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Original Article

Optimization of urinary small extracellular vesicle isolation protocols: implications in early diagnosis, stratification, treatment and prognosis of diseases in the era of personalized medicine

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Abstract: Extracellular vesicles isolation from urine was severely interfered by polymeric Tamm-Harsefall protein due to its ability to entrap exosome. Studies had been reported to optimize the extraction of urine extracellular vesicles by using reducing agents, surfactants, salt precipitation or ultrafiltration, but rarely based on highly specific purification methods. We optimized the density gradient centrifugation method for the isolation of urinary small extracellular vesicles (sEV) and compared seven differential centrifugation protocols to obtain the high-yield and high-purity sEV isolation procedures. Our study showed Tris sucrose gradient centrifugation at 25°C had more concentrated distribution of exosomal marker in the gradient compared to Tris sucrose gradient centrifugation at 4°C and PBS sucrose gradient centrifugation. Dissolving the 16000 g pellet using Tris, Nonidet™ P 40 or Dithiothreitol then pooling the supernatants did not increase the exosomal markers and number of nanoparticles in sEV preparation compared to the control and PBS groups. Differential centrifugation at room temperature without ultrafiltration recovered more exosome-like vesicles, exosomal markers and nanoparticles than that at 4°C or combining ultrafiltration. Differential centrifugation at RT without ultrafiltration and salt precipitation recovered the highest number of nanoparticles than other protocols. However, differential centrifugation at RT combining 100 kd ultrafiltration obtained the highest purity of sEV calculated by Nanoparticle number/Total protein. In conclusion, we had established two urinary sEV isolation procedures that can recovered higher yield of sEV and more pure preparation of sEV. It is not recommended to treating 16000 g pellet with reducing agents or surfactants to increase the yield of sEV.

Keywords: Small extracellular vesicle, gradient centrifugation, Tamm-Harsefall protein

Introduction

Extracellular vesicles (EVs) are heterogeneous populations of lipid-enclosed structure secreted by essentially all cell types. According to the mechanism of formation, EVs can be classified into three categories with overlapping size and density: exosomes, microvesicles and apoptotic bodies. EVs act as mediators of intercellular communication by transferring biological information between cells in physiological and pathophysiological conditions [1, 2]. EVs were found to play important role in the early diagnosis, stratification, treatment and prognosis of diseases [3]. However, many studies about EV used low-specific separation methods, which could lead to erroneous experimental conclusions.

According to the statement of the International Society for Extracellular Vesicles [4], all EVs isolation methods can be divided into three categories according to the recovery and specificity. The “high recovery, low specificity” methods will also enrich a large number of non-vesicular components, such as precipitation kits. The “intermediate recovery, intermediate specificity” methods separate vesicles while

enriching some proteins, such as size exclusion chromatography and differential ultracentrifugation using intermediate time and speed. The "low recovery, high specificity" methods recover a subtype (or a few subtypes) of EVs with rare non-vesicular components. This type of method includes filtration combined with another method such as density gradient separation, immuno- or other affinity isolation including flow cytometry. Density gradient centrifugation is still the most widely implemented method for recovering EVs. The EVs isolated by this method are small in diameter and contain more exosomes, more and more scholars call it small extracellular vesicle (sEV) [5-7].

Urine is an ideal non-invasive diagnostic specimen. Urine as a waste excreted from the body also contains a certain amount of EVs. Uromodulin (UMOD), also known as Tamm-Horsfall urinary glycoprotein (THP), is a membrane protein synthesized only in the ascending segment of the medullary sputum, and is the most abundant protein in urine under physiological conditions [8]. Polymeric UMOD can entrap urinary extracellular vesicles [9], which may cause vesicles to be removed with precipitation after three-step differential centrifugation. In addition, UMOD can adsorb on EVs and change their distribution in the density gradient, thereby reducing the yield and purity of the sEV. It has been reported that the use of surfactant or reducing agent Tris to dissolve or disrupt UMOD can increase the yield of EVs [10-12], and the use of salt to precipitate protein can reduce the contaminant of UMOD [13]. However, there protocols for isolating EVs were based on low-specificity methods such as polymer-based precipitation and ultracentrifugation, so the conclusion is not convincing.

Another issue is that the relative centrifugal forces, temperatures and durations of differential centrifugation were not uniform in urinary EVs isolation methods [14]. Due to the low concentration of EVs in urine, there are many researches using ultrafiltration to concentrate urine during the process of differential centrifugation [14]. However, ultrafiltration concentrates proteins above the molecular weight cut off, which will contaminate EVs without further purification steps [15]. In addition, ultrafiltration membrane is capable of adsorbing EV and reducing the yield of EVs.

This research aims to optimize the density gradient centrifugation method for urinary sEV isolation and compare seven differential centrifugation protocols to obtain the high-yield and high-purity sEV isolation procedure.

Methods

Collection and preprocess of urine samples

Urine was collected from healthy individual without adding protease inhibitor, pooled together and stored at 4°C no more than 24 h before differential centrifugation. Aliquots of urine for differential centrifugation at RT were pre-warmed at RT for 1 h and swirled before centrifugation, and those for differential centrifugation at 4°C were kept at 4°C throughout the process of differential centrifugation.

