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ORIGINAL RESEARCH

Isolating Astrocyte-Derived Extracellular Vesicles From Urine

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Introduction: Brain-derived extracellular vesicles (BDEVs) can cross the blood-brain barrier and enter the periphery. Therefore, quantifying and analyzing peripherally circulating BDEVs offer a promising approach to directly obtain a window into central nervous system (CNS) pathobiology in vivo. Rapidly evolving CNS diseases require high-frequency sampling, but daily venipuncture of human subjects is highly invasive and usually unfeasible.

Methods: To address this challenge, here we present a novel method for isolating astrocyte-derived extracellular vesicles from urine (uADEVs), combining urine concentration, ultracentrifugation to isolate total EVs, and then glutamate-aspartate transporter (GLAST) EV isolation using an anti-GLAST antibody.

Results: The identity of these GLAST+EVs as uADEVs was confirmed by transmission electron microscopy, nanoparticle tracking analysis, western blotting, and assessment of astrocyte-related neurotrophins.

Conclusions: Leveraging the convenience and availability of urine samples, the non-invasive uADEV approach provides a novel tool that allows high-frequency sampling to investigate rapidly evolving CNS diseases.

Keywords: urinary astrocyte-derived extracellular vesicles, human in vivo, non-invasive, central nervous system, high-frequency sampling

Introduction

Central nervous system (CNS) and neuropsychiatric diseases, such as epilepsy, neurodegenerative diseases, migraine, schizophrenia, depression, and bipolar disorder, remain a management challenge, in part due to the complex and incompletely understood underlying pathobiology.[1] Although direct sampling of the CNS, such as through brain biopsy, could provide tissue for diagnostic and research purposes, the procedure is highly invasive and potentially harmful to patients. Cerebrospinal fluid (CSF) can be collected through lumbar puncture for biological analyses, but again the procedure is invasive and complications, although rare, can be serious. There is therefore a need for non-invasive biomarkers of CNS disorders.

Other characteristics of the CNS, notably the blood-brain barrier (BBB), challenge both the diagnosis and treatment of CNS disorders.[2] The BBB is inherently selective, resulting in significant disparities in the expression levels of molecules found in the CNS and the periphery, making their interpretation difficult, so peripheral blood sampling, although routine, provides only limited representation of the CNS.[3] Furthermore, many CNS diseases like Alzheimer's disease (AD) and Parkinson's disease (PD) have protracted diseases courses; others like multiple sclerosis can relapse and remit; and some, like depression, bipolar disorder, and encephalitis, can rapidly fluctuate in terms of symptom severity. Therefore, while snapshots of reliable protein biomarkers for AD and PD such as Aβ,[4] tau,[5] and α-synuclein[6]

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taken annually or half-annually may be suitable for monitoring the disease course, for disorders with rapid symptom fluctuations, more frequent sampling may be necessary to capture highly dynamic changes. Except for in special settings like the intensive care unit (ICU), daily sampling of peripheral blood or CSF is impractical. Therefore, a new, rapid, and non-invasive method for capturing CNS signals is crucial for both clinical and research purposes.

Extracellular vesicles (EVs) are found in various bodily fluids, including blood, urine, tears, and saliva.[7] EVs have emerged as a promising source of disease biomarkers, serving as "liquid biopsies".[8] Notably, EVs can cross the BBB bidirectionally,[9] making brain-derived EVs (BDEVs) a potential "window to the brain".[10] BDEVs in peripheral blood have been examined as biomarkers of CNS diseases. In a large-sample trial, the concentrations of T-tau, P-T181-Tau, and $A\beta_{1\text{-}42}$ in serum neuro-derived EVs (NDEVs) were linearly associated with their concentrations in CSF, with correlation coefficients close to 0.9.[11] Furthermore, these NDEVs predicted the onset of AD.[12] Similarly, in PD, α-synuclein was increased in NDEVs in patients with PD,[13] and the area under the receiver operating characteristic curve (ROC) exceeded 0.9.[14] In animal studies, plasma astrocyte-derived EVs (ADEVs) and their contents are correlated with brain homogenous (BH) levels.[15] BDEVs in peripheral blood could therefore be good proxies of CNS status.[16] While peripheral blood collection is relatively minimally invasive, daily blood sampling by venipuncture is also usually not feasible nor acceptable to patients. To overcome this limitation, we focused on another body fluid - urine - which also contains a large amount quantities of EVs.[7]

