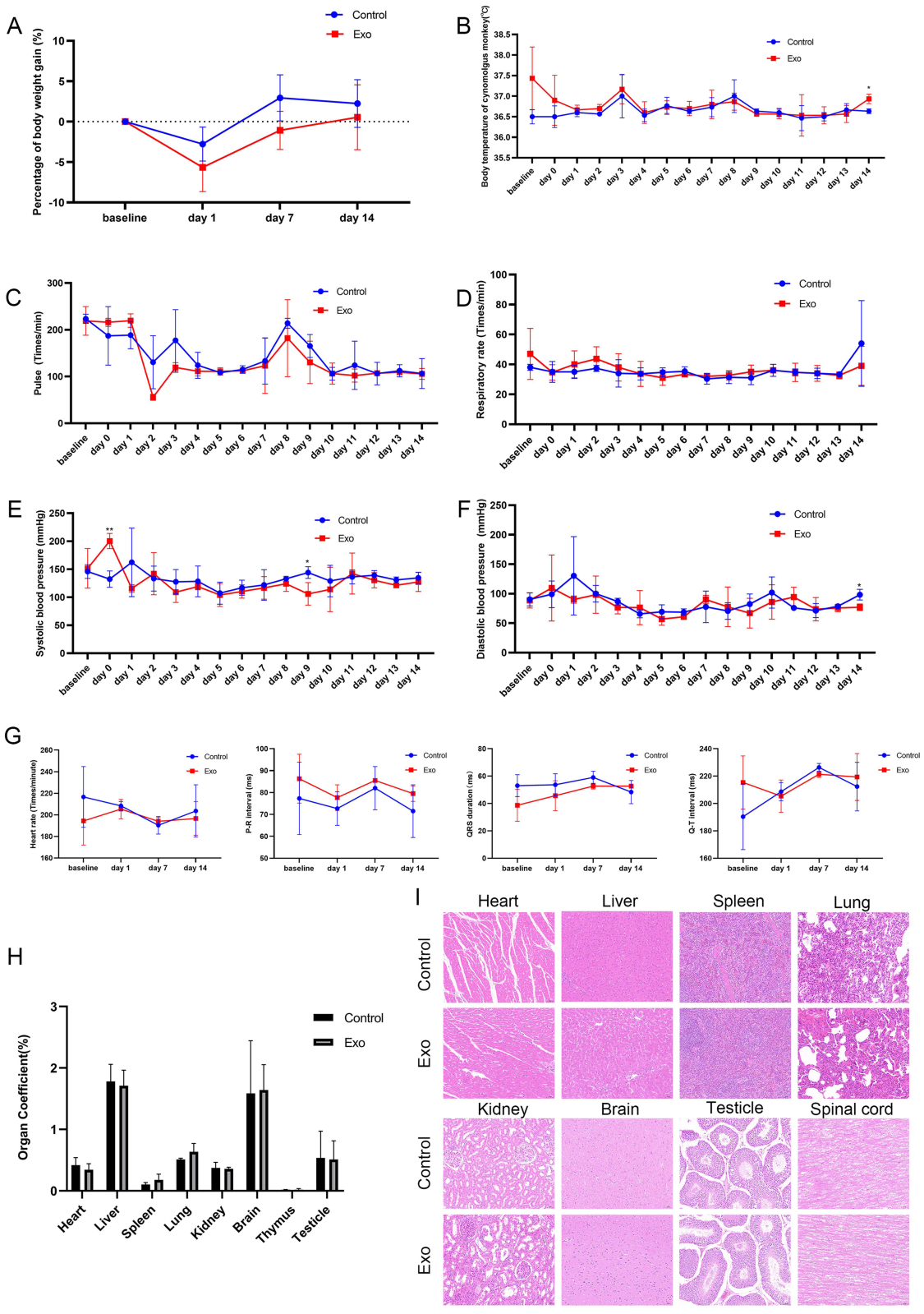

Figure 2 General clinical features and pathological changes.

Notes: (**A**) Percentage of body weight gained. (**B**) Body temperature. (**C**) Pulse. (**D**) Respiratory rate. (**E**) Systolic blood pressure. (**F**) Diastolic blood pressure. (**G**) Electrocardiogram changes. *$P < 0.05$ and **$P < 0.01$ compared with control group. (**H**) Organ coefficient. (**I**) Representative graphs of HE staining results of main organs of cynomolgus monkey. Scale bar = 50 μm. n = 3 monkeys.