Differential centrifugation

Urine samples were centrifuged at 500×g for 10 mins and 2,000×g for 20 mins. For salt precipitation, sodium chloride (NaCl) was added to the 2000×g supernatant to a concentration of 0.58 M to precipitate urinary mucoproteins, including THP. Except for temperature-sensitive proteins that need to be operated at low temperature (4°C), it can generally be carried out at RT, which is beneficial to protein precipitation. After 2 hours of precipitation at RT, the 2000×g supernatant was centrifuged at 2000×g for 20 mins again. The supernatant was subsequently centrifuged at 16,000×g for 20 mins in fixed-angle rotor. Acceleration was set at level 9 and deceleration set at level 1. The supernatants (SN1) were sucked out of the tube by disposable pipette to prevent any pellet contaminating the supernatant and pellet obtained in the next centrifugation or ultracentrifugation.

Ultrafiltration

The 2,000 g supernatant was concentrated ten times by using 15 ml Millipore Amicon Ultrafilter with 30 kDa or 100 kDa molecular mass cut-off. After the concentrating, the ultrafilters were washed with ddH_2O and soaked with 70% alcohol for 5-10 mins, then centrifuged at 3000×g for 5 mins. The ultrafilters were soaked in 20% alcohol and stored at 4°C.

Dissolve 16,000 g pellet

The 16,000 g pellets from urine of equal volume were resuspended in 0.5 ml PBS, 20 mM

Tris-HCl (PH 8.6), 0.05% NP40 or 100 mg/ml DTT Tris-HCl solution (20 mm Tris-HCl PH 8.6), respectively. The suspensions were incubated at 37°C for 5-10 min, and vortexed every 2 min. After incubation, the suspensions were centrifuged at 16,000 g for 20 min at room temperature (RT). The supernatants (SN2) were sucked out of the tube by disposable pipette to avoid any pellet, pooled with SN1 or diluted to 8 ml with PBS in ultracentrifuge tube for sEV isolation.

Isolate crude urinary EVs

The 16,000×g supernatants were spun at 200,000×g for 1 hour at max acceleration and low deceleration in SW41Ti swing rotor using Beckman XL-80 Ultracentrifuge (Beckman Coulter) to pellet the crude EV. The pellet was resuspended in 3 ml PBS or 20 mm Tris (PH 8.6).

Isolate sEV by discontinuous sucrose gradient

Sucrose gradients were built manually as described by Christopher Y. Chen [16], with some modification. Briefly, 5% and 60% sucrose solution in PBS D_2O or 20 mM Tris-HCl D_2O (PH 8.6) were used to prepare 10%, 20% and 40% sucrose. The 1 ml each of the five sucrose solutions (from 5% to 60%) were sequentially injected into the SW41Ti tube from the bottom using a flat needle syringe. Crude EV pellets were loaded on top of the gradient. Tubes were centrifuged for 5 h at 110,000 g (25300 rpm) at max acceleration and low deceleration levels. After stopping the centrifuge, top 3 ml of liquid was siphoned off and 12 fractions were collected from the top of the sucrose gradient without breaks. Each fraction was diluted to 8 ml with PBS or 20 mm Tris-HCL (PH 8.6) in the SW40Ti tube to prevent the tubes from collapsing during the spin, and centrifuged for 1 h at 200,000×g (34100 rpm). The pellets obtained from the centrifuging were resuspended in PBS or 20 mM Tris-HCl. Programmed cell death interacting protein (Alix), tumor susceptibility gene 101 (TSG101) and aquaporin-2 (AQP2) were detected by Western blot to identify the fractions containing sEV.

One-dimensional gel electrophoresis and Western blot

Protein quantification was determined by Coomassie. Pellets or sEV preparations from equal urine were resuspended in 7 M Urea, 2 M thiourea, 5% (w/v) SDS, 40 mM Tris-HCl, pH 6.8, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 20% (v/v) glycerol and 50 mM dithiothreitol (DTT) in a ratio of 0.25 mg of protein per ml of solution. Protein was dissolved and denatured after overnight incubation at RT. Protein was separated by 4-12% and 12% polyacrylamide gel, followed by staining with G-250 or transferring to 0.22 μm nitrocellulose membranes, respectively. Nitrocellulose membranes were blocked in 5% milk and 0.05% Tween-20 in TBS for 1 h. Then, the primary antibodies were added and incubated overnight at 4°C, followed by TBST wash and incubation of secondary HRP-conjugated antibody for 1.5 h at RT. The primary specific antibody used in this study included mouse anti-Alix (Abcam, ab117600), mouse anti-TSG101 (Santa cruz, sc-7964), rabbit anti-UMOD (Affinity, DF6692), mouse anti-AQP2 (Santa cruz, sc-515770), mouse anti-syntenin-1 (Santa cruz, sc-515538). Chemiluminescence detection of bands was visualized using ChemiScope western blot system 3300 (Clinx, Shanghai, CN).