Here, we hypothesized that BDEVs transfer from the bloodstream to urine after plasma filtration in glomeruli, so can be isolated and analyzed as CNS-related molecules.[17,18] Indeed, studies in rats have demonstrated that EVs injected into peripheral blood can be subsequently detected in urine.[19] In humans, neuronal proteins have been detected in urinary total EVs (uTEVs),[20] including elevated levels of ser(P)-1292 LRRK2, a PD-associated protein, in uTEVs of PD patients, which correlated with cognitive and daily function impairments.[21] We therefore developed a protocol that enables enrichment of glutamate/aspartate transporter $(GLAST)^{+}$EVs, believed to be ADEVs,[22–30] from urine (uADEVs).

Materials and Methods

This study was conducted at Renmin Hospital of Wuhan University (Mental Health Center of Hubei Province, Wuhan, Hubei, China) in compliance with the Declaration of Helsinki (revised edition, 2013). The study protocol was approved by both the Human Ethics Committee of Renmin Hospital of Wuhan University. All participants provided informed consent and were free to withdraw from the trial at any time for any reason.

Protocol Summary

Urine samples were first concentrated and uTEVs isolated using ultracentrifugation (UC). uADEVs were isolated using biotin-anti-GLAST-antibody, similar to the isolation of ADEVs from plasma or serum.[22–29,31] A flow chart of this protocol is depicted in Figure 1A.

Isolation of uTEVs

Nine healthy volunteers, six males and three females, participated in the study. The median (IQR) age of the participants was 25.0 (4.0) years 300–600 ml of fresh morning urine was collected from each participant and promptly delivered to the laboratory. Samples were processed within two hours of collection. The urine sample was centrifuged at room temperature (RT) for 30 minutes at 2,000 g, and the supernatant was collected. Subsequently, sodium chloride (NaCl) was added to a concentration of 0.58 M and the urine incubated at RT for 2 hours to eliminate urinary mucoproteins, including Tamm-Horsfall protein.[32,33] The mixture was then centrifuged again at RT for 30 minutes at 8,000 g, and the supernatant was collected. The sample was filtered using a 0.45 μm filter membrane (Millipore, MA, USA, Catalog# HVLP07625), and then loaded into a concentration device (Amicon® stirred cell, Millipore, MA, USA, Catalog# UFSC40001) and ultrafiltered to a volume of 3–4 ml using a 10 kDa NMW ultrafiltration (UF) disc membrane (Millipore, MA, USA, Catalog# PLGC07610). Next, 200 ml of PBS was added, and the sample was ultrafiltered to a volume of approximately 3–4 ml, resulting in a concentrated component. The concentrated component was transferred to an ultracentrifuge tube and centrifuged at 150,000 g at 4°C for 150 minutes (SW60Ti, OptimaXE-100, Beckman Coulter, Fullerton, CA, USA). The parameter UC (duration of ultracentrifugation) was determined in a pilot study (Supplementary Material 1. The Determination of the

