Altered Amyloid-β and Tau Proteins in Neural-Derived Plasma Exosomes in Patients with Type 2 Diabetes and Orthostatic Hypotension

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Research

Abstract

Background

Emerging evidence suggests a role for orthostatic hypotension (OH) in contributing to the progression of Alzheimer disease (AD). The aim of the study was to investigate whether neural-derived plasma exosomal amyloid-β and tau protein levels are associated with OH in diabetes mellitus (DM) patients.

Methods

There were 274 subjects without dementia included in the study: 81 control participants (controls), 101 normotensive patients with DM without OH, and 92 patients with DM and neurogenic OH (DMOH). Neuronal-derived exosomal proteins were measured by ELISA kits for amyloid-β and tau.

Results

The neuronal-derived exosome levels of Aβ42, T-tau, and P-T181-tau in the DM with OH group were higher than those in the DM and control groups. Multivariable linear regression analysis showed that the presence of OH in patients with DM was associated with elevated exosomal Aβ42 (β = 0.172, P = 0.018), T-tau(β = 0.159, P = 0.030), and P-T181-tau (β = 0.220, P = 0.003) levels after adjustment for age, sex, APOE ε4, duration of type 2 diabetes, HbA1c and cardiovascular risk factors. Furthermore, the levels of Aβ42, T-tau, and P-T181-tau in neuronal-derived exosomes were correlated with HIF-1α levels and the drop in mean cerebral blood flow velocity from the supine to upright position.

Conclusions

The presence of OH in DM patients was independently associated with elevated the Aβ42, T-tau, and PT181-tau levels in neural-derived plasma exosomes. Cerebral hypoperfusion from DM with OH are likely candidate mechanisms.

Trial registration

Chinese Clinical Trial Registry (Identifier: ChiCTR1900021544). Registered 27 February 2019.

Introduction

Diabetes mellitus (DM) has become a major public health concern worldwide, with an increasing number of instances of diabetes and possible severe diabetes-related complications. Autonomic dysfunction is a common and serious complication of DM. Orthostatic hypotension (OH), *as a hallmark of diabetic autonomic neuropathy, is usually irreversible and difficult to manage with medications [1]*. Some studies have demonstrated that OH is associated with an increased risk of mild cognitive impairment (MCI) and Alzheimer's disease (AD) [2, 3], as well as substantially accelerated progression from MCI to AD [4], suggesting that OH may promote AD pathogenesis. OH leads to cerebral hypoperfusion, which, if

regularly occurring, may cause the accumulation of amyloid and tau hyperphosphorylation in the brain and thereafter cognitive decline or dementia [5, 6].

Exosomes are one class of endosome-derived membrane vesicles shed by most cell types that contain various molecular constituents, including proteins of their cellular origin [7]. Neurally derived exosomes are released into not only the cerebrospinal fluid(CSF) but also the blood under physiological and pathological conditions [8, 9]. The levels of plasma exosomal biomarkers reflect pathological brain changes. Jia et al. [10] reported that the levels of Aβ42, T-tau, and P-T181-tau in blood-neuronal-derived exosomes were highly correlated with their levels in CSF in amnestic mild cognitive impairment and AD patients. Moreover, studies demonstrated that Aβ42, P-T181-tau, and P-S396-tau in blood neuronal-derived exosomes can predict the development of AD up to 10 years before clinical onset [11]. In this study, we hypothesized that one mechanism underlying the association between OH and AD is OH leading to cerebral hypoperfusion and increased Aβ and tau protein levels in the brain. We tested this hypothesis by examining neural-derived plasma exosomal amyloid-β and tau protein levels in patients with type 2DM with and without OH.

Methods

Study subjects

Subjects were prospectively recruited from Weihai Municipal Hospital between February 2019 and February 2020. The Mini-Mental Status Examination(MMSE) was used as a general cognitive screening with a cut off of 27 for controls and patients with diabetes mellitus. There were 274 subjects without dementia included in the study: 81 control participants (controls), 101 normotensive patients with DM without OH, and 92 patients with DM and neurogenic OH (DMOH). All groups were matched for age, male: female ratio, and education. Controls were not excluded for taking antihypertensive medications as long as they were normotensive at the time of testing and had no evidence of OH. OH was defined as an orthostatic drop in the systolic blood pressure (BP) of at least 20 mmHg and/or in the diastolic BP of at least 10 mmHg during the first 3 minutes of standing or being positioned with a head-up tilt on a 60-degree tilt table [12]. Personal backgrounds, *any* medications, and current and past medical histories were recorded for all subjects. Participants also received the Hamilton Anxiety Rating Scale (Ham-A) and the17-item version of the Hamilton Rating Scale (Ham-D17). The *Toronto Clinical Neuropathy Score (*TCNS*) was used to* evaluate neuropathy. The exclusion criteria were as follows: (1) a concomitant neurological disorder that could potentially affect cognitive function or a family history of dementia; (2) a history of cardiovascular problems and stroke or other factors that may influence cerebral blood flow; (3) an abnormal finding on routine transcranial Doppler (TCD), such as middle cerebral artery (MCA) stenosis or vasospasm; (4) a poor temporal window on conventional TCD; (5) patients who were unable to continue TCD monitoring with head-up tilting(HUT) due to severe symptoms associated with orthostasis, such as syncope/presyncope, headache, faintness, dizziness, or significant tachycardia (> 150 beats per minute); and (6) other serious heart, lung, liver, kidney, or brain diseases that affect quality of life.

This study was approved by the Institutional Review Board of Weihai Municipal Hospital. In addition, written informed consent was obtained from every participant.

Assessment of orthostatic hypotension

Participants were instructed to remain on all medications as prescribed and to eat a light breakfast the morning of testing. Testing was scheduled for an 11 A.M. start time to minimize diurnal effects on hemodynamics. Before testing, all participants were allowed a 20-minute period of rest in the supine position to establish physiological and psychological equilibration. The assessments were performed in a quiet room by the same examiner. Blood pressure and heart rate were measured in the supine position after lying down for at least 5 minutes, and measurements were repeated at 1 minute and 3 minutes after standing using a fully automatic electronic sphygmomanometer (Omron HBP-1300; Omron Healthcare, Inc, Dalian, China). OH can be categorized into early OH occurring only at 1 minute after standing and delayedand/or prolonged OH occurring at 1 and 3 minutes after standing [13].

Head-up tilt test with cerebral blood flow measurements

All Doppler measurements were continuously monitored by a Digi - Lite TCD (RIMED, Israel). The 2 - MHz probes were fixed bilaterally over the temporal bone windows using a stable headset (RIMED PW SN12 - 2516). Cerebral blood flow velocity (CBFV) was taken from the mean values of the envelope curves registered simultaneously in the M1 segment of the left MCA at a depth of 50 - 60 mm as well as in the P2 segment of the right posterior cerebral artery (PCA) at a depth of 60 - 65 mm. After a resting period of 15 minutes in the supine position, the recording of CBFV was performed. Then, the subjects were tilted to an 80°head-up position with the use of a tilt table. Measurements of CBFV were repeated 3 minutes after the head-up positioning. Testing was performed at 10 A.M. and 12 P.M. in a quiet air - conditioned room at 23°C standard temperature.

Measurement of serum concentrations

Blood samples were obtained from patients between 6 and 7A.M. after overnight fasting. Samples were centrifuged (402 g, 10 min) to segregate serum and then stored at -70°C until assayed. The serum hypoxia-inducible factor-1α (HIF-1α) levels were measured according to a standard enzyme-linkedimmunosorbent assay (ELISA) kit (RayBiotech, Inc., Norcross, GA, USA) according to the manufacturer's instructions. The interassay and intraassay precisions were < 10%.

