Investigating human milk extracellular vesicles as therapeutic agents: a study on their efficacy in *in vitro* hit models of premature encephalopathy

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Abstract

Background Encephalopathy of prematurity (EoP) pertains to the injury of both white and grey matter in preterm infants. Currently, there are no curative treatments proven for patients. Therefore, novel treatment is needed urgently. Furthermore, human breast milk is recognized already as one of the main contributors to healthy neonatal brain development. One of milk's poorly understood, regenerative components are milk extracellular vesicles (EVs). EVs are phospholipid bilayer-enclosed nanoparticles that are secreted by all cell types and play a critical role in bodily homeostasis. These vesicles carry a bioactive cargo, including RNAs, lipids, and proteins, with functional proteins on their surface capable of interacting with cell surface receptors of recipient cells. Recent literature indicates that milk EVs might have the potential to influence the phenotype of injured neurons, thereby they may provide neuroprotection against EoP. As of now, the neuroregenerative and neuroprotective effects of milk EVs on neuronal injury have not yet been investigated. Therefore, this study is the first of its kind to explore the protective effects of milk EVs in different *in vitro* hit models of neuronal injury.

Methods Human breast milk was used to obtain the EVs and EV-depleted controls in this study. Isolation of EVs and EV-depleted controls was performed in a multi-step process involving differential centrifugation, Optiprep density gradient ultracentrifugation and size exclusion chromatography. For the in vitro hit models, SH-SY5Y neuron cell lines were used, and cell viability was measured with an MTT assay. One *in vitro* hit model explored the pre-treatment and co-treatment effects of milk EVs in an oxidative stress model using H2O2. Additionally, milk EV treatment effect was investigated in an oxygen-glucose deprivation hit model during the reoxygenation phase. Besides, neuron-EV association was assessed using flow cytometry with fluorescently-labeled EVs.

Results Nanoparticle tracking analysis indicated that the concentration range of EVs were measured from 7.33 x 10 to 2.2267 x 10^9 particles/mL, while the EV-depleted control concentrations were measured within the range of 9.016 x 107 to 3.96 x 10^8 particles/mL. Pre-treatment of milk EVs cannot increase neuronal cell viability in an oxidative stress model using H2O2. On the other hand, co-treatment of milk EVs indicated increased neuronal cell viability in a dose-response relationship. Milk EV treatment during reoxygenation in the oxygen-glucose deprivation hit model do not prevent neuronal cell death. Flow cytometry using fluorescently-labeled EVs indicated a visible association between SH-SY5Y neurons and EVs after 4h incubation.

Conclusion This pioneering study demonstrates a treatment with milk EV increases neuronal cell survival in a co-treatment *in vitro* oxidative stress model with H2O2. The treatment effect might be attributed to either EV-SHSY-5Y association or potential scavenging activity of milk EVs for ROS. Future research is needed to understand of milk EVs' mechanisms of action and to investigate their contribution in the protection against neurodevelopmental impairments in preterm infants.

Keywords: Encephalopathy of prematurity; breast milk; milk EVs; SH-SY5Ys; H2O2; oxygen-glucose deprivation; flow cytometry.

Plain Language Summary

A baby can be born too early, leading to problems in the oxygen and sugar supply towards their brain. This can lead to brain damage with long-term problems with muscle movements and thinking abilities. Currently, there is no recovering treatment available for these babies. Furthermore, it is known that breast milk can support the healthy development of the baby's brain by providing nutrients, protecting the children from inflammation and improving the baby's gut health. One component that is abundant in milk and little known about is extracellular vesicles (EVs). EVs are small, enclosed lipid bubbles with healthy RNAs and proteins and are produced by all types of cells. Once these bubbles interact with other cells, they can change cell functionality or improve their condition. It is believed that these lipid bubbles that are present in breast milk, called milk EVs, have the potential to interact with injured brain cells in babies and improve their functionality. Therefore, this study aimed to investigate the ability of these milk EVs to restore injured brain cells, namely neurons.

First, the milk EVs are isolated from breast milk and used in different neuron-injury models to observe whether the milk EVs have protective effects. The neuron-injury models consist of neurons that are grown in a plate and exposed to hydrogen peroxide (a toxic molecule) or an oxygen-glucose shortage, which deliberately kills half of the neurons. In addition, flow cytometry is performed, involving the tagging of EV with glowing markers to assess whether these EVs attach to the neurons.

