Storage stability and delivery potential of cytochalasin B induced membrane vesicles

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ABSTRACT

Cell-free therapies based on extracellular vesicles (EVs) derived from mesenchymal stem cells (MSCs) are considered as a promising tool for stimulating regeneration and immunomodulation. The need to develop a practical approach for large-scale production of vesicles with homogenous content led to the implementation of cytochalasin B-induced to induce microvesicles (CIMVs) biogenesis. CIMVs mimic natural EVs in size and composition of the surrounding cytoplasmic membrane. Previously we observed that MSC derived CIMVs demonstrate the same therapeutic angiogenic and immunomodulatory activity as the parental MSCs, making them a potentially scale-free therapeutic option. However, little is known about their storage stability and delivery potential. We determined that different storage conditions alter the protein concentration within the solution used to store CIMVs over time, this coincided with a decrease in the amount of CIMVs due to gradual degradation. We established that freezing and storage CIMVs in saline at -20 °C reduces degradation and prolongs their shelf life. Additionally, we found that freeze-thawing preserved the CIMVs morphology better than freeze drying and subsequent rehydration which resulted in aggregation of CIMVs. Collectively our data demonstrates for the first time, that the most optimal method of CIMVs storage is freezing at -20 °C, to preserve the CIMVs in the maximum quantity and quality with retention of effective delivery. These findings will benefit the formation of standardized protocols for the use of CIMVs for both basic research and clinical application.

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1. Introduction

Extracellular vesicles (EVs) are spherical membrane-protected vesicles ranging in size from 50 to 2000 nm, released by almost all human cells and capable of delivering signals to neighboring and distant cells within an organism [1]. EVs can act as paracrine effectors, controlling a variety of biological processes by transferring biologically active molecules (e.g. nucleic acids, proteins and lipids) to recipient cells [2], changing their phenotype, modulating the microenvironment [3] and/or triggering receptor-mediated cell signaling [4–6].

Stem cell (SC) derived EVs carry transcription factors [7–11], cell surface receptors [12–14] and a molecular content of growth factors, cytokines and chemokines that reflect the parental cells. Numerous studies have shown that EVs recapitulate the biological activity of SCs by stimulating cell proliferation, increasing viability, inducing chemotaxis [15,16], coordinating recovery processes after tissue damage by transferring microRNAs [17] and promoting angiogenesis [18–20], as well as demonstrating immunosuppression activity [21]. It is hypothesized that EVs can act as a functional extension of SCs, and the EVs regenerative potential of stem cell EVs has been used to treat heart, nerve, kidney and liver tissue damage [22–29].

Cytochalasin B induced membrane vesicles (CIMVs) were developed as a therapeutic tool in order to allow large-scale production of vesicles that imitate natural EVs in size and composition of the surrounding cytoplasmic membrane. CIMVs also contain surface receptors and cytoplasmic content that reflects the parental cells and are able to maintain cellular signaling [30]. CIMVs have been used to study the function of
membrane receptors and cell signaling [30], to deliver drugs and nanoparticles in vivo [31], and to stimulate angiogenesis [20]. Furthermore, their use for drug delivery has also been postulated. Peng et al. proposed loading of antitumor drugs into CIMVs to reduce unwanted toxicity effects [32].

In our previous work, we isolated CIMVs from neuroblastoma SH-SY5Y cells, prostate PC3 cells [33] and human MSCs [20] and showed that the size of CIMVs ranges from 100 to 2000 nm, with the majority (89.36 %) ranging from 100 to 1200 nm [20] and that the greatest impact on CIMV entry into target cells is the heterophilic interaction of CIMV membrane receptors with the surface proteins of target cells [33]. We also demonstrated that CIMVs produced by SH-SY5Y cells contain biologically active molecules derived from the parental cells and can stimulate the formation of capillary-like structures in vitro and angiogenesis in vivo. More recently we have shown that human CIMVs-MSCs retain the molecular content, immunophenotype, and angiogenic activity of parental MSCs [20].