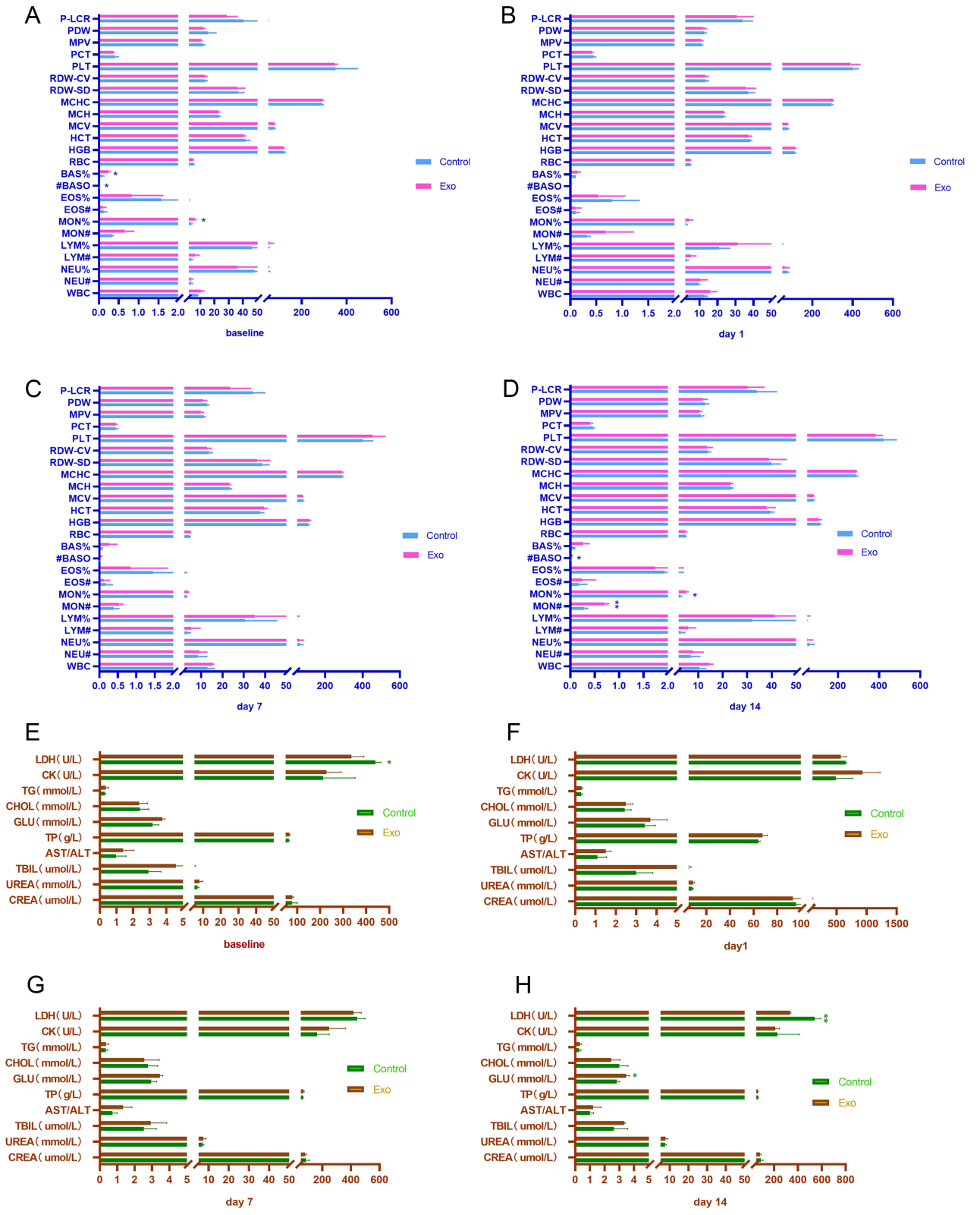

Figure 3 Routine blood routine tests and blood biochemistry results of cynomolgus monkeys at different time points.
Notes: (**A-D**) Routine blood test. (**E-H**) Blood biochemistry. n = 3 monkeys. *P < 0.05 and **P <0.01 compared with control group.

