Title page

Title：Storage stability of exosomes in different buffers with/without lyophilization

Running head: Optimizing storage conditions of exosomes

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Highlights

PBS outperformed NS and 5%GS in maintaining ultrastructure and concentration.
Lyophilization could maintain size integrity of exosomes with acceptable concentration loss.
NS and 5%GS exhibited progressive aggregation of exosomes.
PBS was considered as the optimal buffer of exosomes for short-term storage.

Abstract

The clinical translation of exosome-based therapies remains constrained by suboptimal storage protocols. While the cryopreservation at -80°C in phosphate-buffered saline (PBS) is widely adopted, emerging evidences have suggested that buffer composition and lyophilization may influence exosome stability. This study aimed to evaluate the effects of three storage buffers (PBS, normal saline [NS], 5% glucose solution [5%GS]) with/without lyophilization on the storage stability of human bone marrow mesenchymal stem cell-derived exosomes. Exosomes were characterized via western blot, transmission electron microscopy, and nanoparticle tracking analysis. Exo/PBS group showed more uniform distribution and biconcave-disk shape. Exo/NS and Exo/5%GS groups displayed shriveled and aggregated vesicles with less biconcave characteristic. Storage stability was assessed through measurements of concentration and size at fresh, 1-week, 2-week, 4-week timepoints, and follow-up intervals including Fresh to 1 w (ΔT1), 1 w to 2 w (ΔT2) and 2 w to 4 w (ΔT3) in both -80°C and lyophilized conditions. PBS showed superior short-term storage stability. Lyophilization induced concentration loss in the Exo/PBS group but preserved size homogeneity. Exo/NS and Exo/5%GS groups exhibited progressive aggregation of exosomes under lyophilization, in contrast with the stable size of exosomes in the Exo/PBS group. PBS was considered as the optimal buffer for short-term storage. Lyophilization showed paradoxical capacity to maintain size integrity despite concentration loss. These findings provide evidence-based guidelines for balancing stability and logistics in the exosome preservation of clinical use.

Keywords: exosome; storage buffer; lyophilization; concentration; size stability

Introduction

Extracellular vesicles (EVs), first discovered in the 1960s, are lipid-bound nanoparticles secreted by diverse cell types[1, 2]. EVs are composed of a phospholipid bilayer containing specific lipids and proteins, capable of mediating molecular signals, transporting targeted cargos and other specialized functions[3, 4]. Exosomes, a subtype of EVs, mediate intercellular communication by conveying nucleic acids, proteins, lipids, and bioactive molecules to neighboring cells[5]. Owing to the small size (typically 30~150 nm) and characteristics such as favorable biocompatibility, circulation stability and inherent targeting, exosomes

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have emerged as increasingly promising drug delivery vehicles[6].

Although exosomes show considerable potential in various biomedical applications, their clinical translation remains constrained by several critical challenges[7, 8]. The development of standardized storage protocol is particularly critical among these challenges, which needs to be capable of preserving exosomal integrity, bioactivity, and therapeutic efficacy throughout long-term preservation[7]. Anticoagulant selection, storage temperature and duration have been shown to induce structural alteration, membrane compromise and functional impairment in exosomes[9, 10]. However, there are currently no sufficiently standardized protocols for optimal exosome preservation[9].

Accumulating evidences have demonstrated that exosomes may undergo structural and functional changes when exposed to unstable storage environments such as temperature variations and freeze-thaw cycles[11]. The International Society for Extracellular Vesicles (ISEV) recommended cryopreservation at -80℃ using PBS as the preferred medium for exosome storage[12]. Nevertheless, emerging studies have suggested this conventional protocol sometimes fail to adequately prevent vesicle aggregation, structural compromise, and fragmentation, which ultimately lead to compromised therapeutic performance[13]. Some studies indicated that exosomes stored at 4℃ exhibited superior bioactivity compared to those cryopreserved at −80℃, suggesting that freeze-thaw cycles may compromise bio-functionalization of exosomes, proposing refrigeration at 4℃ as a more viable strategy for short-term storage (⩽72 hours)[14]. It has been revealed that storage at both 4℃ and −80℃ induced significant increases in the hydrodynamic diameter of exosomes compared to freshly isolated specimens[15]. It has been also demonstrated that different storage temperatures and durations affected the stability and function of exosomes, and the optimal storage conditions for exosomes may differ based on research purposes[16].

Meanwhile, different storage buffers also have a significant impact on the stability of exosomes. PBS, the most commonly used buffer for cryopreservation, may cause damage to extracellular vesicles during storage, leading to the loss of certain functions[17, 18]. Several additives such as bovine serum albumin (BSA) and trehalose have been evaluated to improve exosome storage stability[19]. It has been reported that compared to PBS alone, the addition of Human Serum Albumin (HSA) and trehalose significantly improved extracellular vesicle quality following short-term and long-term storages[20]. When compared to exosomes stored in PBS, exosomes stored in plasma exhibited superior performance in ultrastructure, size distribution, and surface protein expression[9]. Storage in sucrose solution at -80℃ was also found to show superior performance in size distribution, particle concentration, surface morphology and membrane protein integrity of exosomes[21]. In order to approximate the composition of physiological fluids and mimic in vivo delivery, this study selected PBS, normal saline (0.9% NaCl), and 5% glucose solution as storage media for exosomes to conduct a comparative evaluation of their effects on vesicle preservation.

In recent years, lyophilization has been established as an alternative to conventional storage at 80℃[22]. Lyophilization not only prolongs exosome storage duration by enabling direct room temperature preservation, but also reduces preservation costs[23]. Lyophilized materials can be stored at room temperature and rapidly reconstituted in water or physiological solution[24, 25]. Although lyophilization offers storage advantages, the process introduces critical challenges including ice crystal formation, dehydration and osmotic effects, which may increase risks of compromising the structural integrity and composition of exosomal cargos and membranes[26-28]. Consequently, lyoprotectants such as trehalose and sucrose have been indicated to enable lyophilized exosomes with comparable characteristics in terms of size distribution, morphological integrity, particle concentration and protein/RNA content retention, when compared to those stored at -80℃[29]. In this study, we incorporated lyophilization of exosomes in

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different buffers and systematically compared its efficacy with conventional cryopreservation at -80℃.