Collection of neuronal-derived exosomes from blood, the detection of *exosomes,* and the quantification of exosomal proteins

Fasting blood was sampled between 6 and 7A.M. and stored in a polypropylene tube containing EDTA. After drawing, the blood samples were centrifuged at 4000 g for 10 min to obtain the plasma. Specific neuronal-derived exosomes were immediately separated for consistency according to a published protocol [14]. Then, 0.5 ml of plasma was incubated with 0.15 ml of thromboplastin-D (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 60 minutes, and 0.35 ml of calcium- and

magnesium-free Dulbecco's phosphate-buffered saline (DPBS)(Thermo Fisher Scientific, Waltham, MA, USA) with protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) was added. After centrifugation at 3000 g for 20 minutes at 4°C, supernatants were incubated with ExoQuick exosome precipitation solution (SEXOQ; System Biosciences, CA) and incubated at 4°C for 1 hour. After centrifugation at 1500 g for 30 minutes at 4°C, each pellet was resuspended in 250 μl of DPBS. Each exosome suspension received 100 μl of 3% bovine serum albumin (BSA) (Thermo Fisher Scientific, Waltham, MA, USA) and was incubated for 2hours at 4°C each with3 μlof rabbit anti-L1 cell adhesion molecule (L1CAM) antibody (clone 5G3; eBiosciences, San Diego, USA). Then, 25 μl of streptavidin - agarose resin (Thermo Fisher Scientific, Waltham, MA, USA) containing 50 μl of 3% BSA was added. After centrifugation at 400g for 10 minutes at 4°C and removal of the supernatant, each pellet was suspended in 50 μl of 0.05 M glycine - HCl (pH 3.0) by vortexing for 10 minutes. Each suspension then received 0.4 ml of M-PER mammalian protein extraction reagent (Thermo Fisher Scientific, Waltham, MA, USA) that had been adjusted to pH 8.0 with 1 M Tris - HCl (pH 8.6).These suspensions were incubated at 37°C for 10 minutes and vortexed for 15 seconds before storage at -80°C until use in enzyme-linked immunosorbent assays (ELISAs).

Western blotting was used to detect the protein marker of exosomes, namely, TSG101, using a monoclonal rabbit anti - human TSG101 antibody according to the manufacturer's instructions (1:500, Abcam, Cambridge - UK). Centrifuged samples and immunoprecipitated samples were used to identify plasma neuronal - derived exosomes, and supernatants were used as negative controls.

Transmission electron microscopy (TEM) was used to identify the exosomes according to a published protocol with minor modifications [15]. After immunoprecipitation, the isolated neuronal-derived exosomes were stored in 1% paraformaldehyde, dehydrated through a graded series of ethanol and embedded in Epon. Ultrathin sections (65 nm) were stained with uranyl acetate and Reynold's lead citrate. Finally, the samples were analyzed by a JEM - 1400 plus transmission electron microscope.

Neuronal-derived exosomal proteins were measured by ELISA kits for human Aβ42 (Thermo Fisher Scientific kit), total tau (Abcam kit), and tau phosphorylated at threonine 181 (Abcam kit). The amount of CD81 protein was measured to normalize the exosomal content. The mean value for all determinations of CD81 in each assay group was set at 1.00, and the relative values for each sample were used to normalize their recovery [11]. Exosomal protein assays were performed by investigators blinded to clinical and OH data.

Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 22.0 (IBM, Armonk, NY). Categorical variables were analyzed using the chi-squared test. Tests on the homogeneity of variances were performed. Numerical data, such as the concentrations of amyloid-β and tau proteins in exosomes and group differences, were analyzed by using analyses of variance with Tukey post hoc analysis. Correlative analysis was performed using a linear regression model. All tests were two-tailed, and the threshold for statistical significance was $P < 0.05$.

Results

Clinical and demographic characteristics of enrolled participants

Table 1 shows the clinical and demographic characteristics of the enrolled controls and patients with DM with and without OH. There were no significant differences in age, sex, years of education, the rate of hypertension, hyperlipidemia, or current drinking and smoking (P > 0.05) between the groups. The DM and DMOH groups were similar with regard to BMI, aspirin or statin medications, and diabetic medication use(P > 0.05). Compared with the control and DM groups, the DMOH group had the lowest antihypertensive medication use (P < 0.05) and MMSE scores (P < 0.05). There were significantly higher TCNS, HIF-1α, and HbA1c levels in the DMOH group than in the control (P < 0.05) and DM groups (P < 0.05). No participants were on antihypotensive medications.

Identification of exosomes

The neuronal-derived exosomes were confirmed by transmission electron microscopy and western blotting. The representative transmission electron microscopy image of an OSA patient's exosomes clearly shows typical exosome - sized (30 - 150 nm diameter) vesicles in harvested exosome pellets (Fig. 1A), which were positive for the exosome marker TSG101 in the exosomal samples but not in the supernatants or negative controls, as confirmed by western blotting (Fig. 1B).

Hemodynamic information while supine and standingin different groups

There were no significant group differences in SBP, DBP, heart rate, or mean cerebral blood flow velocity (mCBFV) in the supine position. After transitioning to the standing position, there was a significantly greater reduction in SBP, DBP, and mCBFV in the DMOH group compared with the control and DM groups (P < 0.05, Table 2). There were no significant group differences in heart rate while standing.

Levels of Aβ42, T-tau, and P-T181-tau in neural-derived plasma exosomes

Cross-sectional comparisons of 101 patients with DM, 92 patients with DM with OH and 81 matched controls revealed that the exosomal concentrations of Aβ42 in the DM with OH group (3.12 ± 0.85 pg/ml) were higher than those in the DM (2.81 ± 0.84 pg/ml, P = 0.012) and control groups (2.62 ± 0.75 pg/ml, P < 0.001) (Fig. 2A). There were no significant differences in the levels of Aβ42 between the DM (2.81 ± 0.84 pg/ml) and control groups (2.62 ± 0.75 pg/ml, P = 0.101) (Fig. 2A). The exosomal concentrations of T-tau in the DM with OH group (166.97 ± 32.06 pg/ml) were higher than those in the DM (154.01 ± 32.15 pg/ml, P = 0.006) and control groups (145.31 ± 32.83 pg/ml, P < 0.001) (Fig. 2B). There were no significant differences in the levels of T-tau between the DM (154.01 ± 32.15 pg/ml) and control groups (145.31 ± 32.83 pg/ml, P = 0.075) (Fig. 2B). Compared to the controls (41.23 ± 10.11 pg/ml), the exosomal concentrations of P-T181-tau in the DM (45.07 ± 11.65 pg/ml, P = 0.019) and DM with OH groups (50.65 ± 12.82 pg/ml, P < 0.001) were significantly higher (Fig. 2C). Furthermore, the exosomal P-T181-tau levels in the DM with OH group (50.65 ± 12.82 pg/ml) were higher than those in the DM group (45.07 ± 11.65 pg/ml, P = 0.002)(Fig. 2C).

Among 92 patients with DM with OH, 27 patients had a diagnosis of early OH and 65 patients had delayed and/or prolonged OH. Compared with the patients with DM with early OH (Aβ42: 2.83 ± 0.78 pg/ml; T-tau: 156.81 ± 23.54 pg/ml; P-T181-tau: 45.93 ± 10.31 pg/ml), the exosomal concentrations of Aβ42 (3.24 ± 0.85 pg/ml, P = 0.030) (Fig. 3A), T-tau (171.18 ± 34.28 pg/ml, P = 0.024) (Fig. 3B), and P-T181-tau (52.62 ± 13.31 pg/ml, P = 0.012) (Fig. 3C) in the DM with delayed and/or prolonged OH were higher.

Relationship between exosomal Aβ42, T-tau, and P-T181-tau levels and the presence of OH in patients with DM

Multivariable linear regression analysis with Aβ42, T-tau, and P-T181-tau as dependent variables and age, sex, APOE ε4, group, HbA1c and cardiovascular risk factors as independent variables. We found that, compared with controls, the presence of OH in patients with DM was an independent factor associated with exosomal Aβ42 (β = 0.269, P = 0.004), T-tau (β = 0.371, P < 0.001), and P-T181-tau (β = 0.296, P = 0.002) levels. In addition, compared with patients with DM without OH, the presence of OH in patients with DM was independently associated with exosomal Aβ42 (β = 0.172, P = 0.018), T-tau (β = 0.159, P = 0.030), and P-T181-tau (β = 0.220, P = 0.003) levels after adjustment for age, sex, APOE ε4, duration of type 2 diabetes, HbA1c and cardiovascular risk factors.