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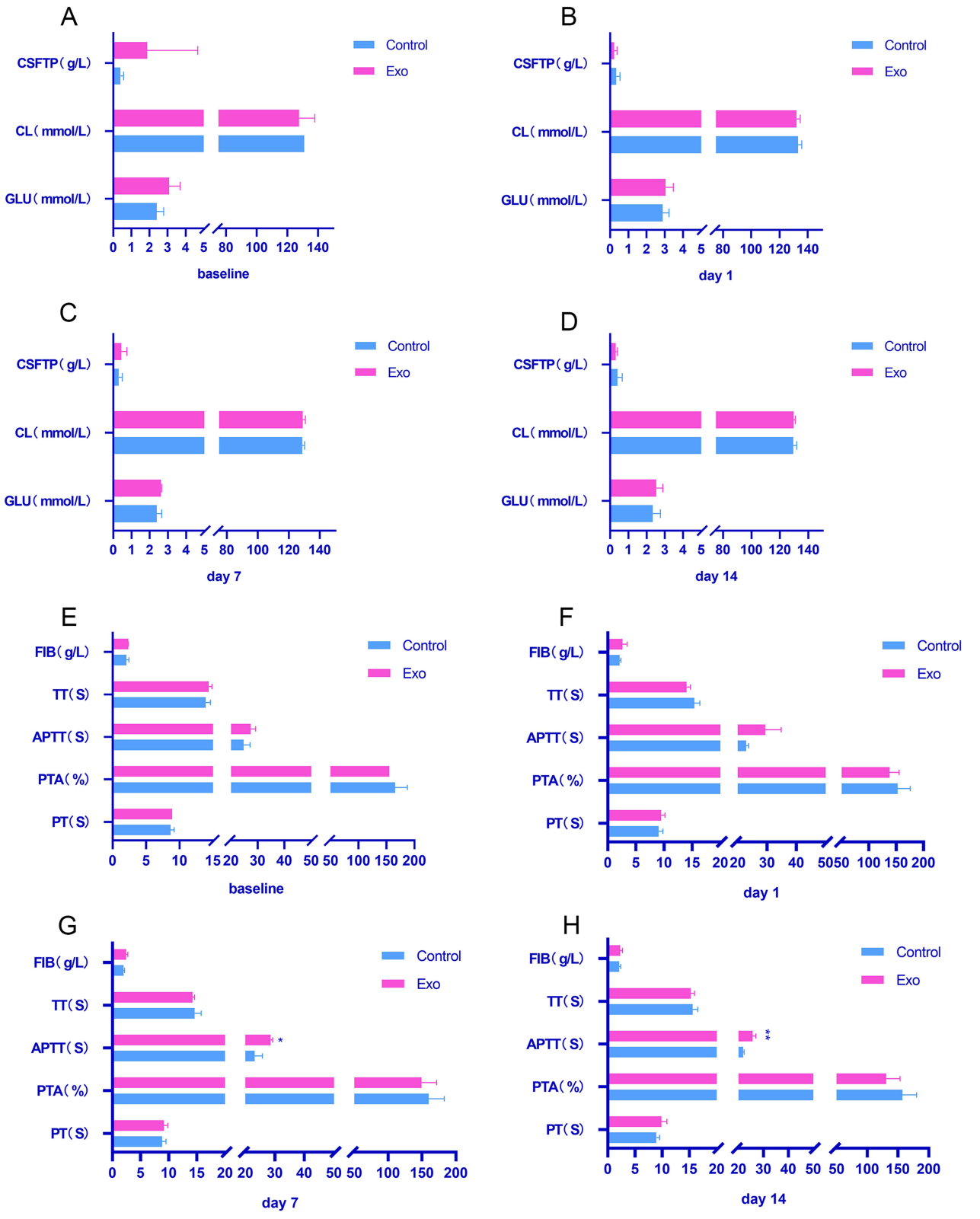

Figure 4 Cerebrospinal fluid biochemistry and coagulation function tests.
Notes: (**A-D**) Results of cerebrospinal fluid biochemistry. (**E-H**) Results of coagulation function. n = 3 monkeys. *$P < 0.05$ and **$P < 0.01$ compared with control group.