These studies demonstrate that CIMVs are a promising tool for clinical application, particularly within the fields of regenerative medicine and antitumor therapy. However, little is known about their storage stability and delivery potential. In this study we determine the effect of storage conditions including freeze-thawing and freeze-drying on the integrity and delivery effectiveness of CIMVs derived from human MSCs.

2. Materials and methods

2.1. MSCs isolation

Human sample collection was approved by the local Ethical Committee of Kazan (Volga region) Federal University based on article 20 of the Federal Legislation on “Health Protection of Citizens of the Russian Federation” 323-FI, 21.11.2011. Signed informed consent was obtained from each donor. To obtain cell suspension the adipose tissue was cut into small pieces and treated with 0.2 % collagenase II (Dia-M, Russia) in a shaker-incubator at 37 °C, 120 rpm for one hour. Cell suspension was pelleted (400 g for 5 min), washed once in PBS (PanEco, Russia) and re-suspended in DMEM (PanEco, Russia) supplemented with 10 % fetal bovine serum (Gibco, UK) and 2 mM L-glutamine (PanEco, Russia). MSCs were maintained at 37 °C, 5 % CO2 with culture medium replaced every three days.

2.2. HEK293FT cultivation and treatment with CIMVs

HEK293FT (Human Embryonic Kidney 293 cells) (ATCC Number: CRL-11268), an immortalized primary human embryonic kidney cell line, was grown in DMEM (Paneco, Russia) supplemented with 10 % fetal bovine serum (Gibco, UK) and 2 mM L-glutamine (Paneco, Russia) at 37 °C with 5 % CO2. Cell passing was performed using a 0.25 % trypsin-EDTA solution (Life Technologies, USA).

HEK293FT cells were seeded in 96-well culture plates at 20,000 cells per well. After 24 h HEK293FT were treated with CIMVs (250 μg/ml) loaded with CFDA SE (65–0850–84, ebioscience, USA) and stored under various conditions to evaluate the delivery effectiveness of CIMVs.

2.3. CFDA SE staining

Cell-permeant dye CFDA SE (65–0850–84, ebioscience, USA) was used to stain donor MSCs and analyze the integrity of CIMVs during storage. MSCs were stained with CFDA SE (65–0850–84, ebioscience, USA) according to the manufacturer’s recommendation. Briefly, MSCs (1 × 106 cells/mL) were incubated with CFDA SE (10 μM; 65–0850–84, ebioscience, USA) for 15 min and washed with DMEM supplemented with 10 % FBS, 2 mM L-glutamine. CIMVs were obtained from CFDA SE stained MSCs.

2.4. CIMVs production and storage

CIMVs were prepared as described previously [20,34]. Briefly, MSCs were washed twice with PBS, and maintained in DMEM supplemented with 10 μg/mL of Cytochalasin B (Sigma-Aldrich, USA) for 30 min (37 °C, 5 % CO2). Cell suspension was vortexed vigorously for 30 s and pelleted (100 g for 10 min). The supernatant was collected and subjected to two subsequent centrifugation steps (300 g for 20 min and 2000 g for 25 min). The pellet from last step, containing CIMVs-MSC, was washed once in PBS (2000 g for 25 min). Isolated CIMVs were resuspended in saline, then divided into separate tubes and placed in various storage conditions: 1) at +4 °C for up to 112 days; 2) at −20 °C for up to 112 days; 3) at 25 °C for up to 28 days; 4) freeze dried and stored at −20 °C for 112 days. CIMVs suspension was added to human serum and stored at 37 °C for up to 7 days.

2.5. Characterization of the CIMVs

2.5.1. Scanning electron microscopy (SEM)

CIMVs were fixed with 10 % formalin for 15 min, then dehydrated using a graded alcohol series and dried at 37 °C. Prior to imaging, samples were coated with gold/palladium in a Quorum T150ES sputter coater (Quorum Technologies Ltd, United Kingdom). Slides were analyzed using Merlin field emission scanning electron microscope (CarlZeiss, Germany).