This study aimed to elucidate the impact of different storage buffers and methods on exosomes' concentration as well as size distribution, thereby establishing an optimal procedure of exosomes for clinical application. Although previous studies focused on individual factors such as temperature or buffer additives, the co-effect of buffer composition and lyophilization remains unclear[30, 31]. This study compared different storage buffers of PBS, NS and 5%GS under both cryopreservation and lyophilization conditions, which may extend the preservation technique of exosomes through lyophilization and help to provide optimal stability and logistics of clinical use.

Materials and Methods

Cell culture

Mesenchymal stem cells (MSCs) derived from human bone marrow (Guangzhou Jennio Biotech Co., China) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Gibco) at 37℃ in a 5% CO_2 humidified incubator (Thermo Fisher Scientific, USA). Cells at passages 3-5 were used for exosome isolation. Exosome-depleted FBS was prepared by ultracentrifugation at 120,000 ×g for 16 h at 4℃ via Optima L-100 XP ultracentrifuge (Beckman Coulter, USA), followed by filtration through a 0.22 μm PES membrane (Millipore, USA).

Exosome isolation and grouping

At 80% confluence, cells were washed thrice with PBS and cultured in exosome-depleted medium for 48 h. The supernatant were collected and sequentially centrifuged: (1) 300 ×g for 10 min (cell removal); (2) 2,000 ×g for 20 min (debris removal); (3) 10,000 ×g for 30 min (apoptotic body removal); (4) final ultracentrifugation at 100,000 ×g for 70 min at 4℃. The pellet was resuspended and subjected to a second ultracentrifugation (100,000 ×g, 70 min, 4℃). The pellet was collected as purified exosomes for the following experiments.

The isolated pellets of purified exosomes were divided into three groups: (1) Exo/PBS group, resuspended in phosphate-buffered saline (Gibco); (2) Exo/NS group, resuspended in 0.9% sodium chloride solution (Daxiang Group, China); (3) Exo/5%GS group, resuspended in 5% glucose solution (Qidu Pharma, China).

Exosome characterization via western blot

Western blot was used to analyze the specific protein markers of exosomes. The isolated pellets of exosomes and their derived MSCs on the cell plate were first lysed with RIPA lysis buffer containing 1% protease phosphatase inhibitors (Beyotime, China). Total proteins of exosomes and MSCs were extracted and qualified via the BCA method (CWBIO, China). Total proteins (20 μg) were loaded on 12% SDS-PAGE gels (GenScript, USA) and transferred to 0.45 μm PVDF membranes (Millipore). Membranes were blocked with 5% skim milk for 1 h and probed overnight at 4℃ with primary antibodies: positive markers, anti-CD63 (1:1,000; Affinity, USA), anti-TSG101 (1:1,000; Wanleibio, China); negative markers: anti-Calnexin (1:1,000; Wanleibio), anti-GAPDH (1:1,000; Servicebio, China). The membranes were incubated with HRP-conjugated secondary antibodies (1:20,000; Zenbio, China) for 1 h and exposed to ECL HRP substrate (Millipore). Chemiluminescent detection were applied via the ChemiDoc Imaging System (Bio-Rad, USA) and analyzed with ImageJ 1.52a software (NIH, USA).

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Characterization of exosomes via transmission electron microscopy

The morphology and ultrastructure of fresh exosomes in three buffers (PBS, NS, 5%GS) were detected via transmission electron microscopy (TEM). Exosomes (10 μL) were adsorbed onto formvar/carbon-coated copper grids (200 mesh) for 10 min, followed by negative staining with 2% uranyl acetate for 5 min. The images were obtained via JEM-1400Flash TEM (JEOL, Japan) at a voltage 80 kV.

Storage procedures

Suspensions of fresh exosomes (200 μL/tube) in three buffers (PBS, NS, 5%GS) were stored in cryovials (Corning, USA) at -80°C and retrieved at 1, 2 and 4 w for analysis. Samples in -80°C conditions were termed -80°C.

Suspensions of fresh exosomes in three buffers were first flash-frozen in liquid nitrogen and lyophilized via vacuum freeze-dryer (ALPHA 2-4 LD plus, Christ, Germany) for 24 h. Lyophilized powders of exosomes were stored in desiccators at 25°C and reconstituted to the primary volume with sterile ddH_2O before analysis. Samples in lyophilization condition were termed Lyo.

Nanoparticle tracking analysis (NTA)

The concentration and size distribution of the exosomes were quantified via ZetaView® (Particle Metrix, Germany). Standardized sample ((100 nm)) was diluted to 1×10^8 particles/mL in ddH_2O and calibrated according to the manufacturer's instructions. The data was processed using ZetaView software. Samples of exosomes were diluted 100 times with their primitive buffers for the detection of the original concentration and particle diameter distribution.

Statistical analysis

The data were presented as the Mean ± SD. The differences among the groups evaluated via two-way ANOVA with the Bonferroni correction (GraphPad Prism 7.0 Software, USA). The level of significance was set at $p < 0.05$.

Results

Identification of protein markers for MSCs-derived exosomes

Western blot analysis confirmed the strong presence of specific markers (CD63 and TSG101) and the absence of negative controls (CALNEXIN and GAPDH) for the isolated MSCs-derived exosomes, and all the markers were expressed in the source cell (Fig. 1), validating the standard-compliant exosomes.

Morphology and ultrastructure of MSCs-derived exosomes in different buffers

TEM results revealed that all the isolated exosomes in three buffers (PBS, NS, 5%GS) exhibited characteristic spherical morphology with intact membrane and expected diameter of 30~150 nm (Fig. 2). Exo/PBS group showed more uniform distribution and biconcave-disk shape (Fig. 2A). Exo/PBS and Exo/5%GS groups displayed shriveled and aggregated vesicles with less biconcave characteristic (Fig. 2B and 2C).

Storage stability of exosomes in different buffers with/without lyophilization at each timepoint

To determine storage stability of exosomes with/without lyophilization, the fresh exosomes in

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different buffers were subjected to NTA analysis, as well as after 1-week, 2-week and 4-week storage timepoints (Fig. 3A-3F).