Transmission electron microscopy (TEM)

For the electron microcopy of urinary sEV, 5 μl sEV Tris-HCl (20 Mm, Tris, PH 8.6) suspension was applied to formvar-carbon films on 200 mesh copper grids (catalog no. BZ11022b; Zhongjingkeyi Technologly, Beijing, CN) and incubated for 1 min. Remove the excess suspension on the grid by contacting the grid edge with filter paper. The grids were negatively stained with 10 μl 1% phosphotungstic acid for 1 min, excess staining solution was removed by filter paper. After drying for 10 mins, the samples were observed using FEI Tecnai G 2 Spirit TWIN 120 kV (FEI, Hillsboro, US).

Tuneable resistive pulse sensing

Nanoparticles were counted by tunable resistive pulse sensing (TRPS) method using qNano instrument (Izon Ltd, Christchurch, New Zealand) according to manufacturer's instructions. Standard polystyrene particles of 100 nm (CPC100b; Izon Ltd) was used to perform calibration. Polyurethane nanopore membrane NP150 (analysis range 70-420 nm) (Izon Ltd) was stretched at 47 mm. Electrolyte solution was made of 50 mM Tris (pH 7.4) and 0.05% (v/v) Triton X 100, and filtered with a Millipore

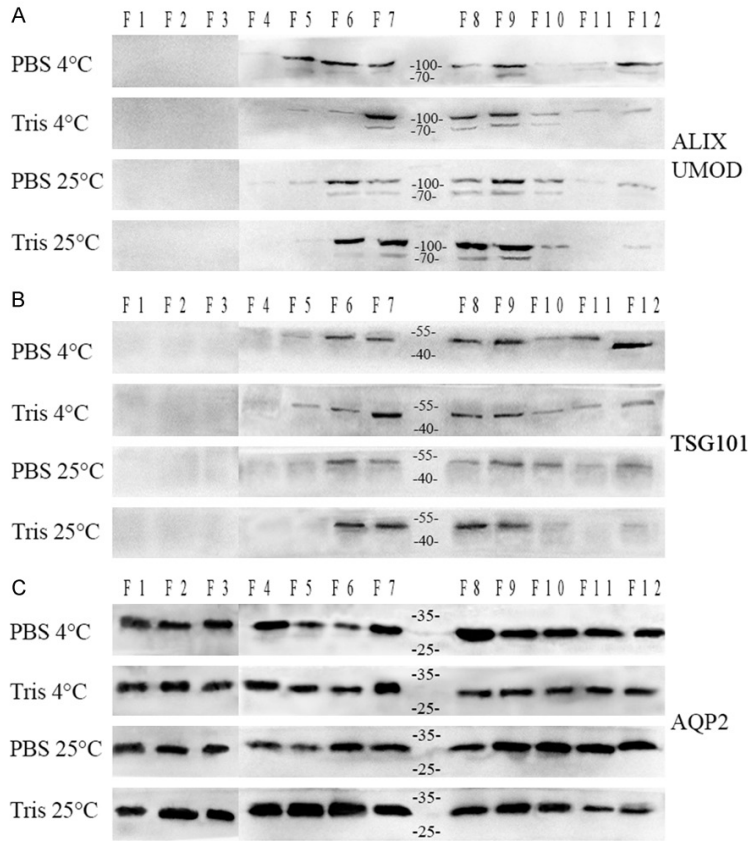

Figure 1. Western blot analysis of twelve fractions recovered after gradient centrifugation by using PBS or Tris D_2O sucrose gradient liquid at 4°C or 25°C. Western blots were performed by using antibodies against indicated proteins. Molecular weights are showed in the middle.

Millex-GS 0.22 μm syringe filter (Merck Millipore). Multi-pressure at 7 mbar and voltage at 0.7 V was applied to determine the particle concentration. Blockade counts set at minimum of 800 events or 10 mins recording. Current pulse signals were collected using Izon Control Suite 3.3 software (Izon Ltd). Each sample was analyzed three times to calculate the average.

Statistical method

The experiments to compare differential centrifugation protocols and the effect of dithiothreitol (DTT) and detergent Nonidet™ P 40 (NP40) were repeated six times. Statistical analyses were performed using Mann-Whitney U test. Differences with *p*-values less than 0.05 were considered significant (**P*<0.05, ***P*<0.001, ****P*<0.0001).

Result

Optimization of discontinuous sucrose gradient centrifugation for urinary sEV isolation

In order to determine the optimum conditions of discontinuous sucrose gradient centrifugation for urinary sEV isolation, 12 fractions obtained from gradient centrifugation by using PBS or Tris D_2O gradient liquid at 4°C or 25°C were analyzed for the presence of sEV protein markers by Western Blot. sEV floated mostly in a continuum of sixth to ninth fractions, especially when centrifuged by using Tris D_2O gradient liquid at 25°C. PBS or Tris D_2O sucrose gradient centrifugation at 4°C and PBS sucrose gradient centrifugation at 25°C brought more Alix and TSG101 positive-sEV to the fourth, fifth, tenth, eleventh and twelfth fractions compared to Tris D_2O sucrose gradient centrifugation at 25°C. All fractions showed strong AQP2 bands. A high degree of correlation between sEV proteins and UMOD (THP) was observed in each fraction (**Figure 1**).