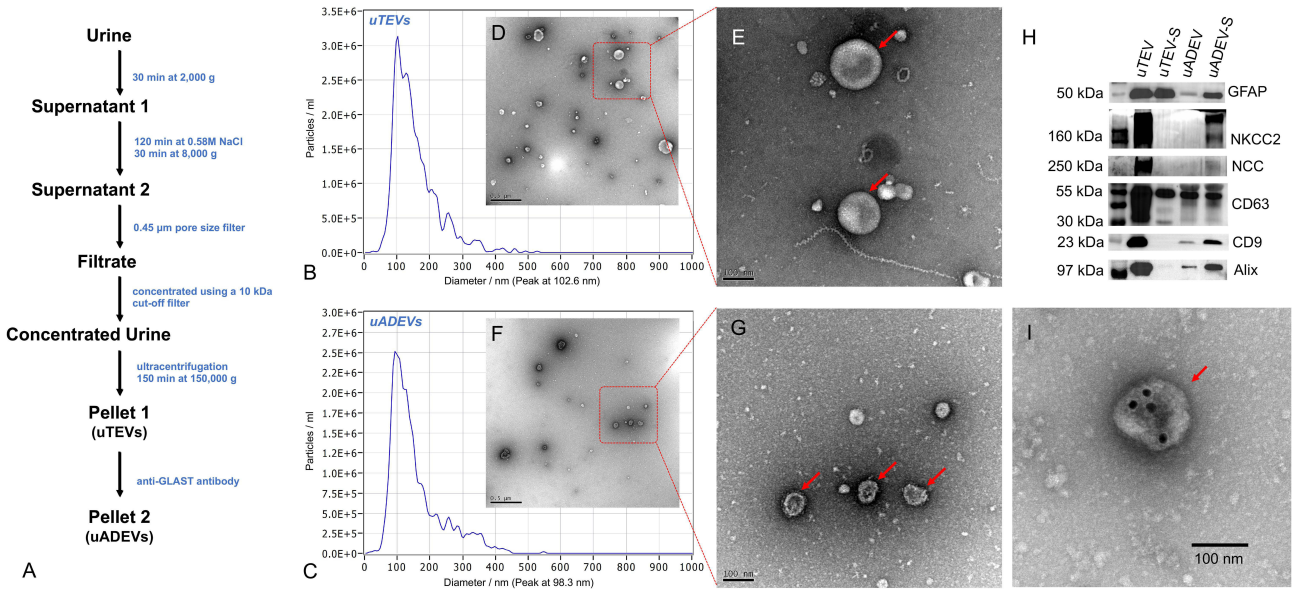

Figure 1 Isolation and validation of uADEVs. (**A**) Schematic diagram of the uADEV isolation protocol. (**B** and **C**) NTA results for uTEVs and uADEVs. The dilution factors for uTEVs and uADEVs were 1,000 and 40,000, respectively. (**D**–**G**) TEM images of uTEVs (indicated by red arrows) and uADEVs (indicated by red arrows). Scale bars: 0.5 μm for **D** and **F**, 100 nm for **E** and **G**). (**H**) Results of western blotting: three EV markers (CD63, CD9 and Alix) and an astrocyte marker (GFAP) were present in ADEV samples, while two kidney markers (NKCC2 and NCC) were absent. The supernatants (-S) were used as controls. The first lane contains the ladder. For each target protein, the protein concentrations were standardized using the bicinchoninic acid assay (BCA): 5.7 μg per lane for CD81, CD9, and ALIX; 1.5 μg per lane for NCC; 4 μg per lane for NKCC2 and CD63; 13.7 μg per lane for GFAP. (**I**) Immunogold-labeled uADEVs (indicated by the red arrow) with anti-GLAST antibody (back dots).

Duration of Ultracentrifugation). The supernatant was discarded, and the precipitate was resuspended in 840 μl Dulbecco's phosphate-buffered saline (DPBS, Beyotime, Shanghai, China, Catalog# C0221D) containing protease and phosphatase inhibitors (PPICs, Beyotime, Catalog# P1046). This resulted in a uTEV sample.

Isolation of uADEVs

Of the 840 μl uTEVs sample, 700 μl was mixed with 100 μl 3% bovine serum albumin (BSA, Beyotime, Catalog# ST023-50g) and incubated for 1 hour at RT with 8 μl of anti-GLAST (ACSA-1)-biotin antibody (Miltenyi Biotec, Bergisch Gladbach, Germany, Catalog# 130–118-984). Subsequently, 20 μl of streptavidin-agarose resin (Thermo Fisher Scientific, Waltham, MA, Catalog# 53116) and 80 μl of 3% BSA were added, followed by incubation for 60 minutes at RT. After centrifugation at 800 g for 10 minutes at 4°C and removal of the supernatant, each sample was resuspended in 200 μl of cold 0.1M glycine-HCl (pH = 3.0) by gently mixing for 30 seconds. The suspension was then centrifuged at 4,000 g for 10 minutes at 4°C, and the supernatant was collected. Several drops of 1M Tris-HCl (pH = 8.0, Beyotime, Catalog# ST780-500ml) was added to adjust the pH to 7.0. This resulted in a uGLAST^{+}EV sample (approximately 216 μl). For western blotting and protein measurements, mammalian protein extraction reagent (M-PER, Thermo Fisher Scientific, Catalog# 78503) with PPICs was added to each uADEV or uTEV sample.