Treatment of hydrogen peroxide-injured neurons with milk EVs increased neuronal survival when EV concentrations were getting higher. Contrary, the neuron-killer model with oxygen-glucose shortage did not appear to have increased neuronal survival. Additionally, the attachment between EVs and neurons was confirmed.

To conclude, this was the first study done regarding the therapeutic effects of milk EVs and milk EVs have shown that they potentially could protect brain cells against injury and stress. Future research is needed to understand the mechanisms of action of milk EVs in restoring injured neurons.

Chapter 1: Introduction

Encephalopathy of Prematurity (EoP) pertains to the injury of both white and grey matter in preterm infants (1–5). Globally, approximately 7%-15% of all births are preterm, which is defined as birth occurring before 37 weeks of gestation. Within the European Union, 400,000 babies are born prematurely each year (6,7). In regions with access to well-equipped healthcare, over 50% of infants born at less than 28 weeks of gestation will survive, and of those approximately 25% will experience severe impairments including cerebral palsy, impaired cognitive function, deafness, blindness, and psychiatric disorders such as attention deficit hyperactivity disorder (ADHD) and autism spectrum disorder (ASD) (6–8). As of now, there are no curative treatments proven to restore brain damage in EoP patients (8).

In fact, various underlying neuropathological mechanisms contribute to the EoP phenotype (Figure 1). One notable mechanism is diffuse white matter injury (dWMI) (4,6,9). dWMI is caused by hypoxia-ischemia and inflammatory processes, leading to the production of reactive oxygen species (ROS) and the activation of microglia. ROS specifically target oligodendrocyte precursors (OPs), resulting in OP cell death and the proliferation of OPs that suffer from maturational arrest (6,9). Another less prevalent form of cerebral injury is intraventricular hemorrhage (IVH), which originates from the germinal matrix (the subventricular region containing multipotent progenitor cells). IVH is caused by hypoxia-ischemia leading to ruptured vessels and subsequent accumulation of blood in the cerebral ventricles (10,11). IVH increases intracranial pressure, reduces cerebral blood flow, and compromises the integrity of the blood-brain barrier (BBB). Additionally, IVH induces inflammation through microglia activation and triggers oxidative stress via ROS production, leading to axonal degeneration and damage to OPs (6,9–11).

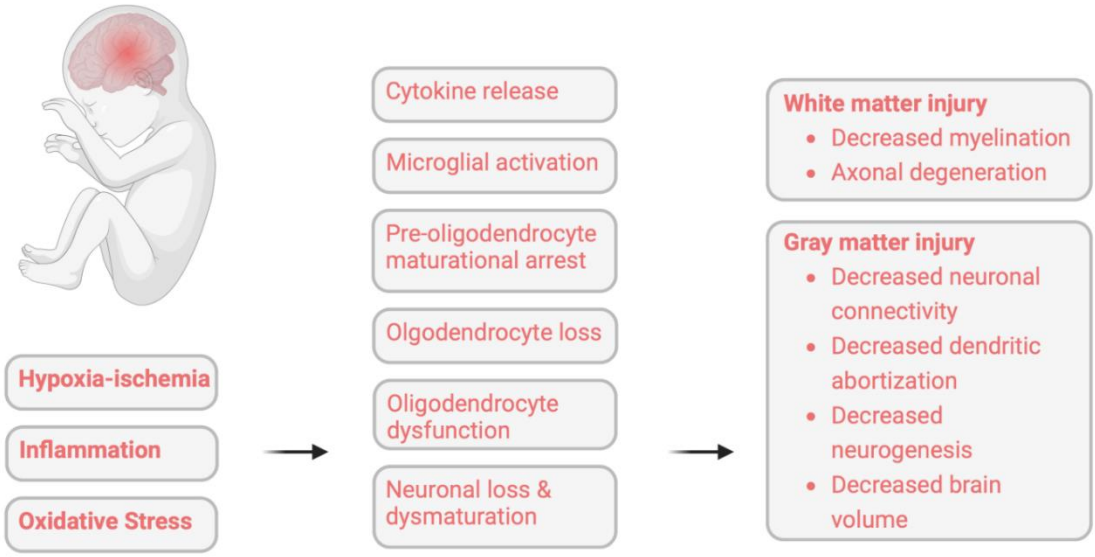


Figure 1. Hypoxia-ischemia, inflammation, and oxidative stress collectively contribute to the development of white and gray matter injury in EoP through various neuropathological mechanisms. These processes lead to long-term cognitive and motor disorders in patients. Figure created with BioRender. Adjusted from Parikh, 2019 (6).