2.5.2. Protein quantification

Pierce™ BCA Protein Assay Kit (ThermoScientific, USA) was used to measure protein concentration in CIMVs storage solutions. Before the BCA testing of CIMVs storage solution, CIMVs were sedimented at 14000 g for 15 min, then storage solution was transferred into a new tube and used for the following analysis. Thus, we excluded the CIMVs from the storage solution avoiding influence on the BCA tests. Further, Protein Assay Kit Reagent A (a solution containing bicinchoninic acid, sodium carbonate, sodium tartrate, sodium bicarbonate in 0.1 N NaOH pH 11.25) and Reagent B (4 % CuSO4 x 5H2O) were mixed in a ratio of 50:1 to prepare working solution. Then the storage solution sample (25 μL) and working solution (200 μL) were mixed and incubated at 37 °C for 30 min. Absorption was measured 30 min later at 562 nm using a microplate reader (TECAN, Switzerland). The protein concentration was determined based on standard dilutions of bovine serum albumin.

2.5.3. Flow cytometry analysis

Quantity of CIMVs was analyzed using flow cytometry (BD FACs Aria III. BD Bioscience, USA). A 405 nm laser was used for CIMVs-MSC detection and compared to a suspension of calibration particles 0.22–0.45 - 0.88–1.34 – 3.4 μm in size (PPS-6 K, Spherotech, USA) prepared in PBS.

2.6. Statistical analysis

Statistical analysis was done using Wilcoxon signed-rank test (R-Studio) with a significance level of p < 0.05. Illustrations were built with “ggplot2” package.

3. Results

3.1. Protein concentration in CIMV storage solution

The influence of storage conditions on the integrity of CIMVs was evaluated by determining the protein concentration in the
CIMV storage solution (Fig. 1, Table 1). At day 0, a freshly prepared suspension of CIMVs was tested to determine the starting protein concentration of the storage solution (28.65 ± 12.1 µg/mL). The same solutions were then tested for the protein concentration at days 1, 3, 7, 14, 28, 56, 84 and 112 to ascertain any changes. In order to characterize what the protein concentration of the solution would be after complete degradation of the CIMVs within the suspension, we used RIPA-buffer to lyse the vesicles and measured the protein concentration at 396.94 ± 14.5 µg/mL (represented as the red line in Fig. 1).

The stability of CIMVs in saline is dependent on temperature and storage time. After 1 day of storage, the protein concentration in the solution increased to 99.6 ± 13.6 µg/mL, 128.5 ± 23.2 µg/mL, 138.1 ± 18.7 µg/mL, 199.2 ± 18.4 µg/mL at +4 °C, −20 °C, +25 °C and after freeze-drying/rehydration, respectively (Fig. 1, Table 1). While the increase in protein concentration was variable across the time course in all samples. The trends exhibited demonstrate that freeze-drying increased the protein content of the storage solution the most, closely followed by storage of the CIMVs in saline at +25 °C up to day 28. The study of the stability of CIMVs in physiological saline at +25 °C was terminated after 28 days, since during storage we observed a substantial decrease in the number of whole CIMVs.

The protein concentration at the measuring point of 28 days at +25 °C was 225.2 ± 19.1 µg/mL, which is more than half of the destroyed CIMVs.

Storage of EVs in saline at +4 °C and −20 °C produced the lowest increases in protein content within the storage solutions across the course of the study, indicating the best preservation of intact CIMVs. After 112 days of storage, the protein concentration in the solution was 119.7 ± 45.2 µg/mL, 87 ± 14.7 µg/mL, 176.3 ± 16.8 µg/mL at +4 °C, −20 °C and freeze-drying/rehydration, respectively.

### 3.2. Influence of storage conditions on amount of CIMVs

The effect of storage conditions and duration on the amount of CIMVs was evaluated using flow cytometry. The results are shown in Fig. 2 and listed in Table 2. The concentration of freshly prepared CIMVs within each storage solution was considered as the control corresponding to the maximum number of intact CIMVs (665.9 ± 158(×10^3)) (Fig. 2). Readings were then taken on days 1, 3, 7, 14, 28, 42, 56, 84, 98 and 112 to ascertain any changes.