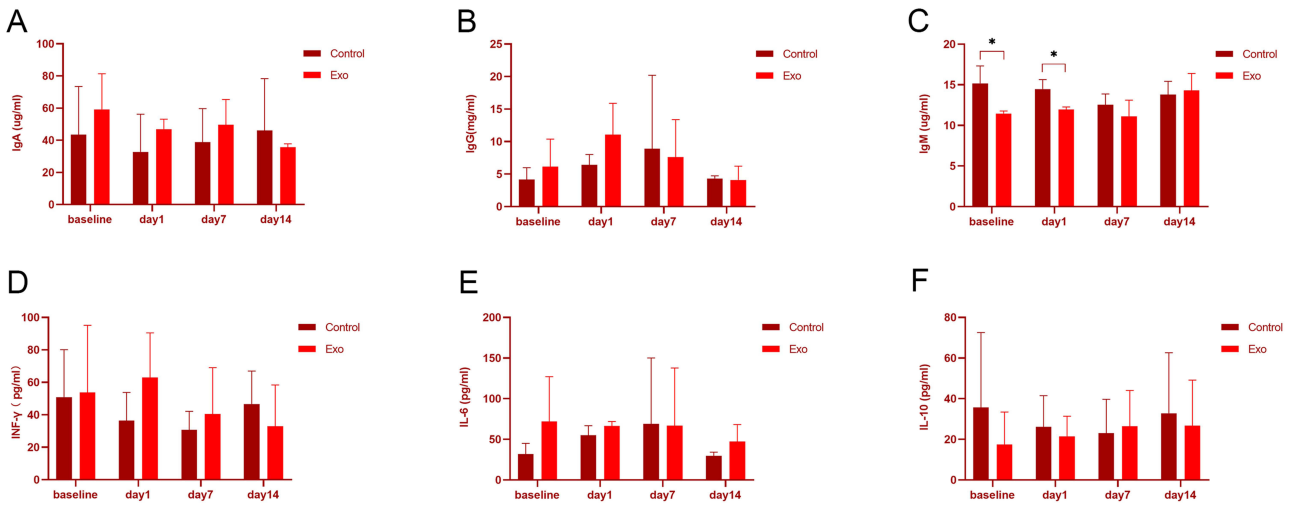

Figure 5 Levels of immunoglobulin and Th1/Th2 cytokines in serum.
Notes: (**A**) IgA. (**B**) IgG. (**C**) IgM. (**D**) INF-γ. (**E**) IL-6. (**F**) IL-10. n = 3 monkeys. *$P < 0.05$ compared with control group.