As showed in TEM image (**Figure 2**), 5-60% discontinuous Tris D_2O sucrose gradient centrifugation removed a lot of TH protein compared with 30-60% Tris D_2O sucrose cushion.

Discontinuous Tris D_2O sucrose gradient centrifugation at 25°C enabled sEV to enter into four centralized and continuous fractions. In contrast, distributions of sEV were more decentralized in PBS or Tris D_2O sucrose gradient when centrifuged at 4°C or in PBS sucrose gradient at 25°C. Thus, the sucrose gradient centrifugations used in the subsequent experiments were all performed by using 5-60% discontinuous Tris D_2O sucrose gradient at 25°C.

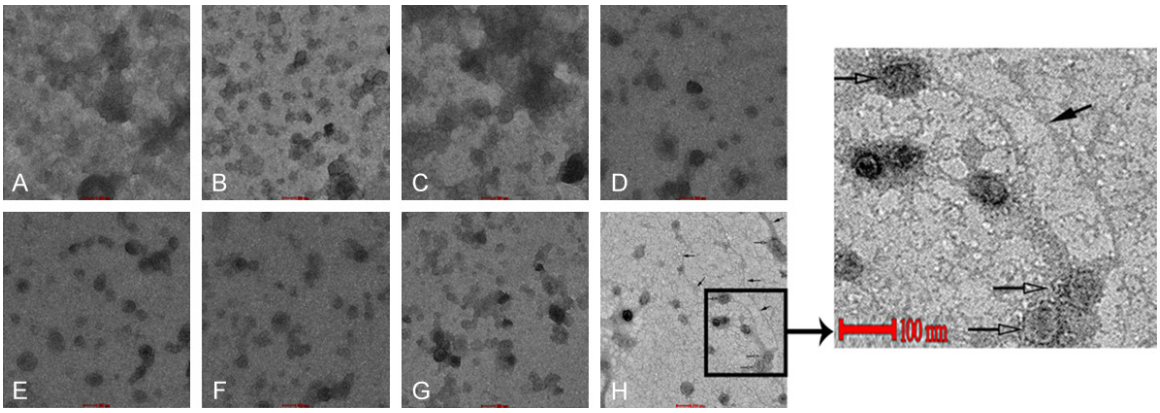

Figure 2. TEM images of urinary sEV enriched by 30-60% Tris D_2O sucrose cushion (H) and seven differential centrifugation protocols (A-G) based on 5-60% discontinuous Tris D_2O sucrose gradient centrifugation at 25°C. The THP network (h: solid arrow) entrapped sEV (h: hollow arrow) in the sample isolated by 30-60% Tris D_2O sucrose cushion.

Table 1. Seven differential centrifugation protocols based on 5-60% discontinuous Tris D_2O sucrose gradient centrifugation at 25°C

	Differential centrifugation protocols
a	differential centrifugation at RT with salt precipitation
b	differential centrifugation at 4°C
c	differential centrifugation at RT
d	differential centrifugation at 4°C with 30 kd ultrafiltration
e	differential centrifugation at RT with 30 kd ultrafiltration
f	differential centrifugation at 4°C with 100 kd ultrafiltration
g	differential centrifugation at RT with 100 kd ultrafiltration

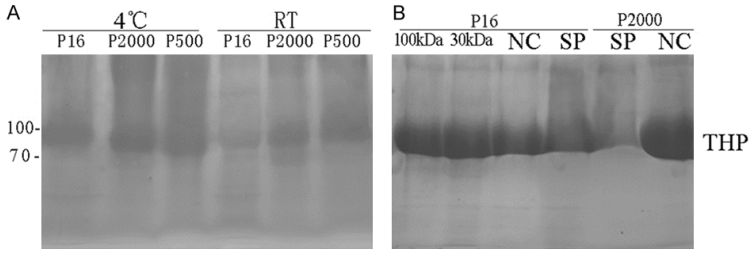

Figure 3. One-dimensional gel electrophoresis of pallets from differential centrifugation protocols. A: Protein pattern of P500, P2000 and P16 obtained after differential centrifugation at 4°C and RT; B: Protein pattern of P16 obtained after differential centrifugation with and without using salt precipitation or ultrafiltration, and P2000 obtained from the second 2000×g centrifugation after salt precipitation. P500: 500×g pallets, P2000: 2000×g pallets, P16: 16,000×g pallets, RT: room temperature, SP: salt precipitation, NC: negative control (protocol without using salt precipitation and ultrafiltration).

The effect of differential centrifugation protocols

To investigate whether the temperature of differential centrifugation and salt precipitation can result in different recovery of urinary sEV, urines of the same volume were centrifuged at 4°C or RT with or without ultrafiltration and at RT with salt precipitation for sEV isolation (**Table 1**). 1-D PAGE showed more THP (large band at around 100 kDa) was precipitated at low speed (500×g and 2000×g) than at 16,000 g. Centrifuging at 4°C precipitated more THP in the three pellets (P500, P2000, P16,000) than at 25°C. THP in P2000 after salt precipitation was much less than that in P2000 before salt precipitation (**Figure 3**). TEM images showed that more exosome-like vesicles were recovered by differential centrifugation without concentration of 30 kd or 100 kd ultrafilter, and the background is darker than centrifugation combining with ultrafiltration (**Figure 2**). Alix, TSG-101, syntenin-1, and THP were more abundant in sEV fractions isolated through differential centrifugation at RT without ultrafilter concentration compared to other protocols (**Figure 4B**).