The concentration stability was shown in Fig. 3A, 3C and 3E. In Exo/NS and Exo/5%GS groups, exosomes of 1 w, 2 w and 4 w with/without lyophilization were found to show significant decreases of concentration to those of fresh exosomes, but no significant differences were shown among 1 w, 2 w and 4 w groups (Fig. 3C and Fig. 3E). As for Exo/PBS group, however, exosomes of 1 w showed higher concentration than those of 2 w and 4 w, indicating a relative concentration stability of PBS for a short-term storage (Fig. 3A). Lyophilization was found to decrease the concentration of PBS groups than that in -80°C condition at each timepoint (Fig. 3A), while no significant differences were shown in NS and 5%GS groups (Fig. 3C and Fig. 3E).

The diameter stability was shown in Fig. 3B, 3D and 3F. No significant differences were shown in Exo/PBS group between -80℃ and Lyo, suggesting a relative size stability of exosomes stored in PBS (Fig. 3B). A growing trend in size of exosomes was shown in both Exo/NS and Exo/5%GS groups over time, suggesting the possibility of exosome aggregation in these two buffers (Fig. 3D and Fig. 3F), which was consistent with the TEM results above. In addition, there was greater size and relative lower concentration of Exo/NS group in lyophilization condition than that in -80℃ condition at 2 w and 4 w, indicating the obvious aggregation of exosomes in lyophilization condition (Fig. 3C and 3D).

To clearly compare and display concentration and diameter of exosomes in different storage buffers at each timepoint, further statistics were applied for the -80℃ and lyophilization conditions respectively (Fig. 3G-3J). As for the fresh exosomes, the incremental concentration of exosomes in PBS, NS and 5%GS buffers were detected (Fig. 3G), but no statistical differences in size were shown (Fig. 3H). This may be attributed to the osmotic pressure differences among three buffers. After one-week storage time, all three groups demonstrated obvious decline in the concentration of exosomes, while Exo/PBS group showed higher concentration (Fig. 3G). However, after two-week storage time and even longer, there were no differences among three groups, indicating the potential advantage of PBS on short-term storage. For the size stability, Exo/5%GS group showed larger diameter of exosomes after being stored for 1 w and 4 w, while Exo/NS group showed smaller diameter at the storage time of 4 w, indicating the better protective effect of PBS than NS and 5%GS after the freeze-thaw cycle (Fig. 3H). Although there were significant concentration differences of fresh exosomes among three buffer groups, no statistical differences were shown among these groups after lyophilization at the timepoints of 1 w, 2 w and 4 w (Fig. 3I). For the size stability, no obvious size differences were found among three buffer groups in lyophilization condition (Fig. 3J).

Concentration and diameter changes of exosomes in different buffers with/without lyophilization based on baseline and follow-up intervals

Concentration and diameter changes of exosomes were analyzed in different buffers with/without lyophilization based on baseline and follow-up intervals including Fresh to 1 w (ΔT1), 1 w to 2 w (ΔT2) and 2 w to 4 w (ΔT3) (Fig. 4). Massive changes of concentration occurred in the follow-up interval of Fresh to 1 w in three buffers with/without lyophilization (Fig. 4A and 4C). Exo/PBS group showed less change of Diameter in the first week with subsequently huge change in the second week in -80℃ condition (Fig. 4A). A stable tendency of diameter distribution appeared in the follow-up interval of 2 w to 4 w in three buffers with/without lyophilization (Fig. 4B and 4D). However, a relative worse diameter stability was shown in Exo/5%GS group (Fig. 4B).

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Discussion

The key challenge of bioactive exosomes for clinical application is the storage stability that maintain the expected biological functions of exosomes without damaging their targeted structure[32]. This requires careful adjustment of three critical factors: cryoprotectant formulation, storage temperature control and lyophilization strategies to effectively prevent aggregation and membrane damage of exosomes during long-term preservation[10]. The present study investigated the stability of MSC-derived exosomes in different storage buffers with or without lyophilization methods, revealing three pivotal findings: (1) PBS demonstrated superior short-term stability in maintaining exosome concentration compared to NS and 5%GS; (2) Lyophilization induced significant concentration loss in PBS-stored exosomes but preserved size integrity of exosomes across all buffers; (3) NS and 5%GS demonstrated progressive exosome aggregation over time, especially under lyophilization.

The superior short-term stability of exosomes in PBS could be attributed to its isotonic properties and absence of divalent cations (Ca^{2+}/Mg^{2+}), which reduce osmotic stress and vesicle fusion—a mechanism consistent with ISEV guidelines for EV storage[12]. However, the observed concentration decline of exosomes in PBS after 2 w aligned with previous reports of PBS-induced vesicle lysis under prolonged storage[33], potentially due to insufficient cryoprotectants. The progressive size increase observed in NS and 5%GS groups suggested solution-specific aggregation pathways: sodium chloride's charge screening effects in NS and excluded volume effect driven by glucose in 5%GS, both of which could promote vesicle aggregation[34, 35]. The observed concentration decrease in lyophilized PBS group resulted from ice crystal-induced structural damage during freeze-drying[36], while the size homogeneity suggested only intact vesicles survived in reconstitution process while damaged ones got eliminated.

It was previously reported that the vesicle enlargement of exosomes was temperature-dependent[15]. The present study demonstrated that the solution composition also participated and influenced this phenomenon. Improved storage stability could be seen in exosomes with HSA/trehalose additives[20]. However, it is unclear whether these supplements have any effect on overall health. Basic buffers like 5%GS outperform PBS in long-term size maintenance, challenging PBS's status as the universal EV storage medium. The persistent aggregation in Exo/NS group of the present study contrasted with the plasma storage result[9], revealing the difference between artificial storage buffers and natural biological environments.

PBS revealed advantages for short-term storage of exosomes, providing actionable procedures for in vivo delivery. The comparable size stability between lyophilized and -80°C-stored exosomes verified lyophilization as a cost-effective alternative in resource-limited situations. This study has the following limitations. First, laboratory-scale testing may not fully replicate preservation dynamics in vivo. Furthermore, the integrity of functional cargos (nucleic acid/protein) was not included to confirm bioactivity retention in the present study. Future validation should incorporate in vitro/in vivo functional tests and industrial-scale lyophilization trials.

Conclusion

In summary, our results demonstrated that PBS outperformed NS and 5%GS in maintaining exosome concentration during short-term storage (≤2 w). Lyophilization showed paradoxical capacity to maintain size integrity despite concentration loss. The observed buffer-specific aggregation highlighted the necessity of purpose-driven buffer selection for clinical applications. Taken together, these findings

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advanced preservation strategies of exosomes via buffer selection based on expected storage duration and lyophilization for size-sensitive applications despite its concentration limitations. Future research is expected to focus on conducting functional validation of therapeutic efficacy of preserved exosomes and industrial-scale procedures for clinical applications.