Validation of uADEVs

Transmission Electron Microscopy (TEM)

Similar to our previous ADEV studies,[29,31] TEM (JEM-1230, JEOL, Tokyo, Japan) was used to image EVs. For direct evidence, immuno-electron microscopy (JEM1400, JEOL, Japan) staining for the astrocyte marker GLAST was conducted using a 10 nm gold-labeled secondary antibody for uADEVs samples.

Nanoparticle Tracking Analysis (NTA)

The diameter (nm) and concentration (particles/ml) of EV samples were determined using the ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) with ZetaView 8.04.02 nanoparticle tracking software (Particle Metrix, Meerbusch, Germany).

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Western Blotting

Western blotting was conducted to detect: (i) three EV markers, using primary rabbit anti-cluster of differentiation (CD)63 antibody (Abcam, Cambridge, UK, Catalog# ab134045), rabbit anti-CD9 antibody (Abcam, Catalog# ab125011), and mouse anti-Alix antibodies (Proteintech, Rosemont, IL, Catalog# 67715-1-Ig); (ii) an astrocyte marker, using rabbit anti-glial fibrillary acidic protein (GFAP) antibody (Abcam, Catalog# ab68428); (ii) and two kidney markers, using Na^+-K^+-Cl^- cotransporter (NKCC) 2 (Abcam, Catalog# ab171747) and sodium-chloride cotransporter (NCC) (Abcam, Catalog# ab95302) antibodies.

Protein Quantification

Astrocyte-related neurotrophins (brain-derived neurotrophic factor (BDNF), epidermal growth factor (EGF), fibroblast growth factor (FGF)-2, glial cell-derived neurotrophic factor (GDNF), GFAP, nerve growth factor beta (NGF-β), and S100 calcium-binding protein B (S100B)) were measured using the Human ProcartaPlex™ Simplex kit (Thermo Fisher Scientific, Catalog# PPX-07).

Statistical Methods

For comparisons between uTEVs and uADEVs, concentrations of neurotrophins (pg/ml) were normalized to a reference of 10E+10 particles/ml, yielding values in pg/per 10E+10 particles, according to MISEV2018.[7] Fold-changes in uADEVs/uTEVs ratios were calculated for both particle and neurotrophin concentrations. Welch's two sample *t*-tests were employed to test differences between the uADEVs and uTEVs samples for each parameter. A two-sided *p*-value of <0.05 was considered statistically significant. All statistical analyses were performed using R version 4.2.0 (R Project for Statistical Computing) within RStudio version 1.4.1106 (RStudio).

Results

Validation of uTEVs and uADEVs

Figure 1 shows the schematic of uADEV isolation and their validation using NTA, TEM, and western blotting. NTA confirmed that the EV diameters were within the expected size range for small EVs. TEM images revealed characteristic EV-like structures in both uTEV and uADEV samples. Western blotting showed positive expression of three EV markers (CD63, CD9, and Alix) in both uTEV and uADEV samples. Additionally, uADEVs exhibited positive expression of an astrocyte marker (GFAP). Notably, two kidney markers, NCC2 and NKCC, were detected in the uTEV sample but not in uADEVs samples. Furthermore, immunogold electron microscopy provided direct confirmation that the GLAST molecule was present on the surface of uADEVs.

Comparisons Between uTEVs and uADEVs

Particle concentrations in uADEVs (5.3 + 1.6E+10/ml) were significantly lower than in uTEV samples (1.9 + 0.78E+12/ml). Given the sample volumes of uADEVs (216 µl) and uTEVs (840 µl), uADEVs constitute an estimated 0.86% of uTEVs. Considering the efficiency of the immune-isolation method, this might be an under estimate of the true proportion. In contrast, neurotrophin levels were significantly higher in uADEVs than in uTEVs, with 23.1 to 88.1-fold increases for the seven neurotrophins (Table 1).