Furthermore, human breast milk is recognized as one of the main contributors to healthy neonatal brain development (12–16). Human breast milk is vital for early human growth and

development as it provides essential nutrients that support infant growth. It also contains important immune factors that help protect against infections and play a key role in building immunity. Substantial proportions of breast milk during the early postnatal period are known to promote behavioral neurodevelopment in preterm infants with EoP (16–18). Despite its recognized benefits, the exact mechanism through which breast milk exerts its positive effects remains unknown.

Recent scientific advancements have positioned EVs as a promising therapeutic strategy for preterm neonates (19–22). EVs are phospholipid bilayer-enclosed nanoparticles that are secreted by all cell types and play a critical role in bodily homeostasis. These vesicles are classified as apoptotic bodies, microvesicles or exosomes and carry a bioactive cargo, including mRNA, miRNA, circular RNAs, lipids, and proteins, with functional proteins on their surface capable of interacting with cell surface receptors of recipient cells (19,23–30). Through the delivery of this cargo, EVs facilitate intracellular communication to modulate cellular physiology, with RNAs primarily epigenetically regulating gene expression in recipient cells (19–22). Given these properties, EVs offer a potential alternative to traditional (stem) cell-based therapies for neonates. Moreover, preclinical studies of preterm disorders including EoP have already demonstrated beneficial outcomes, further supporting the therapeutic potential of EV administration (19–30). Namely, researchers have explored the regenerative potential MSC-EVs, aiming to mitigate associated risks of stem cell therapy such as rejection, toxicity, and tumorigenicity (19–30).

It is hypothesized that milk EVs could predominantly have protective benefits as they are one of breast milk's regenerative components that both survive the infant's gastro-intestinal (GI) tract and are able to cross the blood-brain barrier (BBB) bidirectionally (14–16). The functional effects of specifically milk-derived EVs have been demonstrated in multiple organs. For example, milk EVs have been shown to prevent gut-liver axis-associated metabolic disorders by protecting epithelial tight junction functionality *in vitro* (31). According to Melnik et al. (2021), milk EVs' miRNA cargo suppresses nuclear factor-κB (NF-κB) signaling, thereby treating necrotizing enterocolitis *in vitro* (32). Liu et al. (2023) demonstrated increased bone formation and reduced inflammation activation through the activation of anti-inflammatory macrophages following milk EV administration *in vivo* (33). As of now, the neurogenerative and neuroprotective effects of milk EVs on the neonatal brain have not yet been investigated. Given these established functional properties, milk EVs might also have the potential to influence the phenotype of injured neurons, thereby providing neuroprotection against the injury of EoP. Therefore, this research contributes to a new theoretical framework in which the regenerative effects of milk EVs are studied on different *in vitro* models of EoP. Potentially, milk EV administration might serve as an appealing alternative for hypothermia or (stem) cell delivery therapies in the proximate future. Within this study, two different *in vitro* injury models of EoP employed SH-SY5Y neuroblastoma cells. The injured neurons received milk EV treatment to determine the neuroprotective effects of milk EVs. The temporal window of milk EV exposure and dose-response relationships pertaining to the neuroprotective effects were assessed as well. Additionally, a functionality assessment was conducted on the association between milk EVs and SH-SY5Y neurons to investigate milk EV-neuron association.