Declarations

CRediT authorship contribution statement

Jiaqi Shen: Writing – original draft, Methodology, Investigation, Data curation. **Yi He**: Methodology, Investigation, Data curation. **Jiali Deng:** Investigation, Data curation. **Wenyi Zeng:** Methodology, Data curation. **Feilong Deng:** Methodology, Data curation, Supervision. **Xiaolin Yu:** Methodology, Data curation, Supervision. **Zhengchuan Zhang:** Funding acquisition, Conceptualization, Software, Writing—review and editing, Supervision. All authors read and approved the final manuscript.

Ethics

Not applicable.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Declaration of competing interest

The authors declare no conflict of interest. Graphical abstract was created by Figdraw (www.figdraw.com).

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Supporting information

None.

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Figure captions

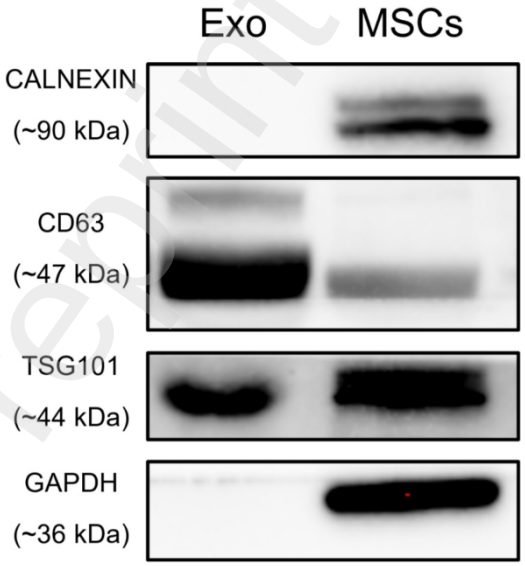

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Figure 1. Identification of MSCs-derived exosomes via western blot. Representative western blots of exosome-positive markers (CD63 and TSG101) and exosome-negative markers (CALNEXIN and GAPDH) for exosomes (Exo) and source cells (MSCs).

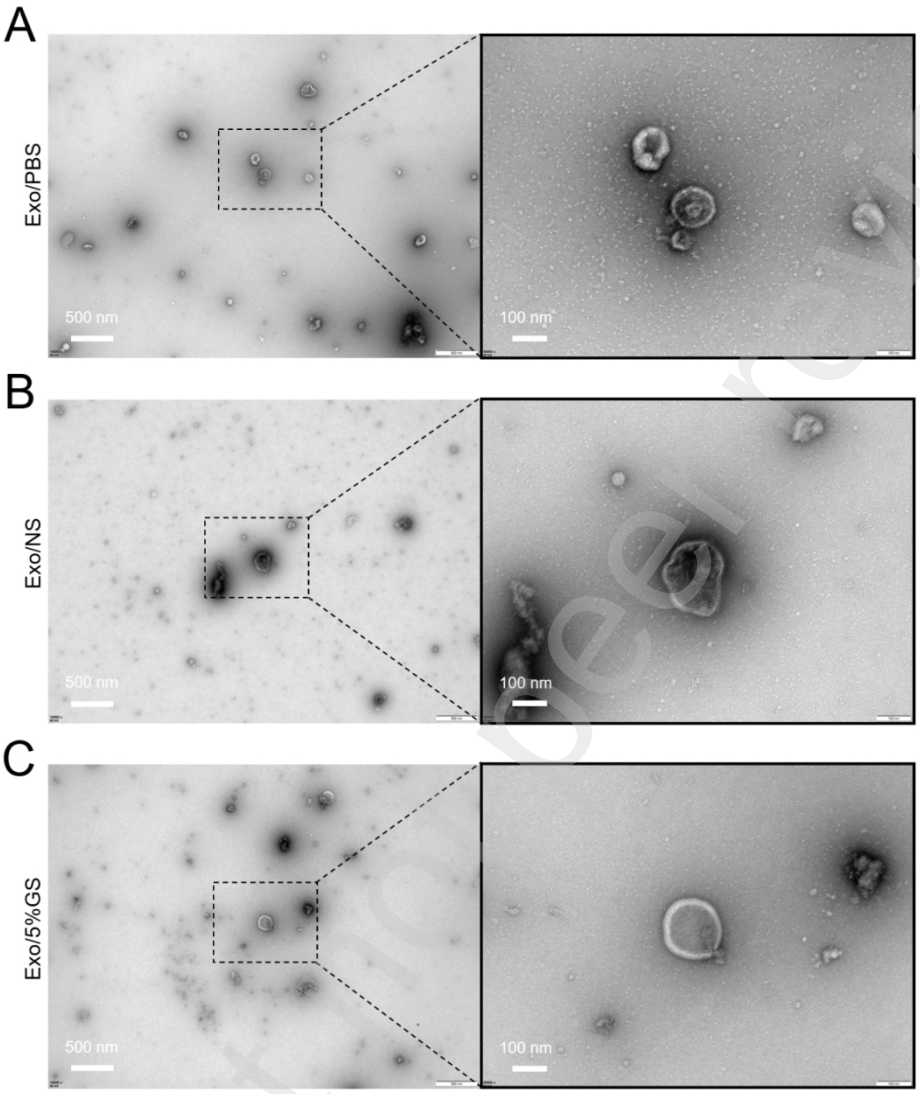

Figure 2. Identification of MSCs-derived exosomes in different buffers via Transmission electron microscopy (TEM). (A) TEM images of exosomes in PBS buffer (Exo/PBS). (B) TEM images of exosomes in NS buffer (Exo/NS). (C) TEM images of exosomes in 5%GS buffer (Exo/5%GS).