Correlation between the levels of Aβ42, T-tau, and P-T181-tau and HIF-1α and mCBFV in patients with DM

We performed correlation analysis and found that the levels of Aβ42 in neuronal-derived exosomes were correlated with HIF-1α levels (R^2 = 0.093, *P*< 0.001) (Fig. 4A) and $^\triangle$mCBFV ($^\triangle$mCBFV was defined as the drop in mCBFV from the supine to upright position) (R^2 = 0.166, P < 0.001) (Fig. 4B). The levels of T-tau in neuronal-derived exosomes were correlated with HIF-1α levels (R^2 = 0.153, P < 0.001) (Fig. 4C) and $^\triangle$mCBFV (R^2 = 0.180, P < 0.001) (Fig. 4D). *In addition*, there was a correlation between the levels of P-T181-tau in neuronal-derived exosomes and HIF-1α levels (R^2 = 0.177, P < 0.001) (Fig. 4E) and $^\triangle$mCBFV (R^2 = 0.226, P < 0.001) (Fig. 4F). There was no correlation between the Aβ42, T-tau, and P-T181-tau levels and the mean cerebral blood flow velocity (mCBFV) in the supine or 80°head-up position.

Discussion

In the present study, we provide additional evidence that DM with neurogenic OH is associated with markers of altered pathological proteins in AD. The neuronal-derived exosome levels of Aβ42, T-tau and P-T181-tau in the DM with OH group were higher than those in the DM and control groups. Furthermore, the exosomal T-tau and P-T181-tau levels in the DM with OH group were higher than those in the DM group. Multivariable linear regression analysis showed that the presence of OH in patients with DM was associated with elevated exosomal Aβ42, T-tau, and P-T181-tau levels. This association was independent of age, sex, duration of type 2 diabetes, HbA1c and cardiovascular risk factors. In addition, the levels of Aβ42, T-tau, and P-T181-tau in neuronal-derived exosomes were correlated with HIF-1α levels and the drop in mean cerebral blood flow velocity from the supine to upright position.

To our knowledge, this is the first study to examine changes inamyloid-β and tau protein levels in neural-derived plasma exosomes in patients with DM with OH. AD-associated proteins, such as Aβ and tau protein, are secreted in exosomes during their formation in the brain [16, 17]. Exosomes can cross the blood-brain barrier and be detected in the peripheral blood [18]. In this study, we isolated neural exosomes from plasma by immunoabsorption of the L1CAM antibody, which mainly represents changes in the nervous system. Our findings supported that OH in patients with DM maylead to increased accumulation of amyloid plaques and tau protein in the nervous system. The exact mechanism governing the link between patients with DM with OH and elevated AD-associated proteins remains unknown. One possibility isthat OH leads to cerebral hypoperfusion, with subsequent consequences on amyloid-β and tau protein levels. Many studies have shown that cerebral hypoperfusion significantly increases β- and γ-secretase activity, consequently increasing Aβ production in the brain [5, 6, 19]. In addition to increased Aβ generation, hypoperfusion affects peptidases that degrade Aβ peptides, thus reducing Aβ clearance [20, 21]. Aβ deposition in small arteries caused by cerebral hypoperfusion could further induce cerebrovascular lesions and worsen cerebral hypoperfusion, finally leading to a vicious cycle and irreversible damage [22, 23]. A possible mechanism by which hypoperfusion upregulates APP processing and leads to Aβ accumulation could be that hypoperfusion induces HIF-1 expression, which then binds to the promoter of β-secretase and consequently increases its expression [24]. HIF-1αis also involved in hypoperfusion-induced blood-brain barrier disruption, which impairs Aβ transport and clearance [25]. In this study, we found that the presence of OH in patients with DM was independently associated with elevated Aβ42 levels in neural-derived plasma exosomes. Exosomal Aβ42 levels were positively correlated with HIF-1α levels in patients with DM.

In the present study, compared to the controls, the exosomal concentrations of P-T181-tau in the DM group were significantly higher. The results are consistent with animal histopathologic data showing that type 2 DM is associated with hyperphosphorylation of neuronal tau [26]. Moran C, et al. also found that there is a strong relationship between type 2 DM and the amount of p-tau in human cerebrospinal fluid [27]. Furthermore, the results of the present study showed thatthe presence of OH in patients with DM was independently associated with elevated T-tau and P-T181-tau levels in neural-derived plasma exosomes. In line with previous studies performed in normal cognition, reductions in cerebral blood flow were associated with increased cerebrospinal fluid total tau and phosphorylated tau [28, 29]. There are several pathways through which cerebral hypoperfusion may contribute to increased levels of neuronal tau in the brain. A previous study showed that tau may be normally modified via the attachment of a monosaccharide to prevent phosphorylation [30]. However, this modification has been shown to be downregulated when cerebral blood flow is reduced, resulting in increased phosphorylation of tau [31]. In addition, the results of a study by Song and colleagues showed that acute cerebral blood flow reductions inhibited the activity of protein phosphatase 2A, which functions to dephosphorylate tau [32]. Our findings suggest that OH effects on neuronal tau protein levels may be independent and possibly additive to the effect of type 2 DM. The *exact* mechanisms through which OH may increase the concentration of tau and affect tau phosphorylation in patients with DM need further study.

Aβ overproduction and Tau hyperphosphorylation may appear to be very sensitive to cerebral hypoperfusion. Koike et al. found that a single, mild reduction in cerebral blood flow has profound and long-lasting effects on Aβ overproduction and tau hyperphosphorylation in 3xTg-AD mice [5]. There were significant correlations between the levels of Aβ42 or tau protein and the severity of hypoperfusion in our study. We found that the levels of Aβ42 and tau protein in neuronal-derived exosomes were correlated with a decrease in the mean cerebral blood flow velocity from the supine to upright position in patients with DM. Moreover, compared with patients with DM with early OH, the exosomal concentrations of Aβ42, T-tau, and P-T181-tau in patients with DM with delayed and/or prolonged OH were higher. This is in line with previous findings that patients with delayed and/or prolonged OH are at a greater risk of cognitive decline or incident dementia in initially non-demented individuals than are patients with early OH [3], as they are more likely to experience longer periods of cerebral hypoperfusion.

Our results have some limitations. First, the results were drawn from a small-scale hospital-based study, and future investigations are necessary to replicate and validate our findings in a large population of patients. Second, the present investigation was a cross-sectional study, and we need to conduct a longitudinal study to investigate the relationship between the levels of exosomal Aβ and tau and the decline in cognitive functions of DM patients. Third, additional information, such as pathology or cerebrospinal fluid data, was not available to confirm the results.

Conclusions

We demonstrated that the presence of OH in DM patients was independently associated with elevated the Aβ42, T-tau, and PT181-tau levels in neural-derived plasma exosomes. Cerebral hypoperfusion from DM with OH are likely candidate mechanisms. Given the high prevalence of OH, if the effects on Aβ and tau could be mitigated with treatment, improving OH diagnosis and treatment could potentially reduce AD risk on a broad scale.

Abbreviations

OH: Orthostatic hypotension;AD: Alzheimer disease; DM: Diabetes mellitus; Aβ: Amyloid-β; MCI: Mild cognitive impairment; CSF: Cerebrospinal fluid; MMSE: The Mini-Mental Status Examination; BP: Blood pressure; Ham-A: Anxiety Rating Scale; Ham-D17: Hamilton Rating Scale; TCNS: The Toronto Clinical Neuropathy Score; TCD: transcranial Doppler; MCA: middle cerebral artery; HUT: head-up tilting; CBFV: Cerebral blood flow velocity; PCA: Posterior cerebral artery; HIF-1α: Hypoxia-inducible factor-1α; BSA: Bovine serum albumin; L1CAM: Anti-L1 cell adhesion molecule; ELISAs: Enzyme-linked immunosorbent assays; TEM: Transmission electron microscopy; BMI: body mass index; TC: Total cholesterol; LDL-C: lowdensity lipoprotein cholesterol; ACEI: angiotensin-converting enzyme inhibitors; ARBs: angiotensin II receptor blockers; CCBs: calcium channel blockers; SBP: systolic blood pressure; DBP: diastolic blood pressure; HR: heart rate; bpm: beats per minute; mCBFV: mean cerebral blood flow velocity.