Chapter 2: Materials & Methods

2.1. Isolation and characterization of human milk EVs and procedural controls

Fresh milk specimens were obtained from healthy mothers that volunteered to donate milk. The procedure for isolating milk-derived EVs used a newly optimized density-gradient protocol (34). First, thawed milk was centrifuged at room temperature (RT) for 10 minutes at 3000 × g (Beckman Coulter Allegra X-12R, Fullerton, CA). This resulted in a visible white pellet and a cream layer. The cream layer with milk fat globules was carefully removed, and the supernatant, free of cell debris, was collected. The supernatant was then poured into fresh tubes and centrifuged again at 3000 × g at RT. The frozen supernatant was moved to polyallomer SW40 (Beckman Coulter) and spun at 5000 × g for 30 minutes at 4°C. After that, it was centrifuged again at 10,000 × g. Then, 6.5 mL of the supernatant was loaded on top of a Iodixanol OptiprepTM 60% (Axis-shield) (Progen 1114542) density gradient in a SW40 tube. This density gradient was made by layering consecutive solutions (500 µL each) of decreasing density. Next, ultracentrifugation was done at 192,000 × g (using a Beckman Coulter Optima L-90K with an SW40 rotor) for 15-18 hours. Following centrifugation, the top 6.5 mL from the gradients was taken and used to produce the EV-depleted milk samples. Then, 500 µL samples were collected from the bottom of the SW40 tube and transferred into Eppendorf tubes. The density of each sample was measured using a refractometer to distinguish those containing milk-derived EVs (with densities ranging from 1.12 to 1.18 g/ml). Subsequently, samples containing milk-derived EVs were pooled together. Size exclusion chromatography (SEC) with Sephadex was employed to isolate EVs in the pooled sample from Optiprep and to collect the 'EV fractions' from the culture medium. For the donor-matched procedural control, milk supernatant depleted of EVs was utilized from the initial density gradient. This sample was subsequently applied to a new density gradient, from which fractions corresponding to EV densities were collected and subjected to SEC to obtain the final EV-depleted controls.

Concentration and particle size distribution of isolated milk EVs and their corresponding EV-depleted controls were assessed across donors using nanoparticle tracking analysis (NTA). Flow mode measurements were conducted using a NanoSight NS300 equipped with a NanoSight Syringe Pump, employing an optimized particles/frame rate ranging from 70 to 110. Each measurement comprised triplicate 60-second captures. In nanoparticle NTA, a particle is defined as a discrete unit of matter with dimensions typically ranging from 1 to 1000 nanometers.

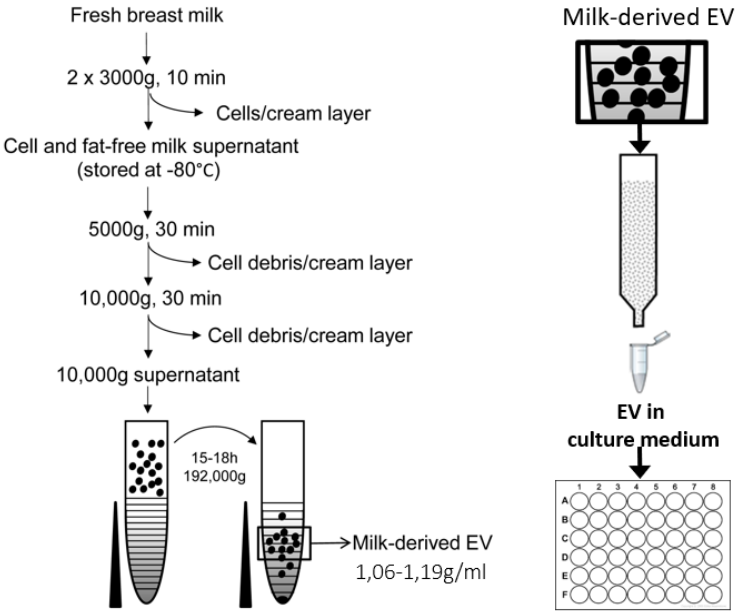


Figure 2. Schematic overview of isolation of milk EVs and EV-depleted procedural control: a process involving differential centrifugation, density gradient ultracentrifugation, and size exclusion chromatography. Figure adapted from Zonneveld *et al.* (2021) (34).