TRPS analysis was performed under the same membrane pore stretch, voltage current and

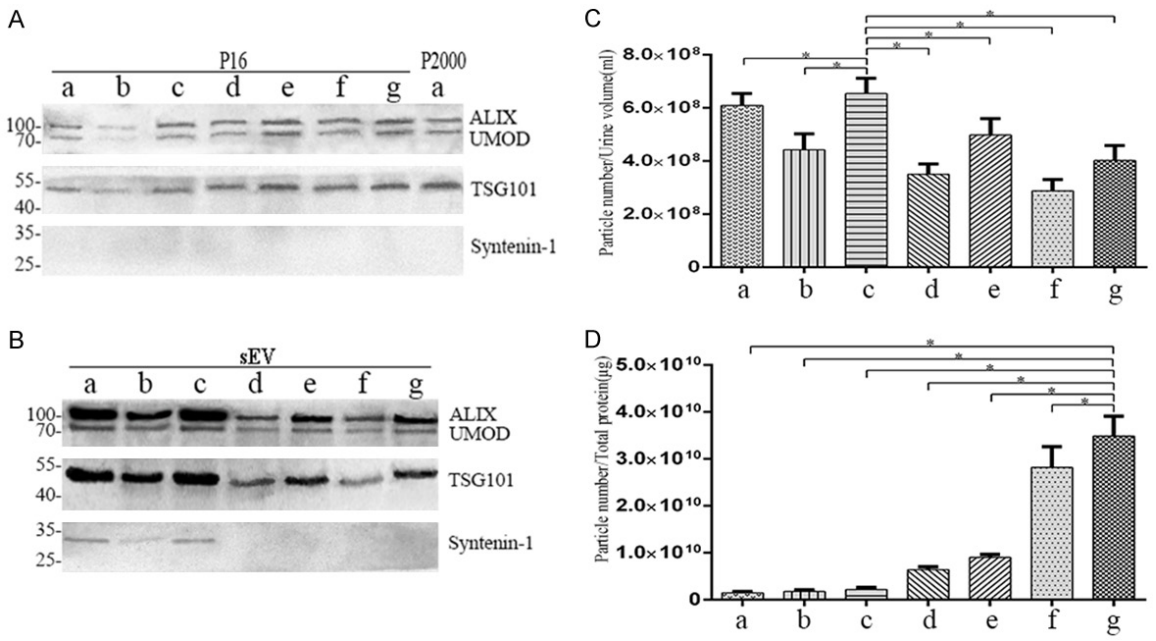

Figure 4. Western blot of pallets and urinary sEV and TRPS particle number counting of urinary sEV sample recovered by seven differential centrifugation protocols (a-g) based on 5-60% discontinuous Tris D_2O sucrose gradient centrifugation at 25°C. A: Western blot of pallets after differential centrifugation. B: Western blot of urinary sEV samples. C: TRPS particle counting of urinary sEV samples. D: Purity of sEV calculated by Particle number/Total protein.

pressure values to get stable and comparable particle-counting results of sEV isolated by different protocols. Differential centrifugation at RT without ultrafiltration and salt precipitation recovered the highest number of nanoparticle, while differential centrifugation at 4°C combining 100 kd ultrafiltration recovered the lowest (**Figure 4C**). However, the purity of sEV obtained through differential centrifugation combining 30 kd or 100 kd ultrafiltration, calculated by nanoparticle number/total protein, was higher compared with differential centrifugation without ultrafiltration. Differential centrifugation at RT combining 100 kd ultrafiltration recovered the highest purity of sEV (**Figure 4D**).

Disrupt the polymeric uromodulin network

To confirm whether reducing agent dithiothreitol (DTT) or detergent Nonidet™ P 40 (NP40) can disrupt the polymeric uromodulin network in the 16,000 g pellet and increase the yield of urinary sEV, SN2 obtained from dissolving the 16,000 g pellet and SN1 were pooled together, followed by using discontinuous sucrose gradient to isolate sEV. Western blot showed the levels of Alix and TSG101 in the crude vesicle were not significantly increased after Tris, NP40 or DTT treatment compared with the PBS group and the untreated group. The levels of Alix and TSG101 in the 16,000 g pellet after treatment were not significantly decreased, but a large amount of AQP2 was transferred from 16,000 g pellet to crude vesicle (**Figure 5A**). Dissolving the 16000 g pellet using Tris, NP40 or DTT and pooling SN1 and SN2 did not significantly increase the level of Alix, TSG101 and syntenin-1 in the final extracted sEV fractions compared with the control and PBS groups (**Figure 5B**). Trps analysis demonstrated there were no quantitative differences between them (**Figure 5C**).