Table 1 Fold Changes in Target Expression in uADEVs Compared With uTEVs

Variable	In uADEV (pg) [a]	In uTEV (pg) [a]	Fold-Change Increase		*p*-value
	Mean ± SD	Mean ± SD	Mean ± SD	Median [IQR]	
BDNF	0.625 ± 0.246	0.010 ± 0.005	88.1 ± 69.3	61.0 [33.8; 112.1]	<0.001
EGF	38.153 ± 9.364	2.761 ± 2.126	23.1 ± 18.1	17.6 [8.3; 32.6]	<0.001
FGF-2	3.747 ± 1.991	0.162 ± 0.124	28.8 ± 16.5	29.9 [19.9; 30.6]	0.001
GDNF	23.355 ± 4.966	0.897 ± 0.685	32.8 ± 11.4	31.0 [28.8; 44.0]	<0.001
GFAP	7.826 ± 6.890	0.285 ± 0.203	29.9 ± 17.3	29.0 [15.0; 33.6]	0.011
NGF-β	0.985 ± 0.879	0.041 ± 0.028	40.4 ± 47.5	13.0 [9.6; 80.7]	0.012
S100β	0.765 ± 0.219	0.029 ± 0.021	43.7 ± 34.6	35.4 [17.8; 59.9]	<0.001

Notes: [a] Normalized to every 10^{10} particles.

Discussion

Here we present a method to extract ADEVs from urine, paving the way for the non-invasive tracking of specific molecular in vivo signals within the CNS using an abundant, readily available biosample. This uADEVs protocol has promise as a groundbreaking, non-invasive approach for daily CNS monitoring, serving as a valuable tool for biomarker discovery and etiological studies of rapidly evolving CNS diseases.

Methodological Considerations for uADEVs Isolation and Validation

Considering the principles of this protocol, uADEVs isolation requires a target protein that is exclusively expressed on the surface of astrocytes and not on the surface of urogenital tract cells. The commonly used target is GLAST.[22–29,31] To confirm the specificity of GLAST, we interrogated the Human Protein Atlas (HPA) database,[34] in which GLAST showed significant expression on the surface of astrocytes in the brain and no evidence of expression by urogenital tract cells. In contrast, the commonly used target protein for extracting neuro-derived EVs, L1CAM, was expressed in the kidneys. Thus, for proof-of-concept of isolating BDEVs from urine, we selected $GLAST^+$ EVs as uADEVs to ensure that they originated from astrocytes.

Since most EVs in uTEVs are derived from the urogenital tract,[35] it is important to assess contamination of uADEVs with urogenital components. To address this, we selected two markers commonly used in urinary EV research, NKCC2 and NCC,[36,37] to evaluate urogenital contamination in uADEVs. Western blot images revealed that NKCC2 and NCC were highly expressed in uTEV samples, as expected, but were barely detectable in uADEV samples (Figure 1H). This suggests minimal urogenital contamination of uADEVs samples. Combined with evidence of neurotrophic factor, these $GLAST^+$ EVs are likely to primarily originate from astrocytes, namely, uADEVs.

Ultracentrifugation has been widely used to enrich for uTEVs, typically using parameters of 100,000 g for 70 minutes, with a second cycle to further isolate uTEVs.[38–44] Although this method yields relatively pure uTEVs, the yield is low. Since uADEVs are relatively rare in uTEV samples, it was crucial to first enhance the yield of isolated uTEVs. A straightforward approach to increasing the yield is by extending the centrifugation time.[45] Therefore, we conducted a preliminary experiment to optimize uTEV yield, finding that 150,000 g for 150 minutes obtained the highest yield of uTEVs (Supplementary Material 1). Moreover, TEM images (Figure 1E–G) suggested that this parameter setting did not significantly introduce contamination.

uADEV Function: Selectively Isolating CNS Signals From uTEVs

Since uADEVs are a subset of uTEVs, and some CNS-related molecules were detected in uTEVs, it might be possible to analyze uTEVs directly rather than isolating uADEVs. However, a key advantage of isolating uADEVs is that they help to ensure that the observed between-group differences in target molecules specifically originate from astrocytes, even if the same molecules are present in other cells, tissues, or organs (Figure 2).