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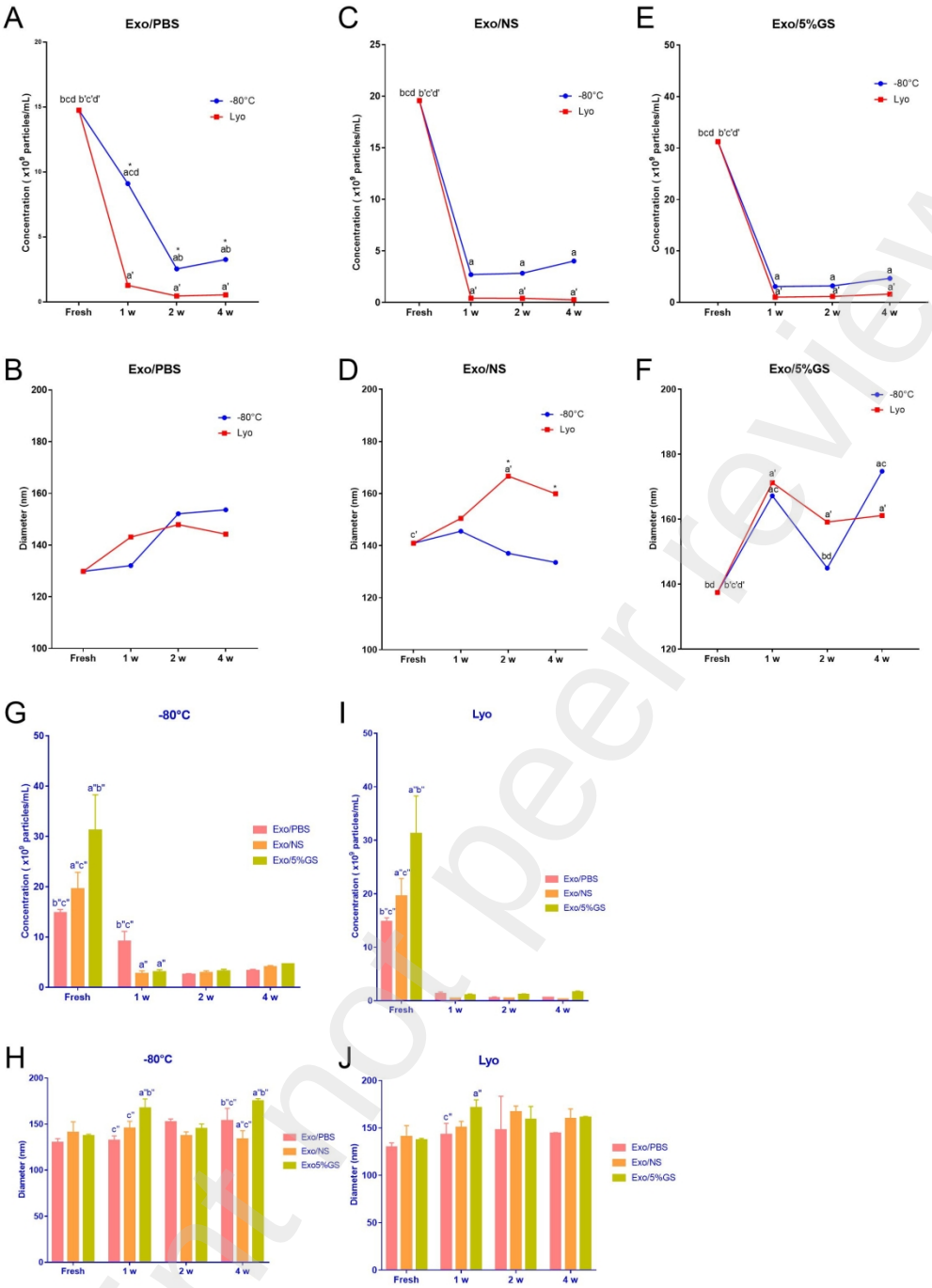

Figure 3. Effect of exosomes in different buffers with/without lyophilization on the storage stability. (A) Concentration of exosomes in PBS buffer with/without lyophilization at Fresh, 1 w, 2 w and 4 w. (B) Diameter of exosomes in PBS buffer with/without lyophilization at Fresh, 1 w, 2 w and 4 w. (C) Concentration of exosomes in NS buffer with/without lyophilization at Fresh, 1 w, 2 w and 4 w. (D) Diameter of exosomes in NS buffer with/without lyophilization at Fresh, 1 w, 2 w and 4 w. (E) Concentration of exosomes in 5%GS buffer with/without lyophilization at Fresh, 1 w, 2 w and 4 w. (F) Diameter of exosomes in 5%GS buffer with/without lyophilization at Fresh, 1 w, 2 w and 4 w. (G) Concentration of exosomes in different buffers without lyophilization. (H) Diameter of exosomes in

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different buffers without lyophilization. (I) Concentration of exosomes in different buffers with lyophilization. (J) Diameter of exosomes in different buffers with lyophilization. Different letters, a, b, c, d above the symbols indicate significant difference compared to the Fresh, 1 w, 2 w and 4 w groups respectively in -80℃ condition, $p < 0.05$. Different letters, a', b', c', d' above the symbols indicate significant difference compared to the Fresh, 1 w, 2 w and 4 w groups respectively in lyophilization condition, $p < 0.05$. *$p < 0.05$, -80℃ condition vs. lyophilization condition. Different letters, a", b", c" above the bar indicate significant difference compared to the PBS, NS and 5%GS groups respectively, $p < 0.05$.