2.2. Milk EV treatment in *in vitro* hit models of EoP

2.2.1. SH-SY5Y cultivation

The human neuroblastoma cell line SH-SY5Y (ATCC, CRL-2266) was cultured in DMEM/F12 medium (Thermo Fisher Scientific, 11330057) enriched with 10% FBS (Invitrogen) and 1:100 P/S (Thermo Fisher Scientific). Cells were cultivated in T75 flasks (Corning Life Sciences) within a humidified incubation chamber (PHCbi) with 5% CO2 at 37 °C. SH-SY5Y cells were collected through incubation in trypsin-EDTA (Invitrogen, 25300-054) for a duration of 2 to 3 minutes. Subsequently, cells underwent centrifugation for 5 minutes (300g) at room temperature to be collected. The number of passages for the SH-SY5Y neurons used in the *in vitro* models did not exceed 9 times. The cells' sensitivity to an *in vitro* hit varies depending on the passage number, as well as the concentration or duration of the hit, which was designed to achieve a 50% reduction in cell viability.

2.2.2. H2O2 hit model (pre-treatment and co-treatment)

SH-SY5Y cells (ATCC) were seeded in a 96-well plate (Thermo Fisher Scientific, 167008) at a density of 5×10^4 cells/well and were permitted to adhere overnight. The subsequent day, cells were exposed to EV-containing DMEM/F12 medium (10% FBS) or EV-depleted DMEM/F12 medium (10% FBS) as a pre-treatment at a concentration ranging from 50% to 100% of the original EV isolation sample (section 2.1.). Control wells and H2O2-hit wells were replaced with standard medium. Subsequently, the cells were treated with varying concentrations (0, 40, 60, 80, 100, 120, 140, 160, or 180 uM) of H2O2 (Merck KgA) in a volume of 100 uL and incubated in a humidified incubation chamber (PHCbi) with 5% CO2 at 37 °C for a duration of 24 hours. The next day, neuronal cell death was evaluated utilizing a 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. In the pre-treatment model, a total of 4 milk EV donors and matched procedural controls were investigated.

In the co-treatment method, cells were exposed to H2O2 a milk EVs concurrently by dilution of H2O2 into the EV-containing DMEM/F12 medium (10% FBS) or EV-depleted containing DMEM/F12 medium (10% FBS). Then next day, neuronal cell death was assessed using an MTT assay, consistent with the pre-treatment approach. In the co-treatment model, a total of 3 milk EV donors and matched procedural controls were investigated.

2.2.3. Oxygen-glucose deprivation (OGD) model

SH-SY5Y cells were seeded in a 96-well plate (Thermo Fisher Scientific, 167008) at a density of 5×10^4 cells/well and were permitted to adhere overnight. The subsequent day, culture medium was substituted with DMEM lacking glucose (Merck KgA, 11966025) supplemented with 1:100 P/S (Thermo Fisher Scientific, 15140122). Cells were incubated in a humidified hypoxic incubator with 1% O2 and 5% CO2 at 37 °C (PHCbi, Cell-IQ 5.7 cu.ft. Multigas CO2/O2 Incubator MCO-170MP-PA) for 16 hours. In parallel, a control plate was cultured in normal DMEM/F12 medium in a humidified normoxic incubator (PHCbi) with 21% O2 and 5% CO2 at 37 °C for 16 hours. Reoxygenation was performed with either EV- containing or EV-depleted DMEM:F12 medium (10% FBS) in a humidified normoxic incubator (PHCbi) with 21% O2 and 5% CO2 at 37 °C for 24 hours following the hypoxic condition. Neuronal cell death was evaluated utilizing an MTT assay. In the OGD model, a total of 3 milk EV donors and matched procedural controls were investigated.

2.2.3. MTT assay

After milk EV exposure and the oxidative hit (H2O2 exposure or OGD), culture medium was replaced by culture medium containing 0.5 mg/ml MTT (Merck KgA, M2128-250MG) in

PBS (Invitrogen). MTT solution was incubated for 3 hours in a humidified incubation chamber (PHCbi) with 5% CO2 at 37 °C. After incubation, medium was aspirated from the wells and MTT crystals were dissolved in 100 µL DMSO (VWR, 23500260). Optical density was measured at 570 nm using a spectrophotometer (Thermo Fisher Scientific, Multiskan GO).