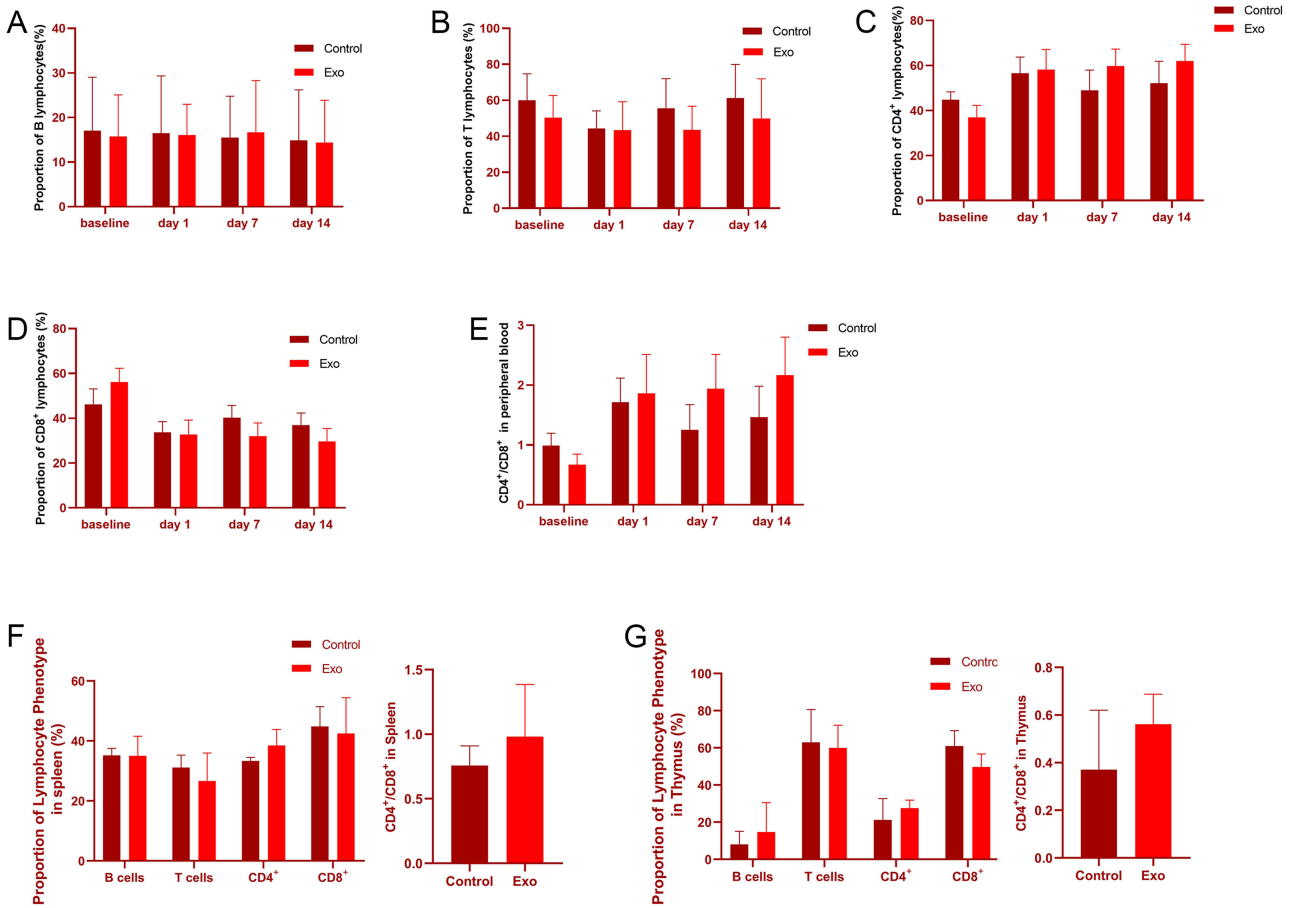

Figure 6 Detection of T lymphocytes and B lymphocyte phenotypes in peripheral blood, spleen and thymus by flow cytometry.
Notes: (**A**) Phenotypic statistics of B lymphocytes in blood. (**B**) Phenotypic statistics of T lymphocytes in blood. (**C**) Statistical charts of $CD4^+$T lymphocytes in blood. (**D**) Statistical charts of $CD8^+$T lymphocytes in blood. (**E**) Statistical chart of $CD4^+/CD8^+$ ratio in blood. (**F**) Phenotypic statistics of T lymphocytes and B lymphocytes in spleen. (**G**) Phenotypic statistics of T lymphocytes and B lymphocytes in thymus. n = 3 monkeys.

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Effect of Exosomes on Main Immune Organs

The results from the granulocyte-macrophage colony formation experiment showed that the CFU-GM colony growth could be observed under a microscope (Figure 7A). The average numbers of CFU-GM and BFU-E colonies in each 35 mm Petri dish in the exosome group were 13.55 and 7.22, respectively, which were significantly higher than those in the control group (5.22 ($P = 0.046$, $P < 0.05$) and 1.28 ($P = 0.018$, $P < 0.05$), respectively) (Figure 7B).

The results of the spleen proliferation test showed that there were no significant differences between the stimulation index of spleen cells between the two groups (Figure 7C), which suggested that the exosomes did not significantly affect the proliferation of splenic T- and B-lymphocytes induced by ConA and LPS.

The bone marrow smear results showed that the lymphatic and bone marrow monocyte systems in the two groups were normal. There were no obvious abnormalities in lymphocyte morphology, and there were no obvious abnormalities in the volume, shape, and differentiation of red blood cells. There were also no obvious abnormalities in the granulocyte morphology or staining (Figure 7D).

The CD4 and CD8 expression results showed that there were no significant differences between the CD4 and CD8 expression in the spleen of the two groups. These findings were consistent with the flow cytometry data, indicating that exosomes had no obvious effect on the spleens of cynomolgus monkeys (Figure 7E and F). TUNEL staining was used to evaluate whether exosomes affected apoptosis in immune organs and the results showed that, compared to the control group, there was no significant difference in the apoptosis ratio of spleen cells in the exosome group, suggesting that exosomes did not cause apoptosis in spleen cells (Figure 7G and H).