Considering the example of cytokines, inflammation plays a crucial role in many CNS diseases, and astrocytes are major cytokines producer within the CNS.[46,47] However, other immune cells throughout the body also produce cytokines. While uTEVs have been used to detect cytokines in urine,[48,49] such measurements might not accurately reflect changes within the CNS due to the presence of cytokines from peripheral sources. However, measuring cytokines specifically in uADEVs could overcome this limitation and allow for more precise tracking of cytokine changes within the CNS. For instance, GFAP is a potential biomarker for diseases such as traumatic brain injury, intracerebral hemorrhage, and stroke, with elevated levels detected in the blood and urine of affected patients.[50,51] However, GFAP levels may show heterogeneity due to interference from non-CNS-derived signals,[52,53] limiting its reliability as a biomarker for CNS-related disease. However, enrichment of ADEVs for GFAP detection would help to reduce interference from non-CNS sources, ensuring that the signal originates specifically from the CNS rather than other cell types.[54]

Additionally, for ubiquitously expressed molecules, such as those in common signaling pathways or some receptors, measuring the concentration of these molecules in uTEV samples is unlikely to accurately reflect CNS levels, as uADEVs only represent ~1% of uTEVs.

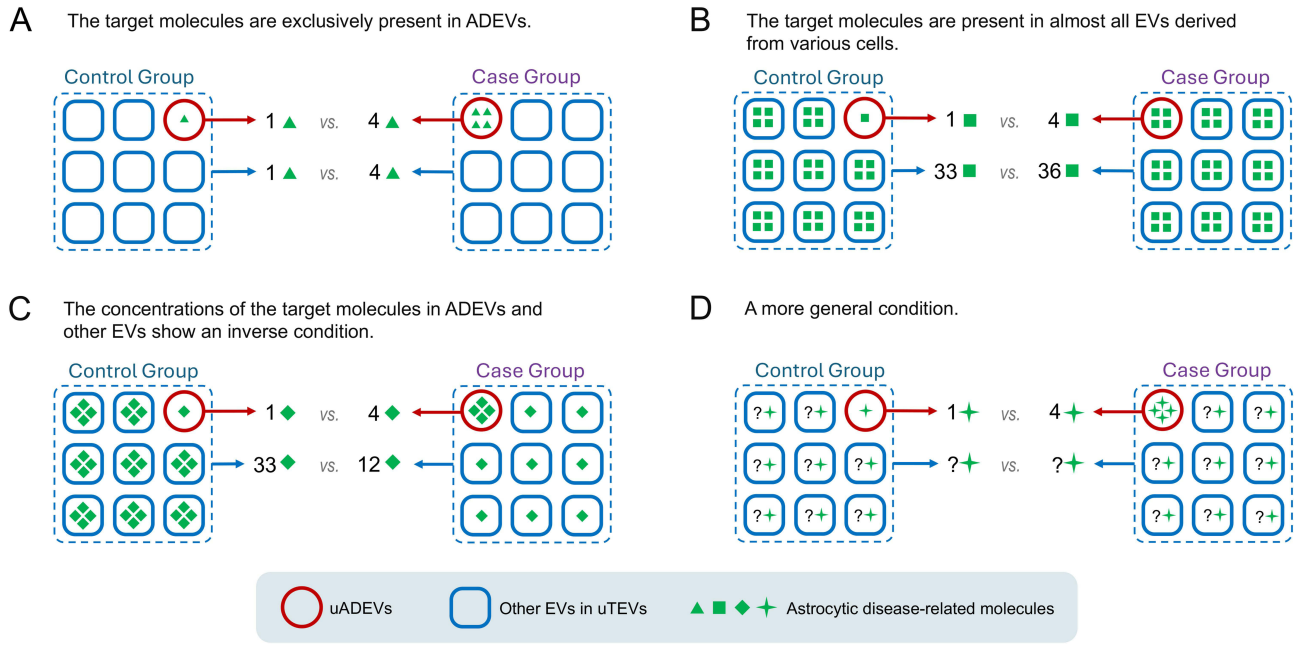

Figure 2 uADEVs ensure accurate measurement of molecular signals from astrocytes. To facilitate discussion, we present a hypothetical scenario in which astrocytic disease-related molecules (represented by green shapes) are overexpressed in the case group compared to the control group. The numbers provided are for illustrative purposes only and do not represent the exact ratios. (**A**) In this scenario that target molecules are exclusively expressed in ADEVs, there is no difference between measuring these green triangles in uADEVs and uTEVs. However, in the real world, many molecules are not exclusively overexpressed in one cell type. Then, (**B**) targeted molecules (green squares) are widely expressed throughout the body and not exclusively in astrocytes. In this scenario, the between-group difference of these