In order to find out why pooling SN1 and SN2 could not increased the yield of sEV, 10 L pooled urine was collected and quartered to perform differential centrifugation. The 16000 g pellets were treated with PBS, 20 mm Tris, 0.1% NP40 and 100 mg/ml DTT respectively. The SN2s were used to isolate sEV separately, but not combined with SN1s first. TRPS analysis showed that sEVs isolated from SN2 were much less than that from SN1, and few exosome-like vesicles could be seen in sEV preparations isolated from SN2 through TEM (**Figure**

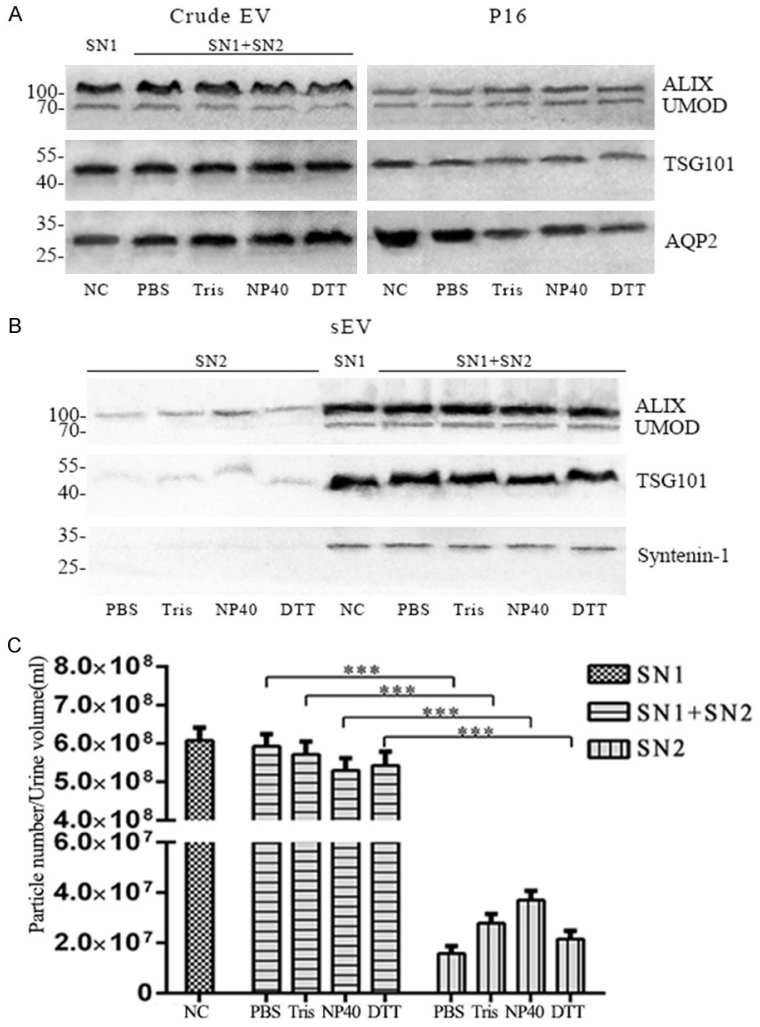

Figure 5. Analysis of sEV-enrichment performance of Tris, DTT and NP40. A: Western blot analysis of P16 and crude EV recovered from SN1 and pooled supernatants (SN1 and SN2). B: Western blot analysis of sEV sample recovered from SN1, SN2 and pooled supernatants (SN1 and SN2). C: TRPS particle counting of sEV sample recovered from SN1, SN2 and pooled supernatants (SN1 and SN2).

6). Tris, NP40 and DTT treatment recovered more Alix and TSG101 than PBS, but the bands were much weaker than that of sEV obtained from SN1 and no syntenin-1 bands was developed. NP40 treatment recovered the highest amount of Alix and TSG101 (**Figure 5B**).

Discussion

The study of extracellular vesicles in urine has been conducted for more than ten years. With the continuous improvement in methodology, methods of EV separation, purification and characterization have been further developed. The experimental requirements for research on EV become more and more rigorous [3, 4]. EV preparation isolated by "High recovery, low specificity" methods, such as PEG precipitation kit, low molecular weight cutoff ultrafiltration with no further separation step, and lengthy or very high speed ultracentrifugation, had been proved to be impure [15, 17-19]. Non-EV components may have an impact on subsequent experiments, and even lead to erroneous conclusions [20, 21]. This is the reason why our research based on the high specificity sEV separation method: density gradient centrifugation. A prominent problem encountered with the separation of sEV in urine is the interference of UMOD. The UMOD can form a reticular and entrap urine EV, which can remove EV during differential centrifugation and also change EV density, thus changing the distribution of EV in the density gradient. Our study found that using PH 8.6 Tris-HCL sucrose gradient solution can obtain more concentrated sEV distribution in gradient at 25°C, indicating that UMOD entrap less EV under alkaline and room temperature conditions compared to neutral and 4°C conditions. Our improvement method can undoubtedly increase the yield of sEV.