Discussion

With more and more exosomes being used in the treatment of diseases, its safety has been paid more and more attention. As a potential treatment, it is very necessary to evaluate the non-clinical safety of exosomes. However, at present, the research on the safety of exosomes is mostly concentrated in mice or rats, and the research results on these animals are difficult to be popularized and applied to humans. Non-human primates, as the animals closest to humans, study the safety of exosomes on them, and the results can be better popularized and applied to humans. Therefore, in this study, we evaluated the safety of exosomes in healthy cynomolgus monkeys with a therapeutic dose higher than that in order to provide scientific basis for the future clinical application of exosomes. According to the requirements of establishing the quality standard of chemical drugs, it is very necessary to establish the quality standard of drugs, and content determination is a part of quality control. Because there is no uniform quality control standard for exosomes at present, in our study, we statistically analyzed the results of the miRNAs and proteins detected by Professor Wang Yue and his team to determine the distribution of miRNAs and proteins within exosomes and to provide a reference basis for the quality control of exosomes in clinical applications. In a pre-experiment conducted by our research group, C57BL/6J mice were injected with 6×10^{10} exosome particles, which was approximately 10 times the therapeutic dose.[20] At this dose, no abnormalities were observed in the mice. Therefore, in this study, we referred to the formula in Experimental Zoology and converted the dose for cynomolgus monkey to 3.85×10^{12} particles according to the body surface area. Following intravenous injection and 14 days of observation, no obvious behavioral changes, abnormal neurological functions, or deaths were reported. In addition, there were no abnormal changes in pulse, heart rate, or electrocardiogram of toxic significance in either the exosome or the control group. Although the weight of the both groups of cynomolgus monkeys decreased on the first day after administration, there was no significant difference between the two groups, and the loss of weight was considered to be a stress reaction caused by injection. In addition, significant differences were observed at times between the body temperature and systolic blood pressure of the two groups. However, our results are consistent with the previous research reports.[21] Therefore, these changes were not considered to be adverse reactions caused by the interventions, and they were likely attributed to normal fluctuations. Although there are significant differences in individual indexes between two groups, these indexes are consistent with the previous results of healthy cynomolgus monkeys,[22,23] and it was considered that the exosomes may not have obvious effect on the blood of cynomolgus monkeys.

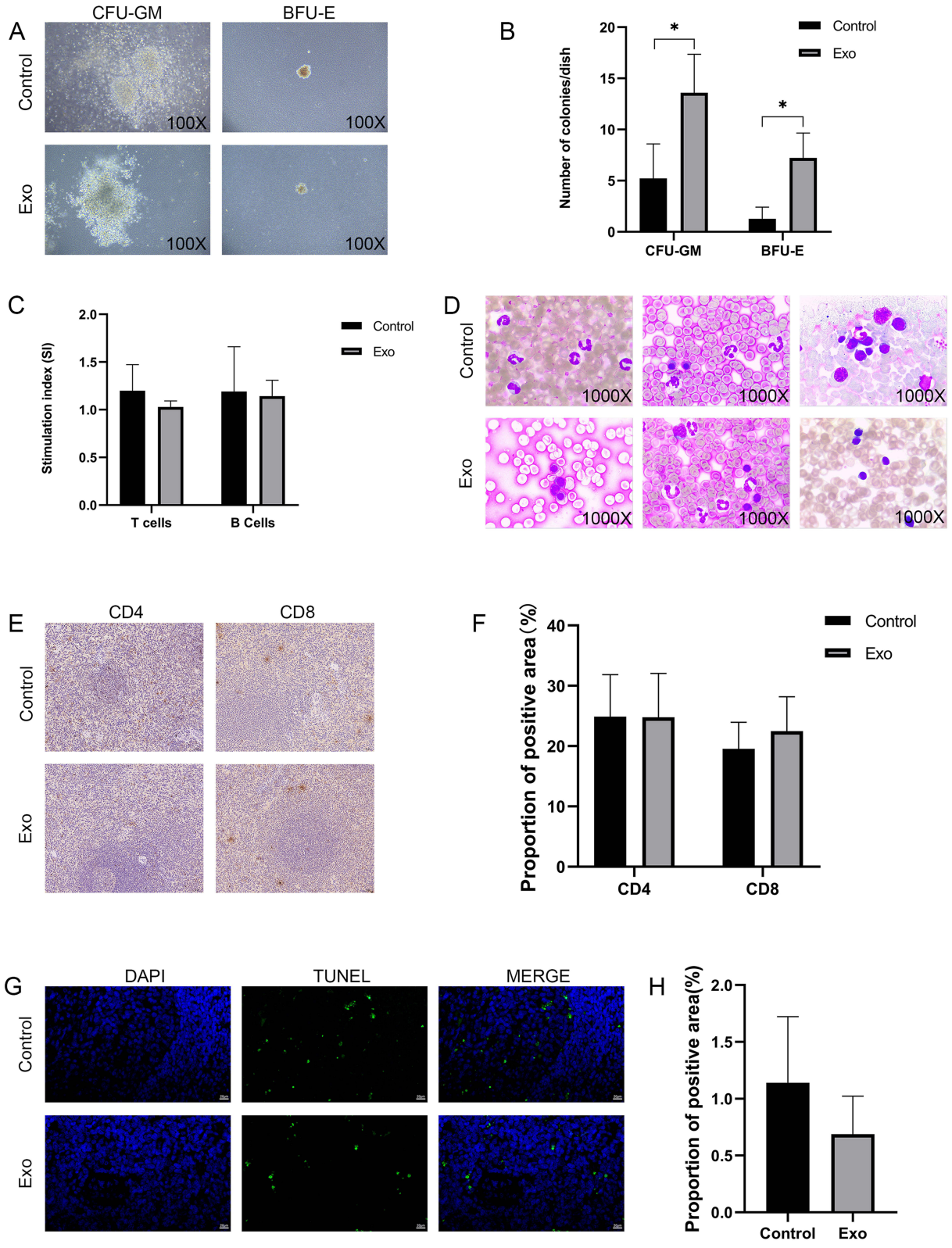

Figure 7 Effects of exosomes on main immune organs.