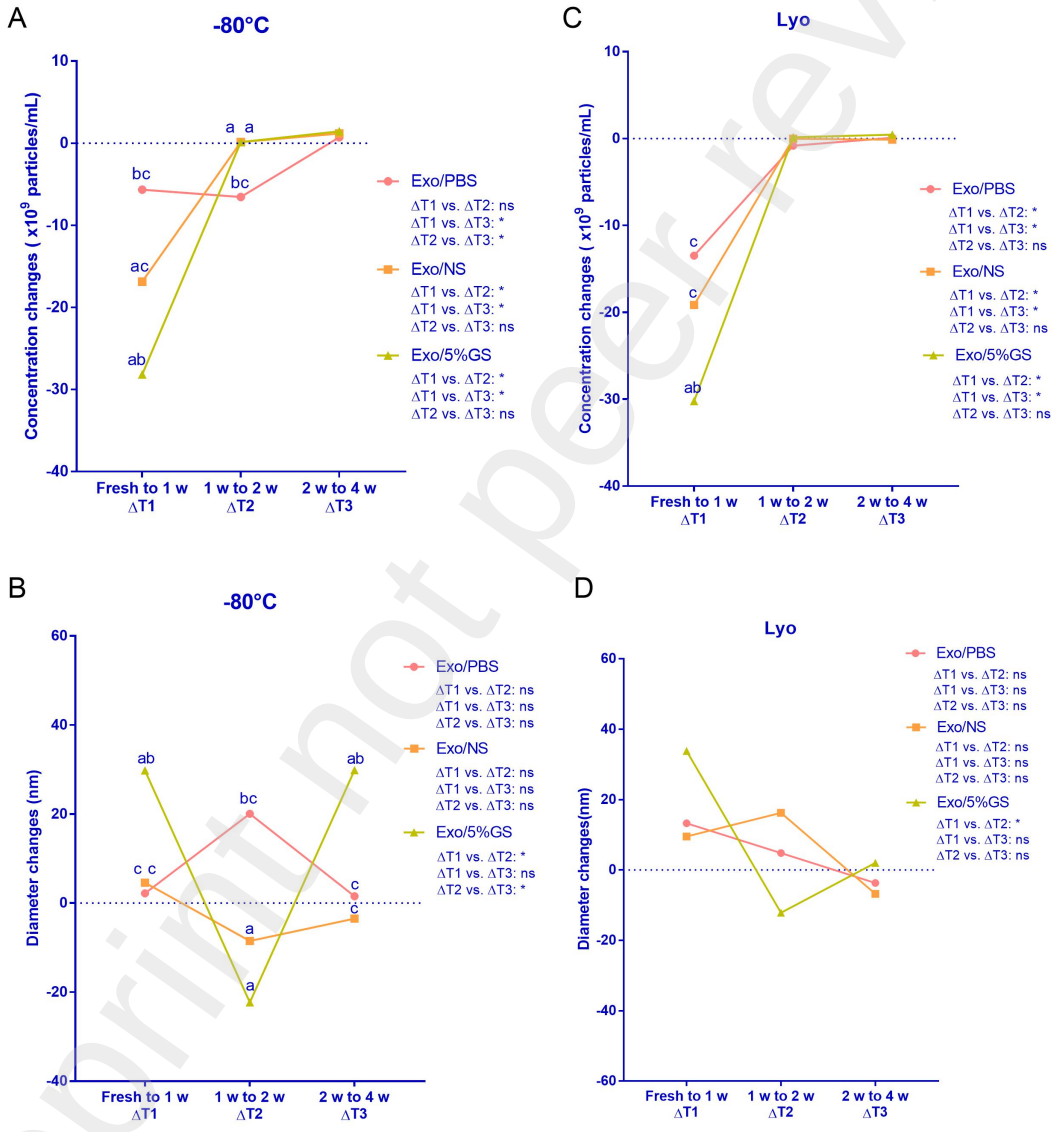

Figure 4. Concentration and diameter changes of exosomes in different buffers with/without lyophilization based on baseline and follow-up intervals including Fresh to 1 w (ΔT1), 1 w to 2 w (ΔT2) and 2 w to 4 w (ΔT3) . (A) Concentration changes of exosomes in different buffers without lyophilization during the periods of ΔT1, ΔT2 and ΔT3. (B) Diameter changes of exosomes in different buffers without lyophilization during the periods of ΔT1, ΔT2 and ΔT3. (C) Concentration changes of

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exosomes in different buffers with lyophilization during the periods of ΔT1, ΔT2 and ΔT3. (D) Diameter changes of exosomes in different buffers with lyophilization during the periods of ΔT1, ΔT2 and ΔT3. Different letters, a, b, c, d above the symbols indicate significant difference compared to the PBS, NS and 5%GS groups respectively, $p < 0.05$. *$p < 0.05$, ns = no significance, ΔT1 vs. ΔT2 vs. ΔT3.

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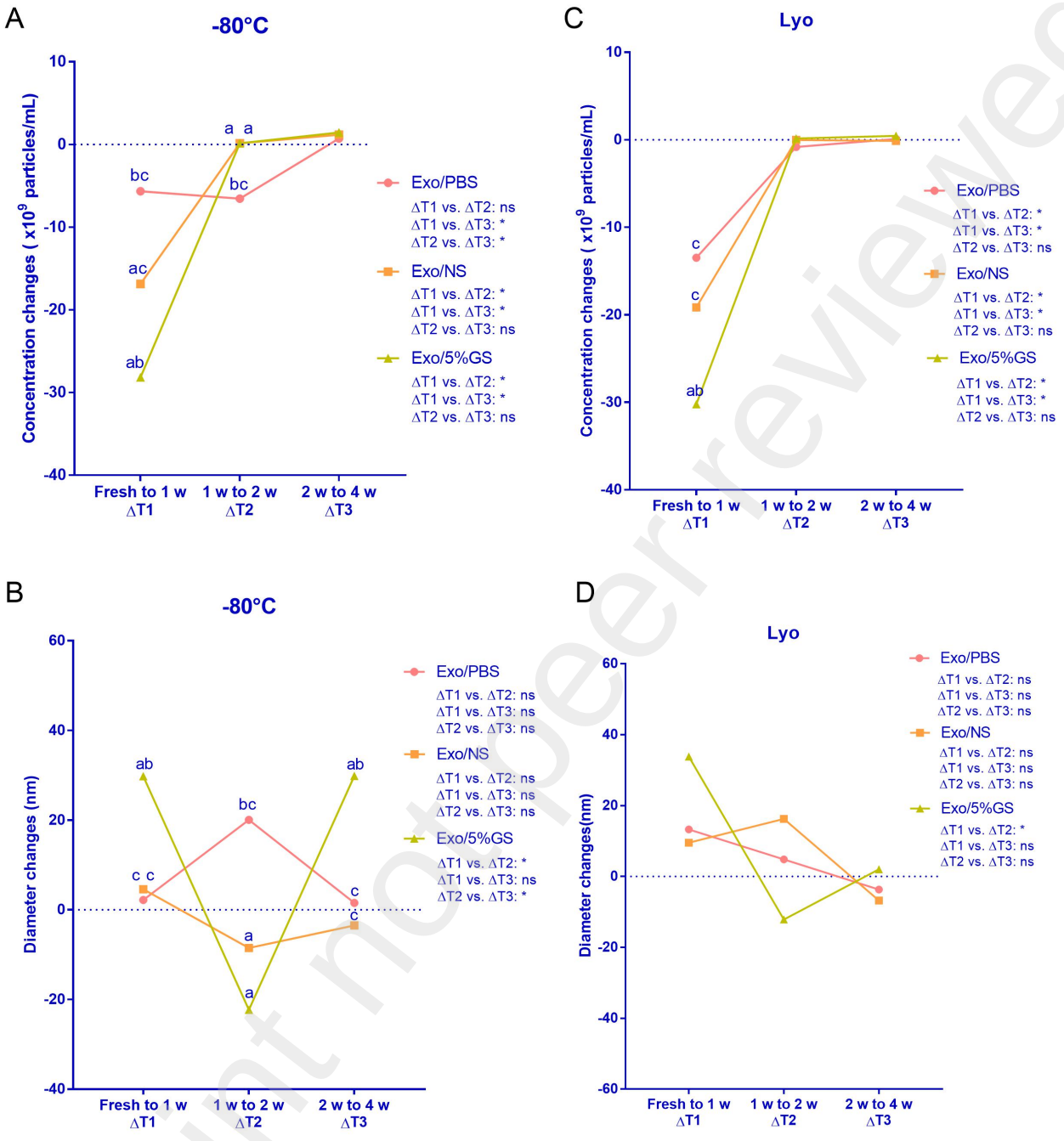