After 1 day of storage, the amount of CIMVs decreased in all conditions to 427.1 ± 19.1(×10^3), 513.9 ± 15.7(×10^3), 153.3 ± 23.5(×10^3), 123.2 ± 17(×10^3), 502.4 ± 36.3(×10^3) at +4 °C, −20 °C, +25 °C, freeze drying/rehydration and in serum at +37 °C, respectively. The rapid decline in the concentration of CIMVs continued for samples stored in serum at +37 °C, decreasing to 218.2 ± 8(×10^3) on day 7. Due to the continuous and rapid decrease in CIMV number, no further measurements of this condition were made following day 7. A similar, but less extreme, continual decrease in CIMV number was also seen when storing the vesicles in saline at +25 °C. For the same reason, the investigation under +25 °C of storage in saline was stopped after 28 days when the concentration of CIMVs reached 317.1 ± 11.5(×10^3).

### Table 1

| Protein concentration in CIMVs storage solution. |
|-----------------------------------------------|
| **Storage, °C** | **+4 °C (µg/mL)** | **−20 °C (µg/mL)** | **+25 °C (µg/mL)** | **Freeze drying/rehydration (µg/mL)** |
| Days | | | | |
| 0 | 1 | 1 | 28.7 ± 12.1 | 199.2 ± 18.4 |
| 1 | 99.6 ± 13.6 | 285 ± 23.2 | 138.1 ± 18.7 |
| 3 | 58.4 ± 24.4 | 84 ± 9.4 | 122.2 ± 18.2 |
| 7 | no data | 101.9 ± 11.7 | 163.7 ± 18.6 |
| 14 | 103.4 ± 17.5 | 95.3 ± 24.7 | 185 ± 40 | 208.9 ± 15.4 |
| 28 | 172.3 ± 19.7 | 126.5 ± 23.2 | 225.2 ± 19.1 | 187.5 ± 40 |
| 56 | 170.8 ± 28.9 | 113.3 ± 21 | – | 209.6 ± 27 |
| 84 | 139.7 ± 20.3 | 133.5 ± 9 | – | 206.4 ± 71.8 |
| 112 | 197.4 ± 45.2 | 87 ± 14.7 | – | 176.3 ± 16.8 |

*Control sample - protein concentration in CIMVs storage solution immediately after the CIMVs isolation; “-“ not evaluated.*
Fig. 2. Influence of storage conditions and duration on the quantity of CIMVs in each storage solution. The data represent mean ± SD of three biological replicates.

Table 2
Amount of CIMVs in storage solutions.

| Day | Storage, °C | Amount of CIMVs, (x10^3) | Freeze drying/rehydration, (x10^3) | Serum at +37 °C, (x10^3) |
|-----|-------------|---------------------------|-----------------------------------|---------------------------|
| 0*  | +4 °C       | 665.9 ± 158               |                                   |                           |
|     | −20 °C      | 665.9 ± 158               |                                   |                           |
|     | +25 °C      | 665.9 ± 158               |                                   |                           |

* Control sample – amount of CIMVs in storage solution immediately after the CIMVs isolation “−” not evaluated.

Between days 7 and 70, the trend in CIMV concentration remains stable for storage conditions using saline at +4 °C and −20 °C and/or CIMVs freeze-dried before storage at −20 °C. However, after day 70, we see a reduction in the number of CIMVs within the freeze-dried storage condition. At the 112nd day of the investigation, the amount of CIMVs was 393.9 ± 73.4(x10^3), 529.5 ± 35.6 (x10^3), 326.6 ± 50.1(x10^3) at +4 °C, −20 °C and freeze drying/rehydration, respectively. These data further confirm our findings that storage of CIMVs in saline at +4 °C or −20 °C generates the best conditions for preservation of intact CIMVs.