green squares will be much higher in uADEVs than in uTEVs. Furthermore, due to the BBB, the concentrations of many molecules in the periphery and CNS are not correlated, and may even be even inversely correlated. As in (**C**), the concentrations of target molecules (green rhombuses) in the periphery and astrocytes are inversely correlated. In this scenario, measuring the between-group difference of these green rhombuses in uADEVs still provides the correct results, while measuring them in uTEVs may even provide opposite erroneous results. (**D**) shows a more general condition, where there is no prior precise distributional information of the targeted molecules (green stars). Nevertheless, we can still obtain the correct between-group differences by measuring uADEVs. In summary, uADEVs ensure that the observed group differences of the target molecules originate specifically from astrocytes in the CNS, without being influenced by the biodistribution of the same target molecules in other cells, tissues, or organs.

Potential Benefits for Trial Design and Data Analyses

Urine is an ideal body fluid for biomarker detection, as it is abundant, easy to collect at regular, frequent intervals, and non-invasive. The enhanced temporal resolution offered by uADEVs could improve CNS disease trial design, especially for rapidly progressing diseases where timely and frequent biomarker identification is critical (Figure 3A). As detection technologies continue to advance, the required urine volume may decrease, potentially enabling hourly sampling and

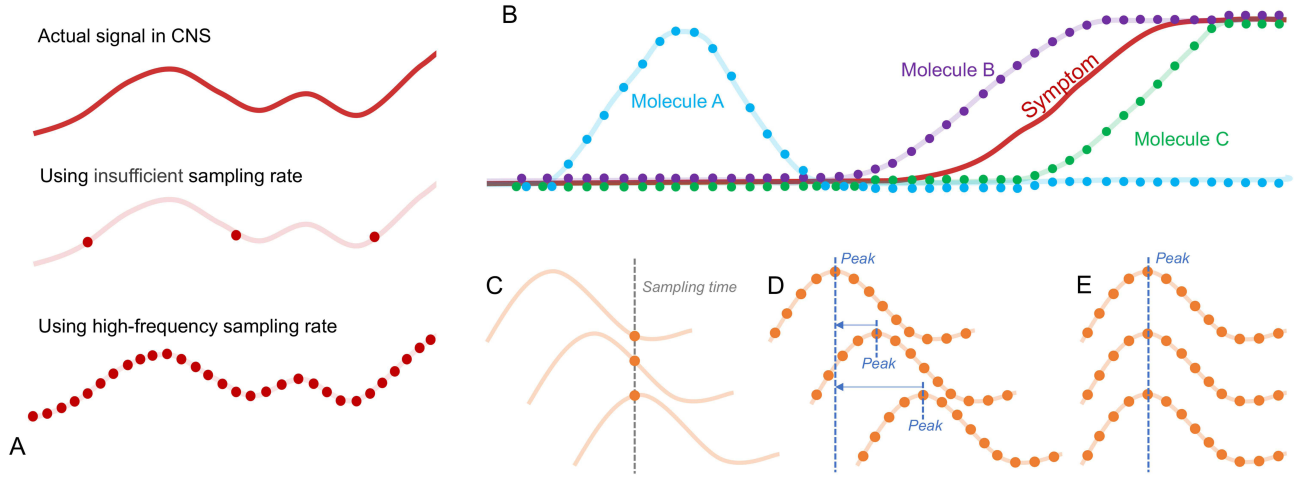

Figure 3 Potential benefits of high-frequency sampling of uADEVs for trial design and data analyses. (**A**) The advantage of high-frequency sampling. (**B**) The detailed trajectories of target molecules may benefit the exploration (Molecules A and B) and rejection (Molecule **C**) of hypotheses. (**C**–**E**) Even under the assumption that all patients have the same molecular trajectories, time-induced heterogeneity may still exist merely due to different sampling points along the trajectory (**C**). However, detailed trajectories (**D**) allow application of *post-hoc* algorithms, such as peak-based realignment (**E**), to reduce the time-induced heterogeneity.