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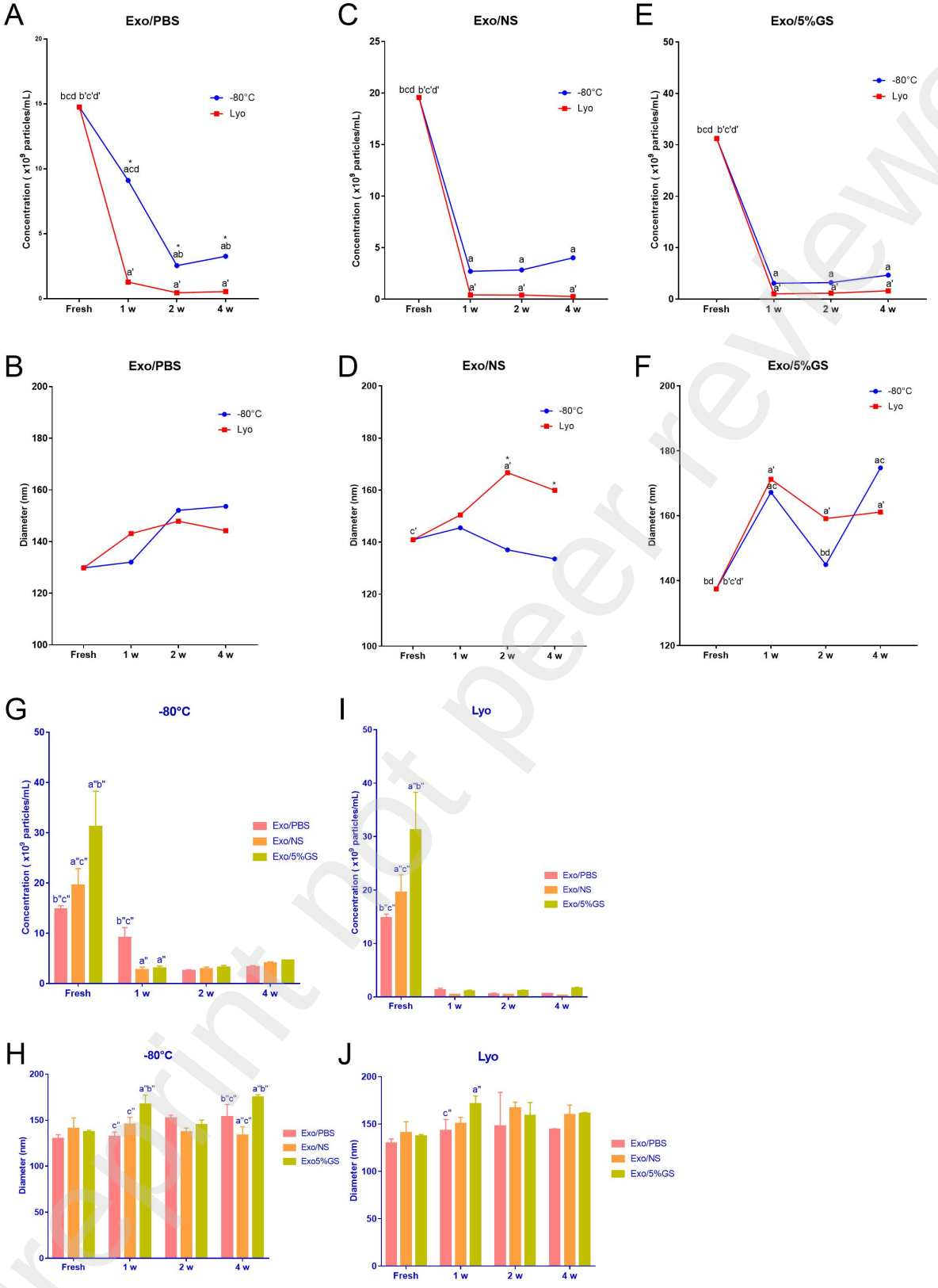