3.3. CIMVs morphology after storage

To further assess the stability of MSCs-derived CIMVs, we investigated the effect of the various storage conditions on the morphology and integrity of CIMVs after 14 days using SEM. We found that CIMVs immediately after isolation represented spherical structures with a smooth surface (Fig. 3A). The same smooth spherical appearance was also seen in CIMVs stored in saline at −20 °C (Fig. 3C). However, we found that after freeze-drying and storage in saline at +4 °C, CIMVs tended to form more aggregates (Fig. 3B and D). The SEM results indicate storage in saline at −20 °C, maybe advantageous in maintaining the original state of CIMVs when freshly isolated.

3.4. Delivery effectiveness of CIMVs after storage

CIMVs loaded with CFDA SE were stored under the various conditions for either 3, 14, 38 or 112 days. The CIMVs were subsequently thawed/rehydrated and added to recipient cells (HEK293FT) to evaluate the ability of the CIMVs to deliver CFDA to the recipient cells. The amount of CFDA-positive recipient cells (CFDA+ cells) was determined 24 h after the addition of the CIMVs by flow cytometry. As a positive control, freshly prepared CFDA loaded CIMVs (250 µg/mL) were added to recipient HEK293FT cells for 24 h, leading to 98 ± 0.14 % of recipient cells becoming CFDA + positive. CIMVs destroyed by ultrasound were used as a negative control.

We had previously seen that storage of CIMVs in human serum at +37 °C leads to a decrease in intact CIMVs, again 7 days of storage at +37 °C in serum led to a decrease in the ability of the stored CIMVs to deliver CFDA. The percentage of recipient cells with green fluorescence (CFDA + cells) decreased from 94.5 ± 2.4 % to 79.1 ± 3.8 % (Fig. 4, black line). No further time points were tested in-line with the termination points of other assays for this condition.
Treatment of recipient cells with CIMVs stored in saline at +25 °C resulted in the percentage of CFDA + recipient cells to decrease to 97.3 ± 0.7% when treated with CIMVs stored for 3 days, to 88.3 ± 1.8% (CIMVs stored for 7 days), 81.8 ± 1.1% (CIMVs store for 14 days) and 63.9 ± 0.8% (CIMVs stored for 28 days) (Fig. 4, green line). Similarly, to storage in serum, storage of CIMVs in salines at +25 °C had previously seen to cause a decrease in intact CIMVs, this assay indicates the same occurrence with the continuous and substantial reduction in CFDA + recipient cells. Therefore, no further data points were measured in-line with the termination points of other assays for this condition.

When applying CIMVs, which were stored in saline at 4 °C, the percentage of CFDA + recipient cells decreased from 97.7 ± 0.17% (CIMVs stored for 3 days) to 93.88 ± 2.18% (CIMVs stored for 14 days), 90.9 ± 1.19% (CIMVs stored for 28 days), 60.2 ± 2.4% (CIMVs stored for 112 days) (Fig. 4, red line).

The effects of storing CIMVs at -20 °C, resulted in the most effective transfer of CFDA to recipient cells. Storage of CIMVs in saline at -20 °C for 112 days followed by a single thaw led to a decrease in the percentage of CFDA + recipient cells to 71.16 ± 3.12% (Fig. 4, blue line). Freeze drying and storage of CMVs for 112 days followed by rehydration led to a decrease in the percentage of CFDA + recipient cells to 86.46 ± 4.04% (Fig. 4, dark green line).

4. Discussion

Here, for the first time, we analyze the storage stability and delivery potential of cytochalasin B induced membrane vesicles.
We observed that using storage the protein concentration in CIMV-containing storage solutions increases over time due to the gradual degradation of CIMVs. The most profound changes in protein concentration were observed when CIMVs were stored in saline at +25 °C, with protein concentrations increasing 7.8 times after 28 days of storage. Similarly, storage of CIMVs in saline at +4 °C also led to increased protein concentration in the CIMV storage solution to 4.1 times. Freeze-drying/rehydration also affected protein concentration in the CIMV storage solution, increasing protein concentration 6.1-fold over the course of the storage period. Our data revealed that the most effective preservation method of CIMVs is storage in saline at -20 °C, since after 112 days of storage the protein concentration in the CIMV's storage solution increased only 3-fold.