capturing CNS dynamics at an unprecedented level. uADEVs therefore allow us to capture in vivo molecular "movies" of the CNS, rather than capturing static "snapshots".

Beyond biomarker discovery, uADEVs offer a valuable tool for testing potential hypotheses related to the underlying pathobiology. As illustrated in Figure 3B, if a candidate molecule's trajectory in uADEVs aligns with, but lags behind, the symptom trajectory, the molecule is more likely to be a consequence or confounding factor rather than the cause of the observed symptom. Conversely, only candidate molecules with trajectories preceding the symptom trajectory are likely to be causal.

Identifying reliable biomarkers for rapidly changing CNS disorders like depression and bipolar disorder is difficult, primarily because both the disease and biomarker expression are highly heterogeneous. Even if all patients with a specific disease shared the same underlying molecular trajectory (unlikely, but useful for illustration), differences in sampling time points also introduce heterogeneity (Figure 3C). This "time-induced heterogeneity" can be a significant obstacle in disease monitoring. However, uADEVs, sampled at high frequency, may offer a potential solution. By capturing individual molecular trajectories over time (Figure 3D), uADEVs could provide more detailed longitudinal data for analysis. This would also allow the use of some *post-hoc* algorithms, like realignment based on peaks (Figure 3E), to reduce time-induced heterogeneity. While real-world data are more complex, increasing the sampling frequency with uADEVs paves the way for exploring various data processing algorithms, ultimately leading to a deeper understanding of CNS disorders. While this represents a simplified model and heterogeneity manifests in various forms with greater complexity in real-world data, we believe that high-frequency sampling provides more opportunities for data processing using multiple algorithms, enabling deeper exploration.

Furthermore, our study not only develops a novel method for CNS monitoring but also suggests a potentially new research paradigm. The uADEVs protocol can be adapted to isolate other specific EVs from uTEVs originating from various cell types, tissues, and organs. If successfully implemented, this approach could unlock the vast potential of urine samples for studying a wide range of diseases beyond the urogenital system.

Limitations

First, while we used UC here, size-exclusion chromatography (SEC) might be suitable for labs lacking this equipment. Additionally, ultrafiltration (UF) can lead to a significant loss of uTEVs. Employing high-capacity UC tubes to directly collect uTEVs without UF may potentially increase uADEV yield, but this requires further investigation. Second, the absolute particle number of uADEVs is low, limiting their use in multi-omics-based high-throughput assays. To address this limitation, we are actively developing methodologies that enable such studies using minimal quantities of uADEVs. Third, the impact of other disease states, particularly urological diseases, on uADEVs remains unclear. Further research is needed to address this question. Fourth, the mechanism by which ADEVs cross the glomerular basement membrane into urine remains unknown. Elucidating this mechanism may significantly enhance the utility of uADEVs. Fifth, this study utilized fresh urine samples, and applicability of the protocol to frozen or concentrated urine samples after thawing needs investigation. Future studies should investigate the effect of sample storage conditions on uADEVs. Furthermore, compared with unconcentrated urine samples, storing concentrated urine samples could increase the storage capacity of biobanks.

Conclusion

Here we propose a simple method for isolating urinary ADEVs, paving the way for non-invasive monitoring of CNS in vivo activity with high sampling rates, up to daily or even more frequently. This approach, coupled with appropriate signal processing algorithms, holds promise for identifying novel biomarkers or exploring the pathology of rapidly evolving CNS diseases. Furthermore, the uADEVs protocol can be further adapted to isolate other specific EVs originating from other cells types from urine samples. This holds promise for using urine to study a wider range of diseases beyond the urological system.

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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.

Disclosure

The authors declare that they have no competing interests.

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