Many studies have reported that the use of surfactants or reducing agents to dissolve or disrupt UMOD can increase the yield of EV [14, 22]. However, most studies have only compared the yield of crude vesicles, without using more specific isolation and purification methods. Our study found that the yield of sEV did not increase but decreased slightly and caused the transfer of non-specific EV proteins such as AQP2 to the crude vesicles when mix together SN1 and SN2 after treating P16 by Tris, Np40 or DTT to isolate sEV. sEVs in SN2 obtained after Tris, Np40 and DTT treatment were more than that in SN2 obtained after PBS

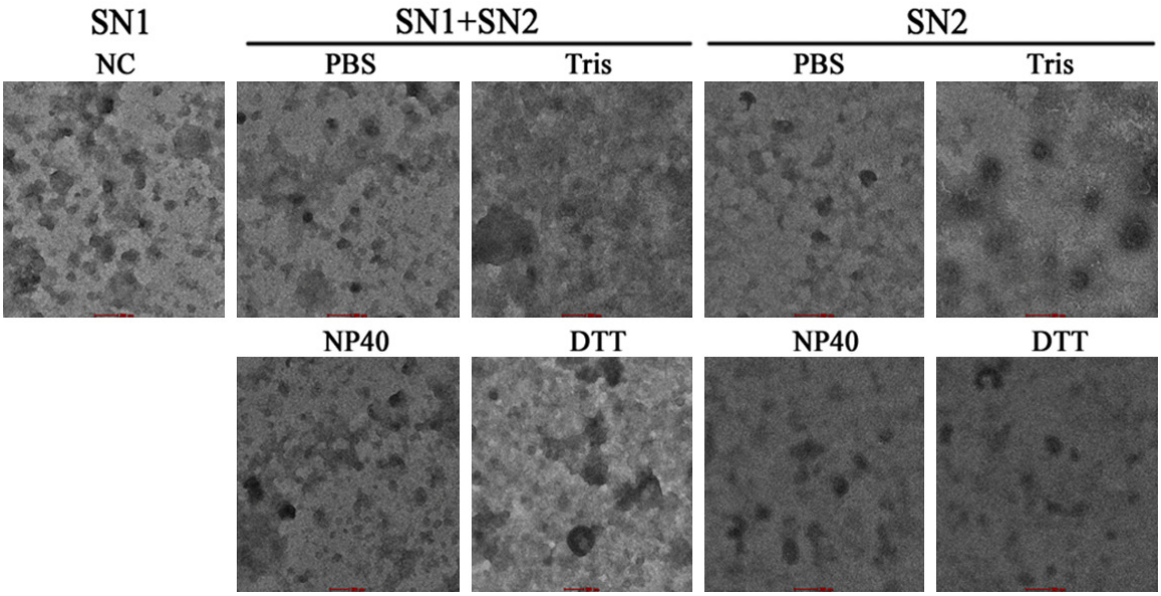

Figure 6. TEM imges of sEV preparation recovered from SN1, SN2 and pooled supernatant (SN1 and SN2) after treatment of PBS, Tris, NP40 and DTT.

treatment, indicating that Tris, Np40 and DTT can release sEV in P16. But the amount of sEV in SN2 is much lower than that in SN1, suggesting that few sEV was released from P16 after Tris, Np40 or DTT treatment. At the same time, we found that the total amount of sEV in the P16 was also very few. 1D gel electrophoresis revealed that most UMOD were removed after the first two steps differential centrifugation, and few UMOD and EV precipitated in P16 after the third step of differential centrifugation. One study used DTT to treat crude vesicle instead of P16 and concluded that the use of DTT did not increase EV production [23]. The reason why the study was inconsistent with most reports was that DTT damaged the UMOD in the crude vesicles, weakening its ability to entrap EVs, so the EV and UMOD obtained after the subsequent ultracentrifugation were correspondingly reduced. Our study found that pooling SN1 and SN2 does not increase the yield of sEV but tends to reduce it. Because the sEV content in P16 is very small, and pooling SN1 and SN2 will cause the reducing agent or surfactant in SN2 to enter into the mixed liquid to destroy the TH protein, then reduce the amount of sEV precipitated in crude EV. Therefore, it is not recommended to using these chemicals to treat P16 to increase the yield of sEV. Not only the effect is not obvious, but also the chemicals may disrupt the biological activity of some EV proteins [22]. We recommended to using 20 mM PH 8.6 Tris-HCL and do not pool SN2 in SN1 when considering the use of chemical reagents to increase the yield of sEV. Crude EVs in SN2 and SN1 should be extracted separately and then combined together to isolate sEV, which may have a certain effect of increasing the yield. And Tris-HCL, as a buffer, have less effect on the activity of proteins than surfactants and reducing agents.