Declarations

Acknowledgements

Availability of data and materials

The data supporting the conclusions of this article are available from the corresponding author upon request.

Contributors

FL, and JZ contributed to the conception and design of the study; HC, TW, TS, BL, and HS contributed to the acquisition and analysis of data; JZ, SZ, and ZL contributed to the drafting of the text and preparation of the figures.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Weihai Municipal Hospital in accordance with the Declaration of Helsinki. Informed written consent was obtained from all subjects.

Consent for publication

All authors approved the final manuscript for submission and gave consent for publication.

Competing interests

The authors declare that they have no competing interests.

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32. Song B, Ao Q, Wang Z, et al. Phosphorylation of tau protein over time in rats subjected to transient brain ischemia. Neural Regen Res 2013; 8: 3173 - 3182.

Tables

Table 1 Demographic and clinical characteristics

	Control(n = 81)	DM (n = 101)	DM with OH(n = 92)
Male/female	36/45	45/56	40/52
Age (years)	68.75± 6.64	67.26± 7.86	68.43± 7.08
Education (years)	8.62± 4.85	8.43± 5.16	8.02± 4.69
APOE ε4 positive, n (%)	12(14.8)	16(15.8)	16(17.4)
Duration of type 2 diabetes (years)	NA	9.46± 3.43	10.71± 3.91 [b]
HbA1c (%)	5.07± 0.98	7.66± 1.76 [a]	8.42± 2.57 [a, b]
TCNS	1.3± 1.0	4.9± 2.7[a]	11.2± 4.1 [a, b]
BMI (kg/m^2)	24.31± 2.86	25.04± 2.75	25.21± 2.98 [a]
Hypertension, n(%)	16(19.8)	24(23.8)	24(26.1)
Hyperlipidemia, n(%)	31(38.3)	43(42.6)	42(45.7)
Current smoker, n(%)	6(7.4)	9(8.9)	6(6.5)
Current drinker, n(%)	11(13.6)	15(14.9)	12(13.0)
Antihypertensive use, n(%)	15(18.5)	19(18.8)	3(3.3) [a, b]
ACEI, n (%)	3(3.7)	2(2.0)	NA
ARBs, n (%)	4(4.9)	4(4.0)	3(3.2)
ARBs plus CCBs, n (%)	2(2.5)	3(3.0)	NA
CCBs, n (%)	6(7.4)	10(10.0)	NA
Diabetic medication			
The use of insulin, n(%)	NA	51(50.5)	51(55.4)
Sulfonylureas, n(%)	NA	19(18.8)	15(16.3)
Nateglinide or repaglinide, n(%)	NA	17(16.8)	15(16.3)
Biguanides, n(%)	NA	57(56.4)	47(51.1)
α-Glucosidase inhibitor, n(%)	NA	52(51.5)	41(44.6)
Aspirin, n (%)	10(12.3)	25(24.8)[a]	26(28.3)[a]
Statins medications, n (%)	14(17.3)	34(33.7)[a]	37(40.2)[a]
Mecobalamin, n (%)	NA	18(17.8)	57(62.0)[b]
Vitamin B1, n (%)	NA	16(15.8)	52(57.1)[b]

HIF-1⍺(pg/ml)	121.4± 11.5	136.3± 12.7 [a]	147.2± 19.3 [a, b]
MMSE	28.6± 1.0	28.4± 0.95	28.0± 1.0 [a, b]

Abbreviations: DM, diabetes mellitus; OH, orthostatic hypotension; NA, not applicable; TCNS, Toronto Clinical Neuropathy Score; BMI, body mass index; TC, Total cholesterol; LDL-C, lowdensity lipoprotein cholesterol; ACEI, angiotensin-converting enzyme inhibitors; ARBs, angiotensin II receptor blockers; CCBs, calcium channel blockers; HIF-1⍺, Hypoxia inducible factor-1⍺; MMSE, Mini-Mental State Examination.

[a] Significant at $P ≤ 0.05$ vs controls.

[b] Significant at $P ≤ 0.05$ vs DM without OH.

Table 2 Hemodynamic information while supine and during standing position

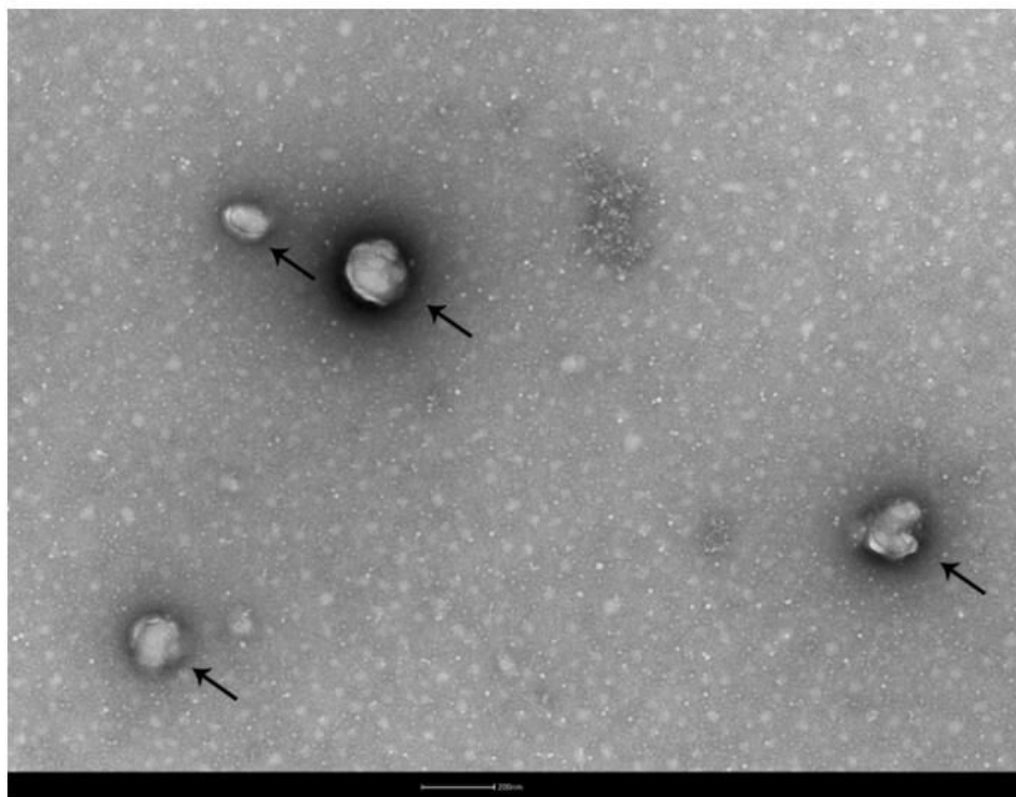|	Control(n = 95)	DM (n = 107)	DM with OH(n = 94)
Supine hemodynamics			
SBP, mm Hg	133.8± 12.6	135.4± 11.9	137.2± 14.4
DBP, mm Hg	79.5± 9.9	80.1± 9.4	82.4± 10.6
HR, bpm	71.9± 8.4	72.3± 9.1	73.8±9.6
mCBFV, cm/s	46.9± 8.8	45.4± 8.5	44.6± 7.8
Change following upright position			
SBP, mm Hg	-1.9± 7.6	-4.8± 5.3 [a]	-27.2± 8.7 [a, b]
DBP, mm Hg	3.4±5.3	4.5± 6.7	-13.7± 4.3 [a, b]
HR, bpm	6.9± 3.2	7.4± 3.8	7.7± 4.0
mCBFV, cm/s	-4.3± 2.3	-5.3± 2.6 [a]	-8.9± 4.4 [a, b]

Abbreviations: DM, diabetes mellitus; OH, orthostatic hypotension; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; bpm, beats per minute, mCBFV, mean cerebral blood flow velocity.