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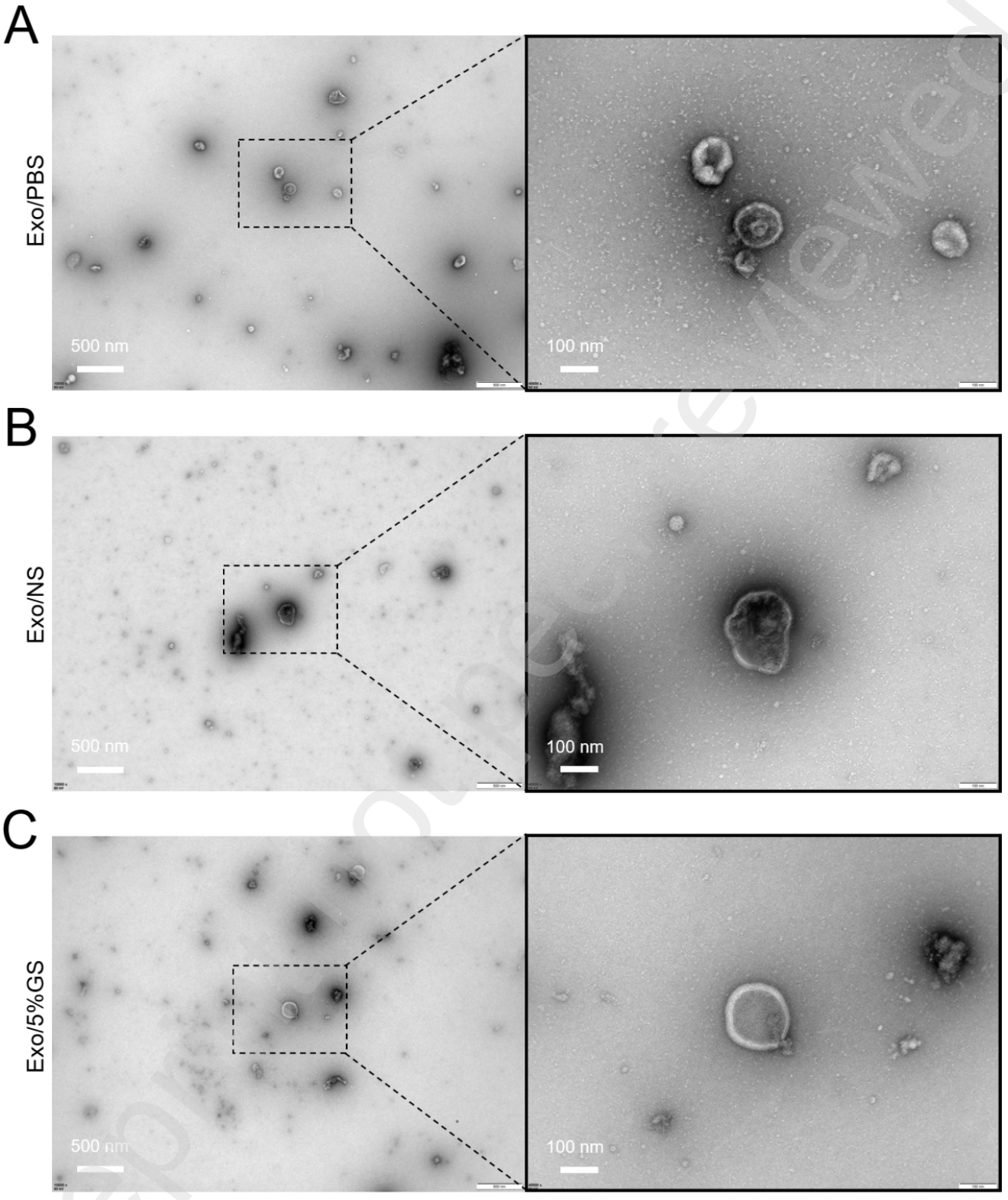

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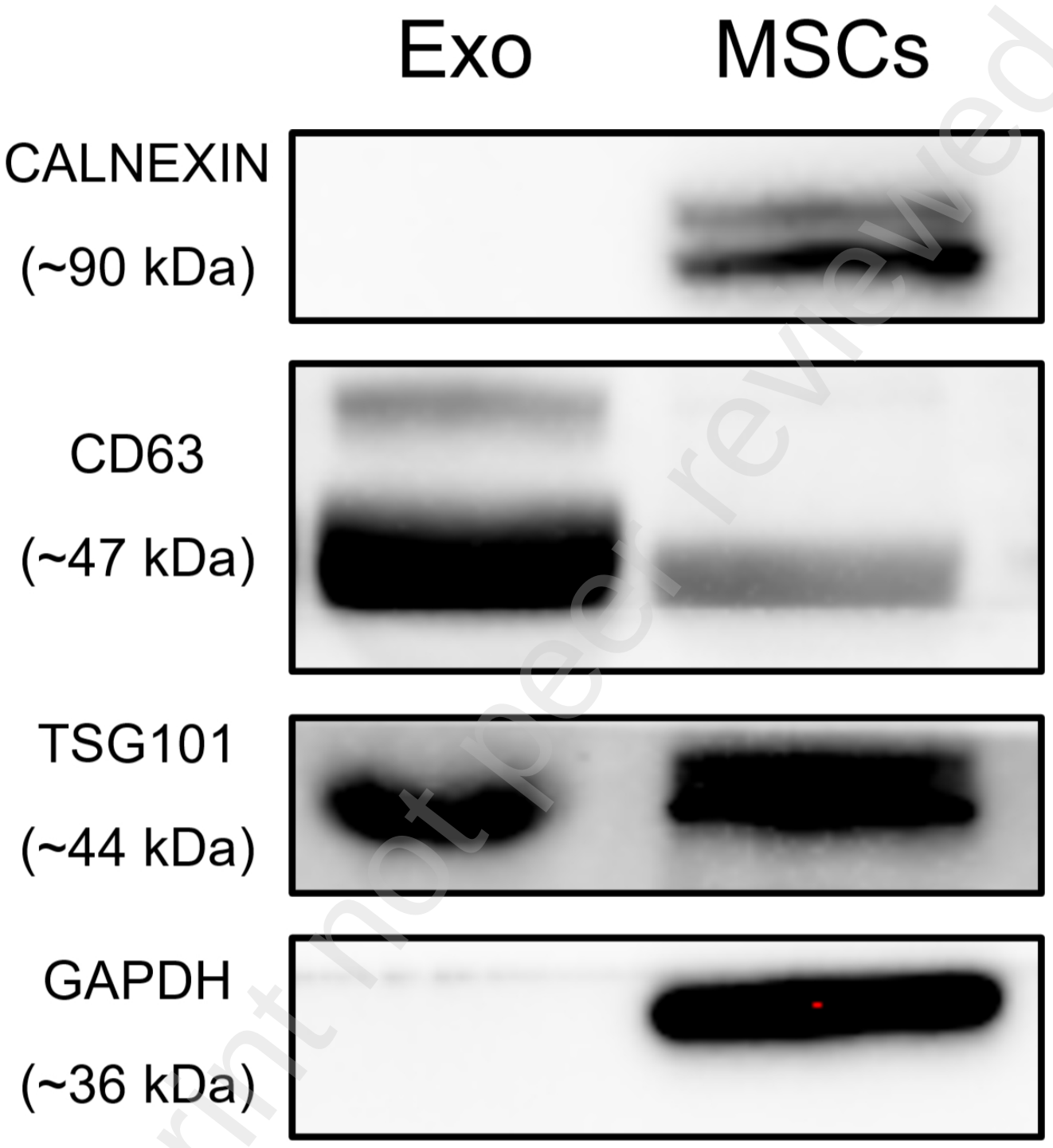

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