Notes: (**A**) Representative graphs of CFU-GM and BFU-E colonies in bone marrow of cynomolgus monkey. (**B**) CFU-GM and BFU-E colony charts in bone marrow. *$P < 0.05$ compared with control group. (**C**) Stimulation index of T and B lymphocytes induced by ConA and LPS. (**D**) Representative graphs of bone marrow cell smear results of cynomolgus monkey. (**E**) Immunohistochemical representative graphs of CD4 and CD8. Scale bar = 50 μm. (**F**) Statistical chart showing proportion of positive areas in CD4 and CD8. (**G**) Representative graphs of TUNEL analysis in spleen. Scale bar = 20 μm. (**H**) Statistical chart of TUNEL positive area percentage. n = 3 monkeys.

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APTT, TT, and PT are routinely measured to assess blood coagulation in toxicity studies of anticoagulants.[24,25] The results of the coagulation tests conducted here showed no significant change between the coagulation function of the two groups. Although the APTT of the exosome group was significantly higher than that of the control group on day 7 and day 14, there was no significant difference between the two groups before and after each time point. This may be because the initial APTT of exosome group is slightly higher than that of control group, so once it fluctuates, it is likely to lead to significant differences between the two groups. Besides, the results of APTT are basically consistent with those of other researchers.[26] Moreover, the main organs of the cynomolgus monkeys were weighed prior to conducting any histopathological studies, and no abnormalities were found in the main organs such as heart, liver, spleen, lung, kidney, or brain. In summary, the exosomes may not have a general toxic effect on the cynomolgus monkeys.

Studying the effects of certain types of drugs on the nature and severity of immune responses is essential in non-clinical safety studies. Lymphocyte subsets and immunoglobulin levels in peripheral blood are important parameters for characterizing cellular and humoral immunity.[27] Cytokines play an important role in the regulation of immune system function and are the link between the immune and other systems.[28,29] Several studies have demonstrated that drug immunotoxicity is associated with the expression of certain cytokines; therefore, we measured the levels of immunoglobulin and Th1/Th2 cytokines in the peripheral blood of cynomolgus monkeys at baseline, day 1, day 7, and day 14 and measured the proportions of T lymphocytes and B lymphocytes in the blood using flow cytometry. No significant difference was observed between the expression of IgA, IgG, and Th1/Th2 cytokines in the control and experimental groups. However, there was a significant difference between the IgM levels in the two groups at baseline and on day 1; however, over time, the IgM levels no longer differed significantly between the two groups. Considering that the IgM of cynomolgus monkeys was different initially, we believe that the exosomes did not have an adverse effect on the immunoglobulin and Th1/Th2 cytokines in cynomolgus monkeys, and the difference was not caused by exosome intervention. In addition, the immunoglobulin contents determined in our study were not consistent with those reported by Voloshina et al,[27] this discrepancy may relate to differences in the detection methods, animal sources, or environments employed, all of which may affect the immune parameters of animals.[27]