[a] Significant at $P ≤ 0.05$ vs Controls.

[b] Significant at P 0.05 vs DM without OH.

Figures

A
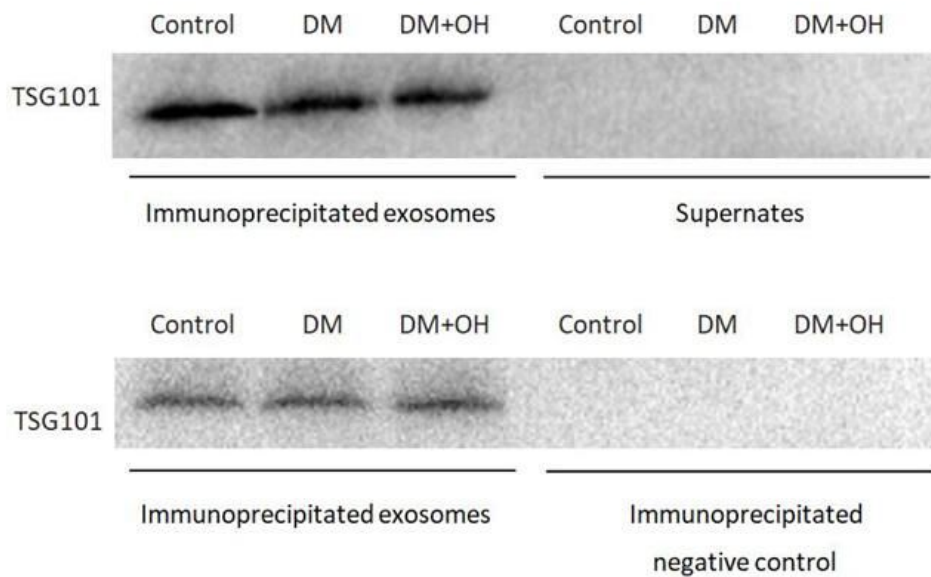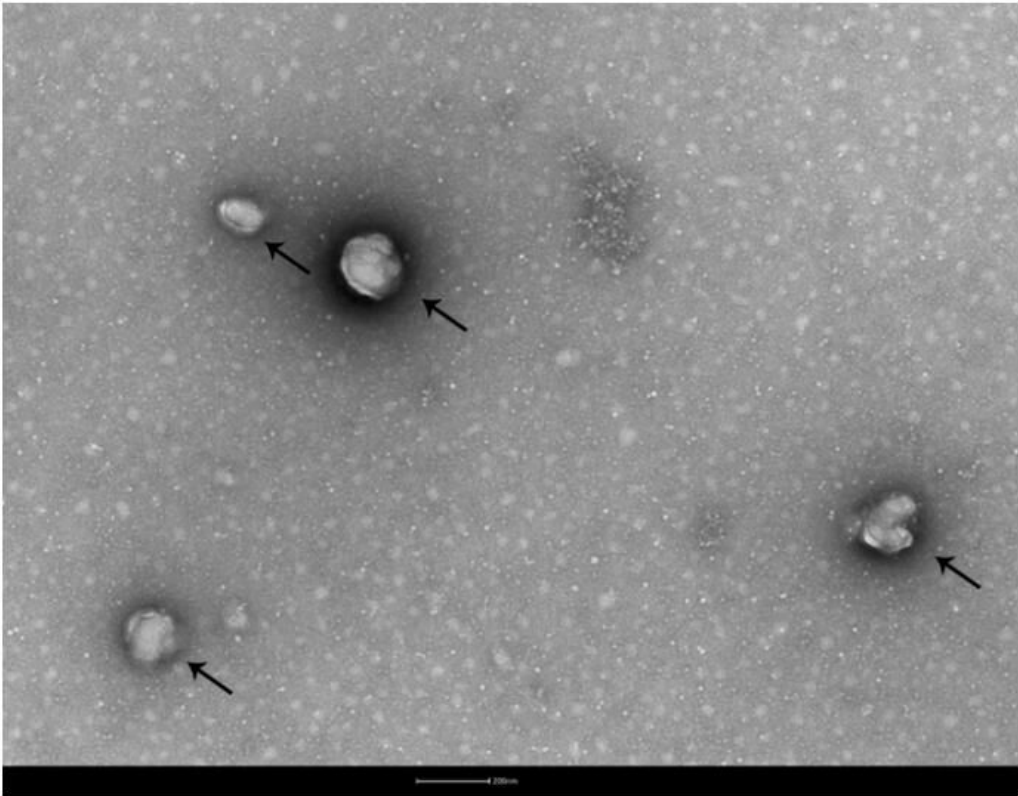

B

	Control	DM	DM+OH	Control	DM	DM+OH
TSG101						

Immunoprecipitated exosomes   Supernates

	Control	DM	DM+OH	Control	DM	DM+OH
TSG101						

Immunoprecipitated exosomes   Immunoprecipitated
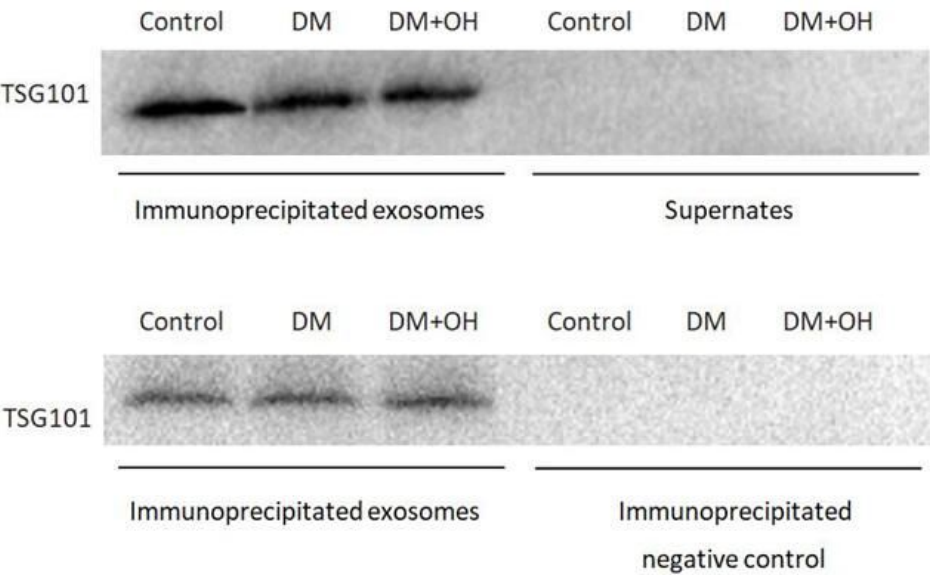   negative control