Based on the improved sucrose density centrifugation method, this study compared different centrifugation protocols and found that differential centrifugation at room temperature can significantly increase the yield of sEV. In low temperature, UMOD protein is prone to aggregate and entrap the EV, which will precipitate and be abandoned after differential centrifugation. Salt precipitation could remove a small part of UMOD, but it also removes a small part of EV, so it had no significant effect on improving the purity of sEV, but increased the total time of isolation. Differential centrifugation combined with ultrafiltration reduced the sEV yield significantly, because the ultrafiltration membrane can adsorb EV [14]. Ultrafiltration can significantly reduce the final yield but improve the purity of sEV sample, especially using ultrafiltration membrane with larger molecular weight cutoff. Crude vesicle was obtained in one ultracentrifugation after the

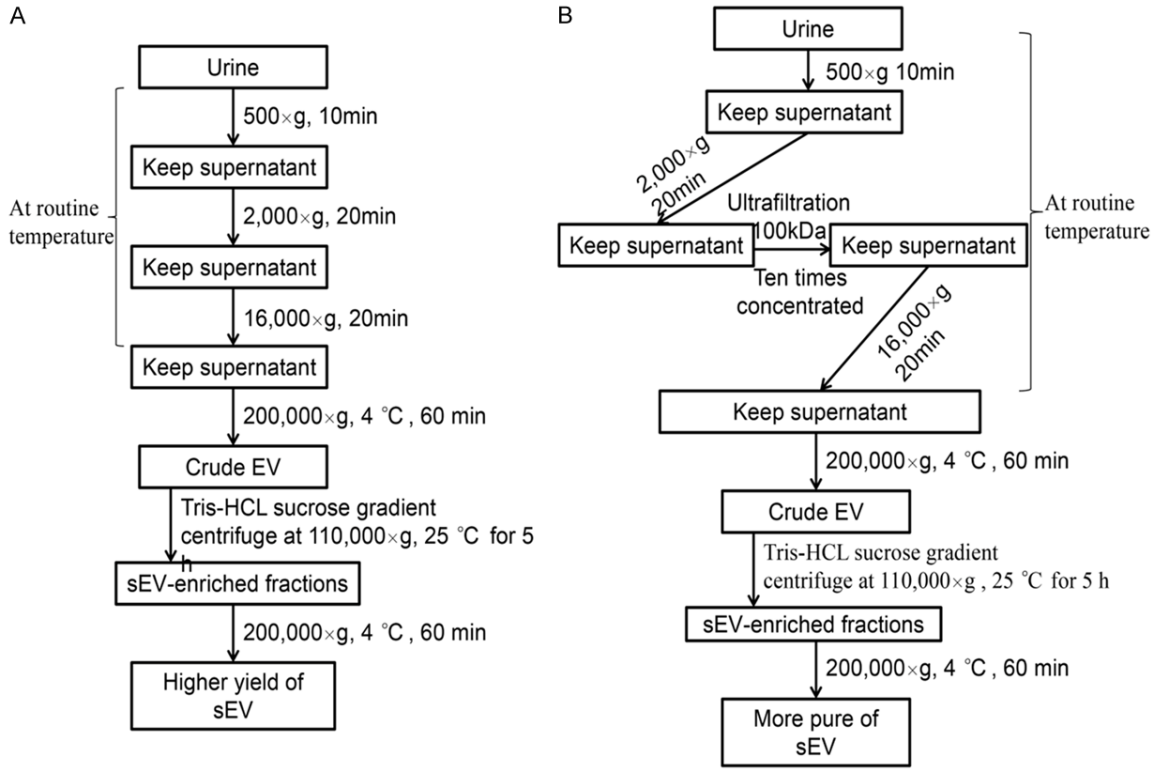

Figure 7. The protocols that achieved higher yield of sEV (A) and higher purity of sEV (B).

ultrafiltration concentration of urine, which can significantly reduce the amount of UMOD in the crude vesicles. At the same time, ultrafiltration can remove small molecular proteins and linear proteins. Without using of ultrafiltration membrane to concentrate urine, more ultracentrifugation times were required to extract crude vesicles, resulting in more UMOD being precipitated in crude vesicles. However, it is not recommended to reuse the ultrafiltration membrane too many times. This will stretch the membrane pore and even destroy the membrane.

In conclusion, our research reveals that modified sucrose density gradient centrifugation method using Tris-HCL sucrose gradient at 25°C can increase the yield of sEV. Differential centrifugation at room temperature without ultrafiltration and salt precipitation can achieve maximum yield of sEV (**Figure 7**). Differential centrifugation combined with 100 kd ultrafiltration at room temperature can obtain the highest purity of sEV. Different from previous discoveries, we found that treating P16 with surfactants and reducing agents and pooling SN2 and SN1 could not increase the yield of sEV. Our findings will contribute to the standardization of urinary sEV isolation and provide rigorous and feasible urinary sEV isolation protocols for the early diagnosis, stratification, treatment and prognosis of diseases in the era of personalized medicine.

Authorship

Qing-Gen Chen carried out the sample preparation, performed the experiment, data preparation and statistics, and drafted the manuscript. Qiong-Hui Zhong, Lian Chen, Lei Zhang and Xia-Hong You Help collecting the specimens and doing experiments. Yu-Huan Jiang, Fan Sun, Shu-Qi Li, Wei-Ming Yang and Qiong-Hui Zhong checked the data and revised the manuscript. Bo Huang and Xiao-Zhong Wang participated in the design and coordination of the study, revised and approved the manuscript. All authors read and approved the final manuscript.

Ethics in publishing

The study was approved by the institutional ethics committees of The Fist and Second Affiliated Hospital of Nanchang University and

all procedures were conducted in accordance with ethical principles.

Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Disclosure of conflict of interest

None.

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