The percentage of peripheral blood lymphocyte subsets in healthy monkeys depends on their sex, age and geographical origin, and immunoglobulin levels may vary significantly according to the analytical method.[30–32] The lymphocyte subset count has been used in preclinical toxicological studies to evaluate the effects of immunosuppressants in rats,[33] and lymphocyte subsets have also been proven to be a reliable endpoint of immunotoxicity in mice.[34] Therefore, we used flow cytometry to detect lymphocyte subsets in the peripheral blood. In our study, the detection results of $CD3^+$ and $CD8^+$ cells in the peripheral blood of cynomolgus monkeys were consistent with these values, and the proportion of $CD20^+$ B lymphocyte subsets was consistent with the results obtained by Voloshina et al[27] $CD3^+$, $CD4^+$, and $CD8^+$ values in the peripheral blood were also consistent with those determined by Zitsman et al,[35] and the ratio of $CD4^+/CD8^+$ was consistent with those reported by Verdier et al.[36] In addition, lymphocyte subsets were detected in the spleen and thymus. We found that exosomes had no obvious effect on T or B lymphocytes in the spleen or on thymocytes. The percentages of $CD3^+$, $CD4^+$, and $CD8^+$ T lymphocytes in the spleen were consistent with those reported by Zitsman et al,[35] but the percentages of T lymphocytes in the thymus were different. It is possible that the cynomolgus monkeys in their study were older and the thymus had gradually shrunk with age, which could have resulted in decreased lymphocytes and an increase in adipose tissue. The cynomolgus monkeys in our study were younger, possibly explaining why the thymus T lymphocytes in our study were higher. In addition, in our study, the percentage of $CD4^+$ lymphocytes in the spleen and thymus of the exosome group were slightly higher than that of the control group, the percentage of $CD8^+$ T lymphocytes was slightly lower than that of the control group, the ratio of $CD4^+/CD8^+$ lymphocytes were higher than that of the control group, and the ratios of $CD4^+$, $CD8^+$, and $CD4^+/CD8^+$ in the peripheral blood of the exosome group also presented the same trend. Although there was no statistically significant difference between the two groups, this may suggest that exosomes could improve immune function. Our results for B lymphocytes were consistent with those of Zitsman et al.[35]

To further study the immunotoxicity of the exosomes, we measured the proliferation of spleen cells, the expression of CD4 and CD8 proteins in spleen tissues, and the apoptosis of spleen cells. We used LPS and ConA to induce the proliferation of T and B lymphocytes in the spleen and found that exosomes did not affect their proliferation ability. In addition, there were no significant changes in the expression levels of CD4 and CD8 proteins in the spleen tissue. Apoptosis plays a key role in the immune response and regulates the number and mode of action of lymphocytes. Therefore, we evaluated apoptosis of spleen cells in this study, and the results showed no significant difference in the percentage of TUNEL-positive cells in the spleen

tissue between the two groups. However, the percentage of apoptotic cells in the exosome-treated group was slightly lower than in the control group, suggesting that exosomes may be involved in inhibiting the apoptosis of spleen cells. Zhang et al showed that exosomes can inhibit cell apoptosis;[37] however, there was no significant difference between the groups in our study, and this could be due to the large individual differences and the small sample size of the cynomolgus monkeys used, which could have led to differences between our results and those of previous studies.

The assessment of bone marrow toxicity provides a broad measure of the potential effects of chemicals on the growth and development of immune cells, since all immune-related cells are developed from pluripotent hematopoietic stem cells in the bone marrow. Therefore, in this study, the bone marrow cells of cynomolgus monkeys were used to conduct colony-forming experiments on granulocyte-macrophages and were stained using Wright's staining. Examination with a microscope showed no obvious abnormalities in the bone marrow morphology between the two groups.

Bone marrow suppression is a common dose-limiting toxicity. When used in vivo, nanoparticles may distribute in bone marrow and release drugs into bone marrow. Therefore, detecting the potential toxicity of nanoparticles or drugs carried by nanoparticles is an important aspect of preclinical safety assessments. Bone marrow suppression can be determined by the quantitative detection of granulocyte-macrophage colony-forming units in the bone marrow. In our study, we observed that there were approximately five CFU-GM colonies in each 35 mm Petri dish in the control group, and this result was consistent with Goto et al.[38] Interestingly, our study found that the number of granulocyte-macrophage colonies in each 35 mm Petri dish in the exosome group was significantly higher than that in the control group, suggesting that exosomes did not inhibit bone marrow cells and might instead promote the growth of granulocytes and macrophages. These results show that it is safe to infuse exosomes into healthy cynomolgus monkeys with normal immune functions under experimental conditions.

Conclusion

In conclusion, in the present study, we evaluated the safety of exosomes with reference to the safety evaluation standards of therapeutic biologics, and the general pharmacological results and immunotoxicity results showed that the injection of this dose of exosomes did not produce obvious adverse effects on the cynomolgus monkeys. The results showed that this dose of exosome was relatively safe for therapeutic use, which provided a basis for the non-clinical safety evaluation of exosome, filled the blank of safety evaluation of exosome, and laid a foundation for the future clinical application of exosome.

Data Sharing Statement

The datasets generated and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Ethics Approval

This study was approved by the Ethics Committee of Thinxon Biomedical Co., Ltd. (SSLI-23001), Nanning, China. The study was performed in accordance with the Guidelines for the ethical review of laboratory animal welfare People's Republic of China National Standard GB/T35892-2018.[39]

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Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis, and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no conflicts of interest in this work.

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