Figure 1

Transmission electron microscopy(TEM) and Western blot were used to identify the exosomes. (A) TEM image showing neuronal-derived exosomes (black arrows) that were successfully collected. (B) Western blot images showing that the exosomal marker TSG101 was highly expressed in exosomal samples but not detected in supernatants. Moreover, an additional negative control was set after the ExoQuick

immunoprecipitation step with beads alone not linked to L1CAM neural adhesion protein. Western blots showing that TSG101 was not detected in the negative control.
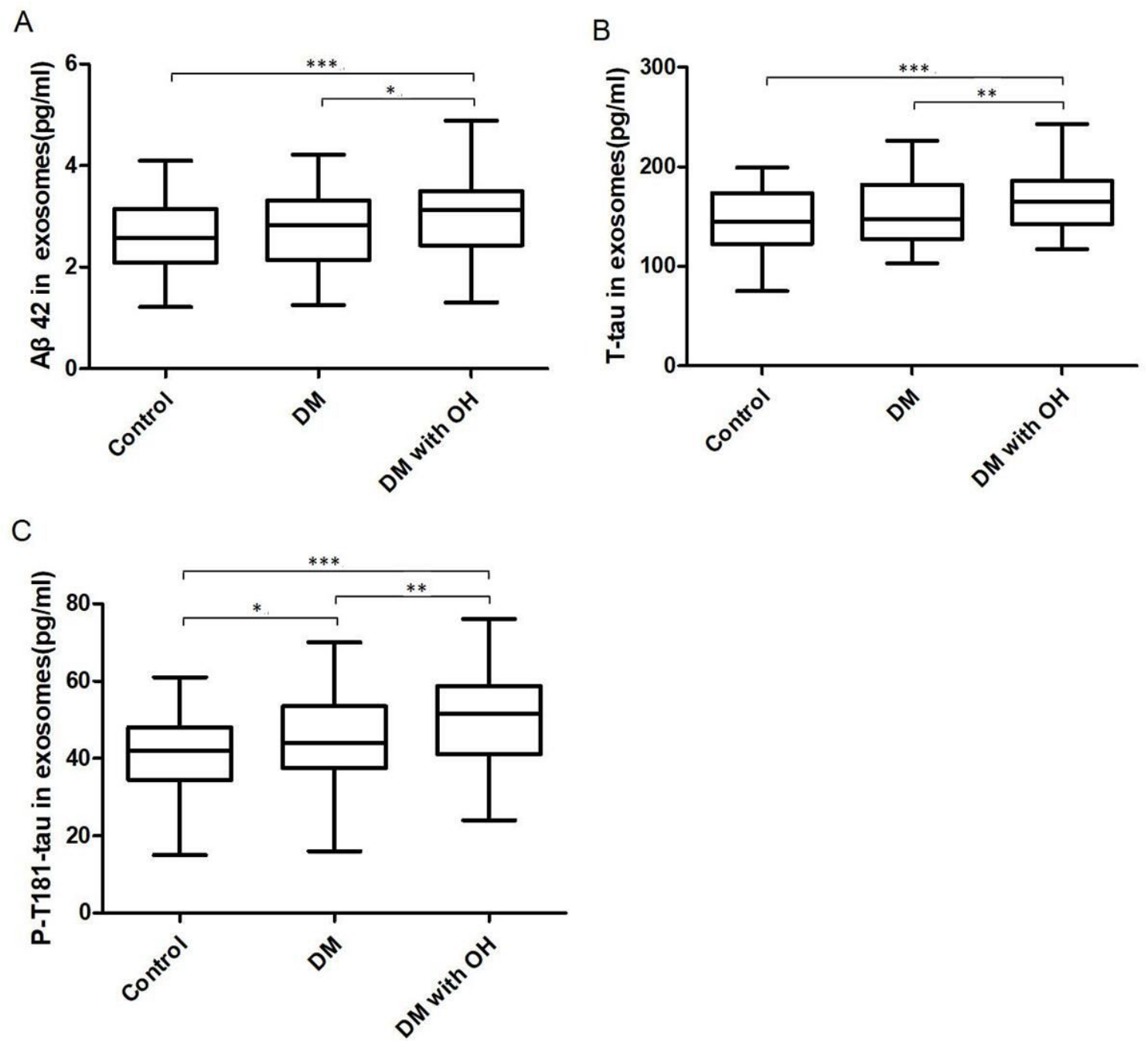
A

B

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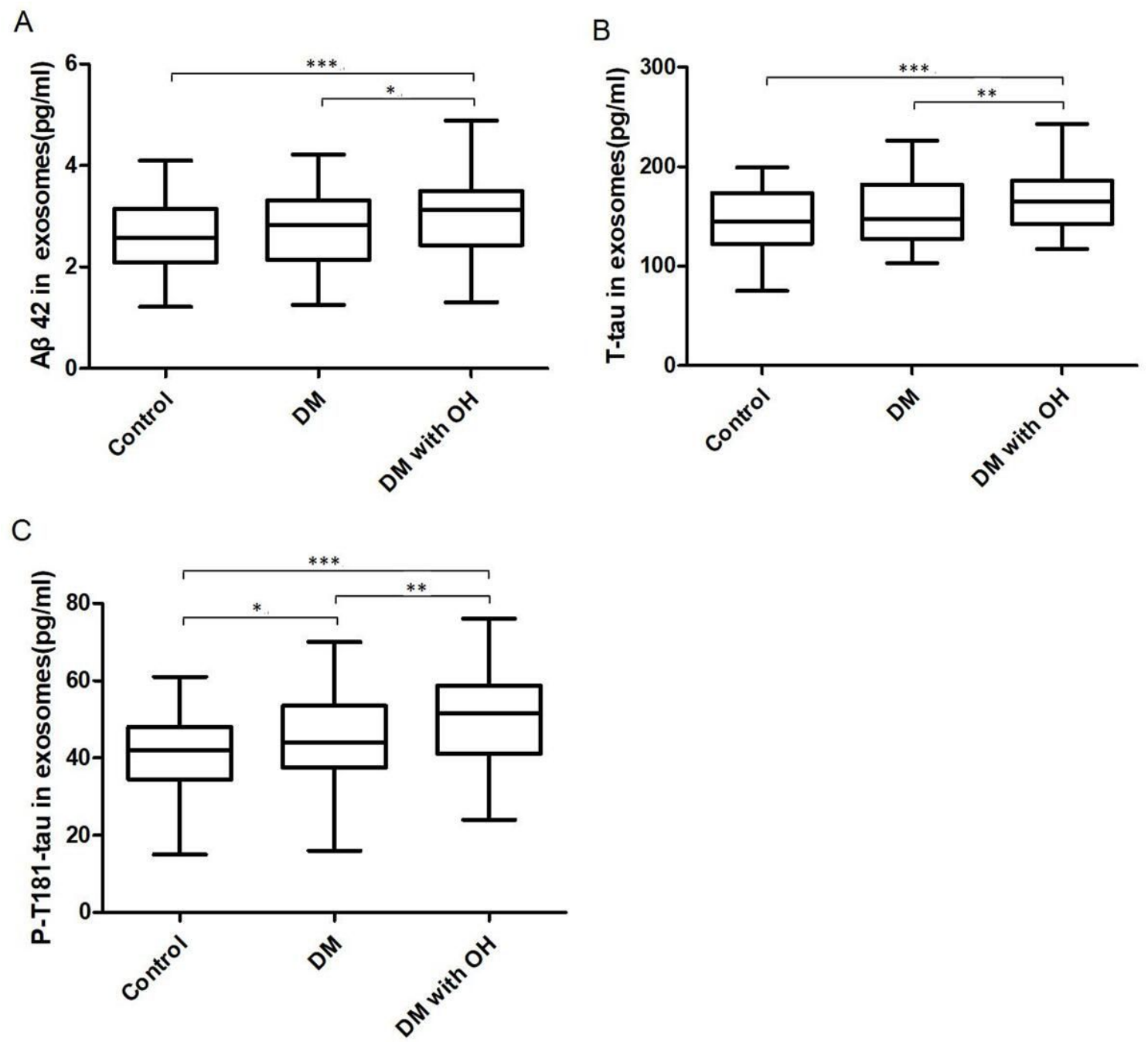

Figure 2

Plasma neuronal-derived exosomes levels of amyloid-β and tau proteins in cross-sectional control, DM, and DM with OH groups. (A) The exosomal concentration of Aβ42 in DM with OH group was higher than those in the DM and control group; there was no significant differences in the levels of Aβ42 between DM and control group. (B) The exosomal concentration of T-tau in DM with OH group was higher than those in the DM and control group; there was no significant differences in the levels of T-tau between DM and control group. (C) The P-T181-tau levels in the plasma neuronal-derived exosomes from DM and DM with

OH patients were higher than those in control subjects; the exosomal P-T181-tau levels in DM with OH were higher than those in DM group. *P < 0.05, **P < 0.01 , ***P < 0.001.