To further determine how storage effects CIMV number, we analyzed CIMVs following storage by flow cytometry. In addition to the storage conditions previously studied, we also evaluated the stability of CIMVs in human serum at 37 °C imitating the internal environment of human body. We found a significant decrease in the amount of CIMVs in human serum during the observation period. After 7 days of storage only 30 % of CIMVs remained intact (CIMVs decreased from 665.9 ± 158 (10^3) to 218.2 ± 8 (10^3)). Storage at +25 °C in saline for 28 days led to preservation of 47.7% of CIMVs, decreasing the temperature to +4 °C prolonged the shelf life of CIMVs to 112 days with preservation of 59 % of CIMVs. Freeze drying led to 49 % of CIMVs remaining intact. Whilst freezing CIMVs suspended in saline at -20 °C preserved 79.5 % CIMVs. In addition, we found that freezing at -20 °C preserved the CIMV morphology, whereas freeze-drying and storage at +4 °C led to aggregating of CIMVs.

Our data corroborate results obtained by Lörenz et al. who demonstrated that storage of EVs derived from neutrophilic granulocytes at +4 °C for 1 day resulted in a significant decrease in EV concentration. The authors also observed that storage at -20 °C did not affect the number of EVs for up to 28 days of storage, but caused a change in the size of the EVs and an almost complete loss of antibacterial function after 28 days [35].

Park and co-authors observed that the stability of EV surface proteins was significantly affected by storage at 37 °C for 16 days, and maintained during storage at +4 °C, -20 °C and -70 °C for 25 days [36]. Previously we have shown that delivery effectiveness of CIMVs greatly depends on heterophilic interaction of CIMV membrane receptors with the surface proteins of target cells [33]. Here we observed that the effectiveness of CIMVs to deliver cargo after the storage in saline under different conditions increased in the following order: storage at +25 °C, +4 °C freezing at -20 °C and freeze drying/rehydration. In this way, our findings are concordant with those of Park et al., and taken together indicate the delivery effectiveness of CIMVs is connected with preservation of surface proteins during storage at decreased temperature. The storage of samples to lower temperatures (e.g. -70–80 °C) as Park et al., used, may replicate the findings we observed at -20 °C. However, use of such conditions is not generally conducive to wide-spread clinical use and as such were not included in our study. Despite of CIMVs demonstrated high delivery efficiency after freeze drying/rehydration, their stability and integrity were lower compared to storing at +4 °C and -20 °C according to protein quantification and flow cytometry analysis (Figs. 1 and 2). We suppose that the observed high delivery efficiency of CIMVs after freeze drying / rehydration might be due to partial degradation of CIMVs and non-specific staining.

5. Conclusions

Taken together, our results indicate that storage temperature is an important factor in maintaining the integrity and delivery potential of CIMVs. We found that optimal method of CIMVs storage is freezing at −20 °C in saline. For the first time we demonstrated that this method allows the preservation of CIMVs in high quantities which retain physically features and delivery effectiveness. Freeze drying prior to storage at −20 °C also produced good results, but issues with aggregation of CIMVs first need to be overcome. The solution to which may lie in the evaluation of the use of cryoprotectants.

Author contributions

Conceptualization, Sevindz K. Kletukhina and Marina O. Gomzikova; Data curation, Marina O. Gomzikova; Formal analysis, Sevindz K. Kletukhina, Olga A. Neustroeva, Sirina V. Kurbangaleeva, Iliru I. Safalutfidinov, Alexey M. Rogov and Marina O. Gomzikova; Methodology, Marina O. Gomzikova; Supervision, Albert A. Rizvanov and Marina O. Gomzikova; Validation, Marina O. Gomzikova; Writing – original draft, Sevindz K. Kletukhina; Writing – review & editing, Victoria James, Albert A. Rizvanov and Marina O. Gomzikova.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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