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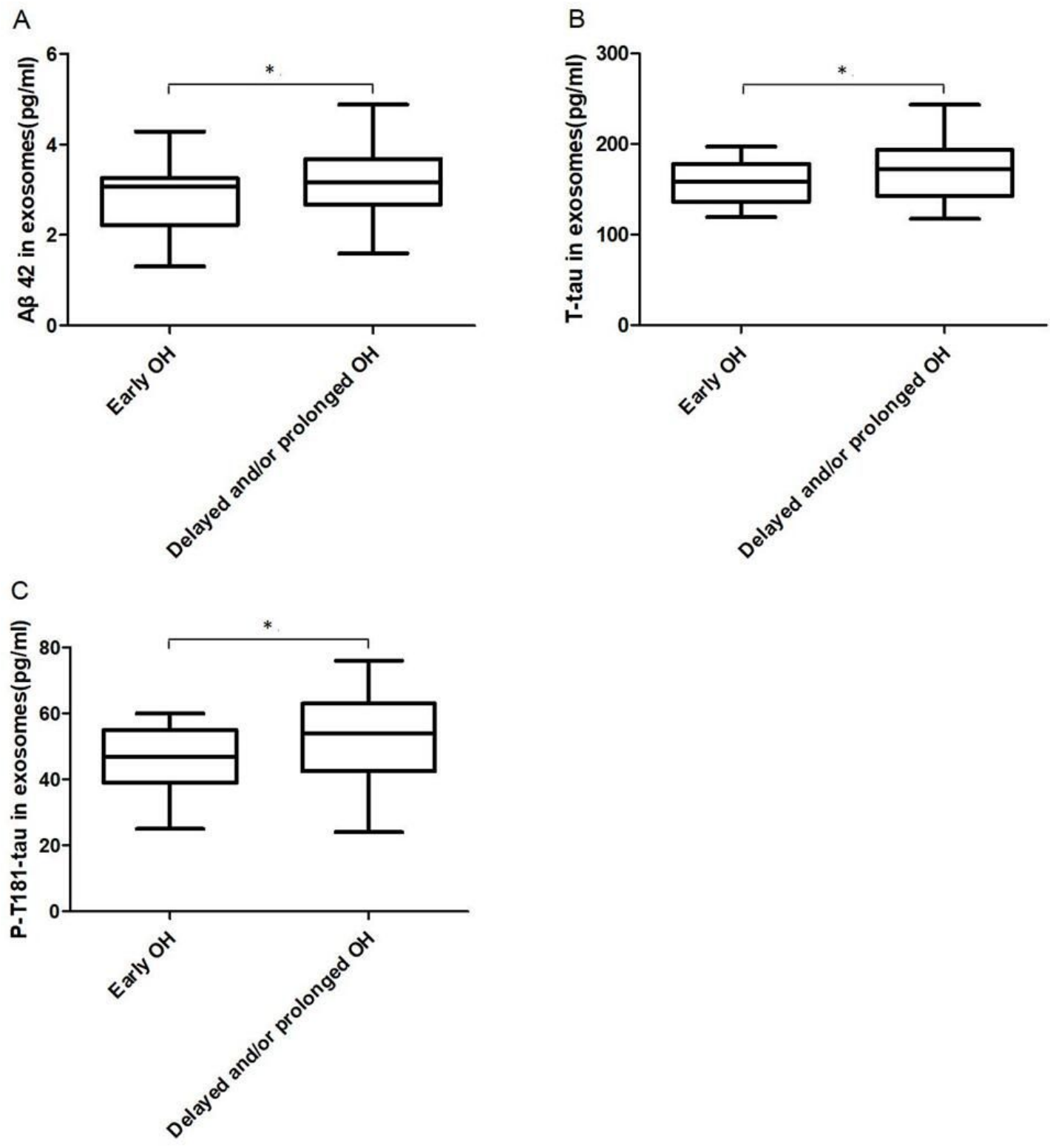

Figure 3

Plasma neuronal-derived exosomes levels of amyloid-β and tau proteins in DM patients with early OH and delayed and/or prolonged OH. Compared with the DM patients with early OH, the exosomal

concentrations of Aβ42, T-tau, and P-T181-tau in the DM with delayed and/or prolonged OH were higher.
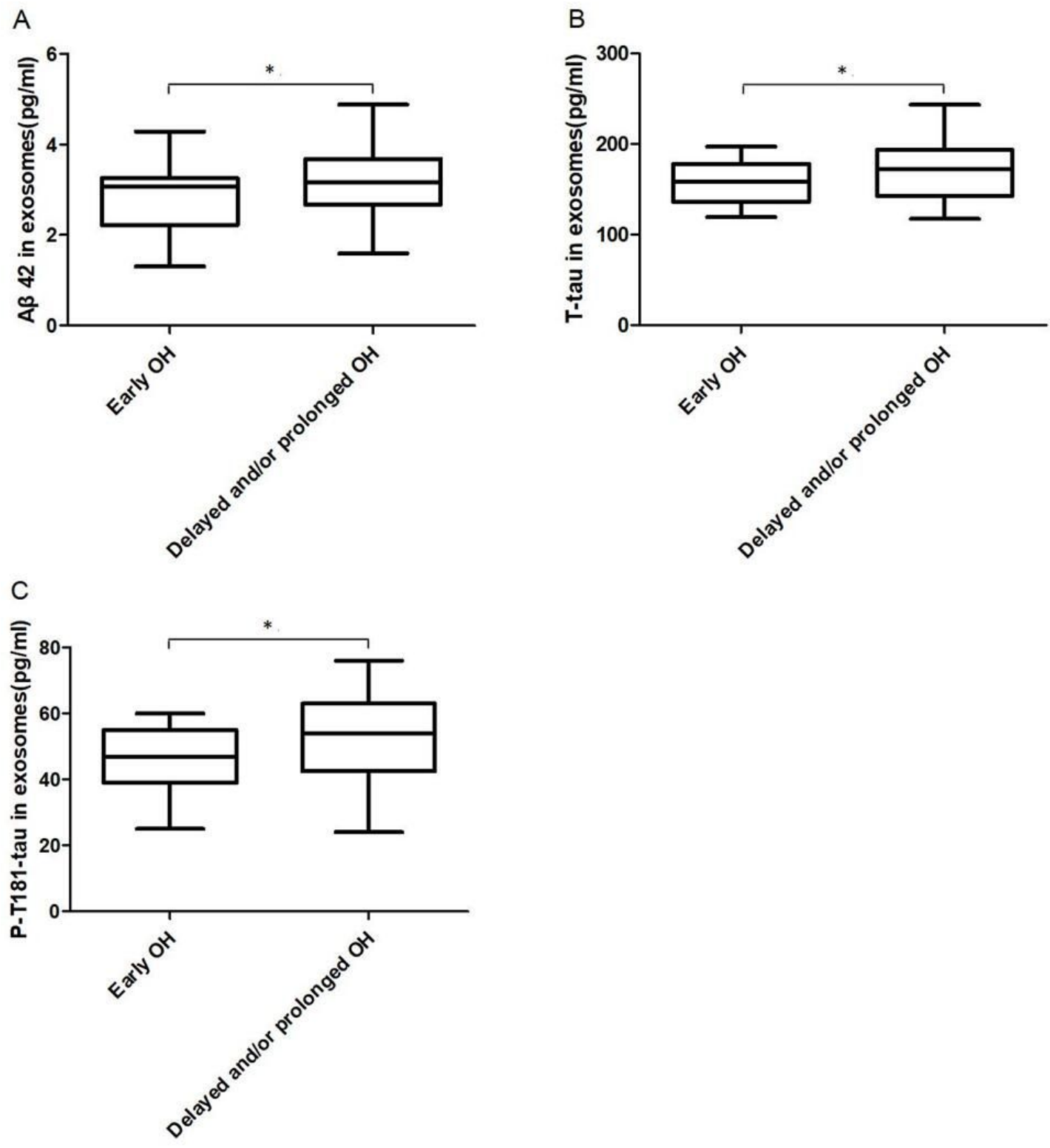*P < 0.05

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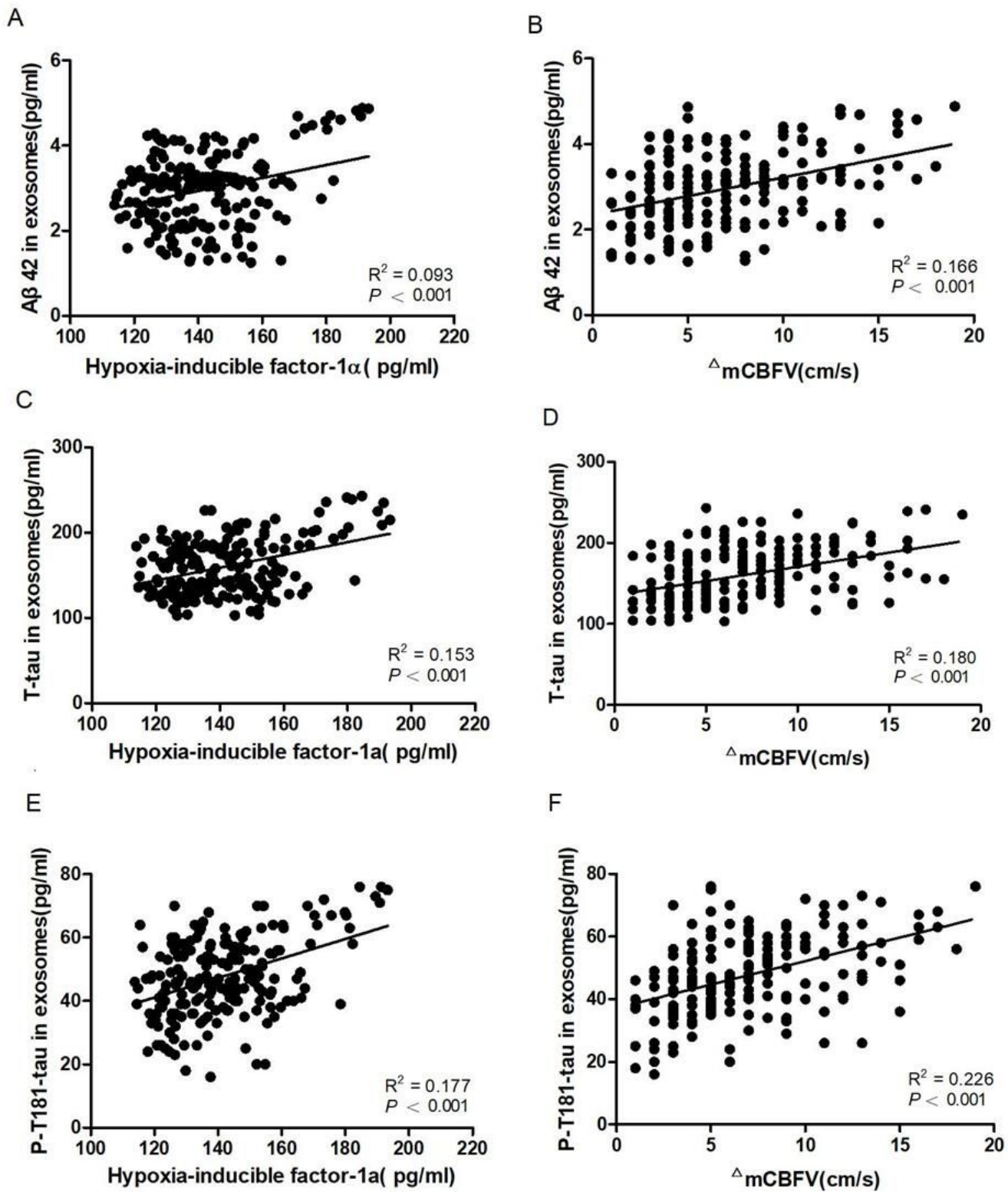

Figure 4

Association between the levels of Aβ42, T-tau, and P-T181-tau and HIF-1α, mCBFV in DM patients. The levels of Aβ42 in neuronal-derived exosomes were correlated with hypoxia-inducible factor-1α (A) and △mCBFV (B). Plasma neuronal-derived exosomes levels of T-tau (C, D) and P-T181-tau (E, F) were

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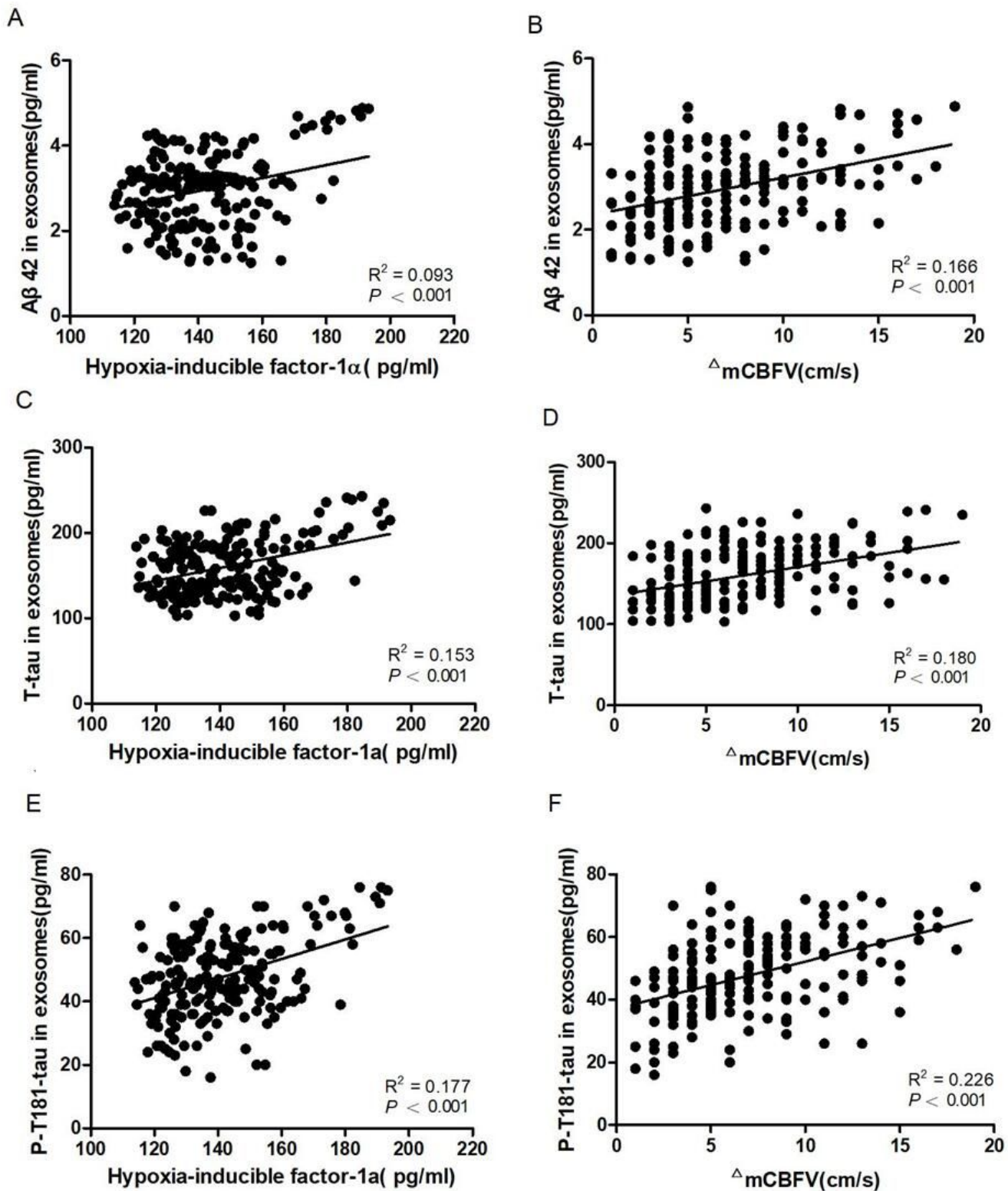

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