2.2.4.Statistical analysis

Statistical analysis was executed employing GraphPad Prism 8.3 (GraphPad Software). If data exhibited normal distribution, statistical analysis was conducted utilizing a one-way ANOVA with Holm-Šidák post-hoc tests. Conversely, if data did not conform to normal distribution, statistical analysis was conducted utilizing a nonparametric test with Dunn's post-hoc tests. Data is expressed as the mean ± SEM and discrepancies of $p \leq 0.05$ were deemed statistically significant.

2.3. Flow Cytometry

SH-SY5Y cells were seeded in a 24-well plate (Thermo Fisher Scientific, 055429) at a density of 1.5×10^5 cells/well and were allowed to adhere overnight. Additionally, milk EV membranes from three donors were labeled with Alexa Fluor™ 488 C5 Maleimide (A10254, Thermo Fisher Scientific). Alexa Fluor™ 488 C5 Maleimide are added to EVs or procedural controls of the original EV isolation sample (section 2.1.) Subsequently, SEC was performed to remove the removal of unbound dye (C5-Maleimide stain) from the EVs, providing a purified EV fraction. EV-depleted samples from three donors were similarly labeled and used as a negative control. The following day, the SH-SY5Y culture medium was replaced with C5 Maleimide-labeled milk EVs and incubated in a humidified incubator (PHCbi) with 21% O2 and 5% CO2 for 4 hours. Subsequently, the neurons were harvested with trypsin, centrifuged at 300xg for 5 minutes, and the supernatant was removed. The cell pellet was resuspended in 250 µL dPBS (1% FBS) (Thermofisher Scientific, 14190144) and placed in a 96-well plate for measurement in the flow cytometer (CytoFLEX, Beckman). Analysis was performed on 25,000 events using FlowJo™ Software.

Chapter 3: Results

Quantification of milk EV concentration through nanoparticle tracking analysis (NTA)

Milk EV concentrations per donor were determined through NTA for both EV samples and EV-depleted procedural controls. The range within which the EV sample concentrations were measured was from 7.33 x 10^8 to 2.23 x 10^9 particles/mL, while the EV-depleted sample concentrations were measured within the range of 9.02 x 10^7 to 3.96 x 10^8 particles/mL. An example of NTA analysis for the EV sample from Donor 43 and its procedural control is shown in Figure 3. NTA analyses for all other donors are presented in the supplementary section (S1). Donor 43's EV concentration was 2.01 x 10^9 ± 1.57 x 10^8 particles/ml (figure 3A), and the EV-depleted sample was 9.38 x 10^7 ± 3.64 x 10^6 particles/ml (figure 3B).

A: D43 EV **B: D43 EV-depleted**

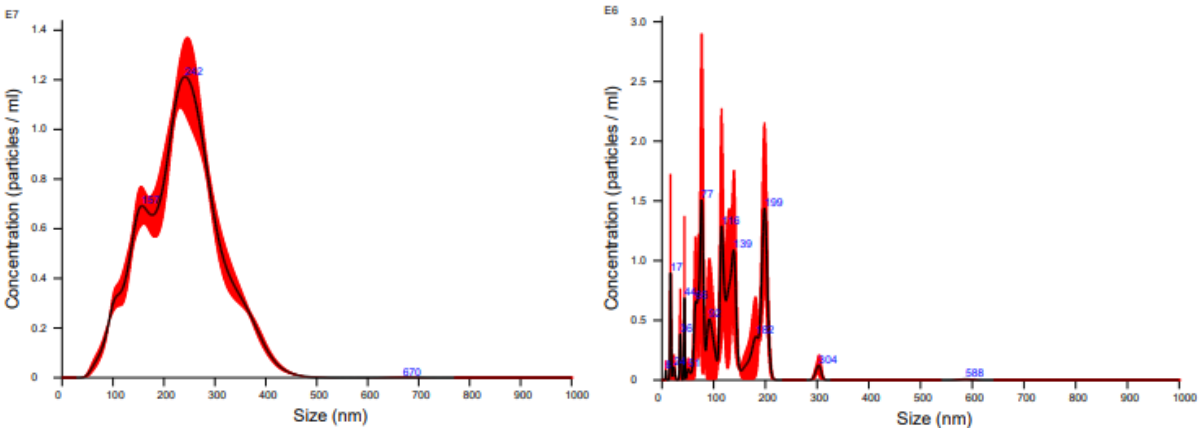
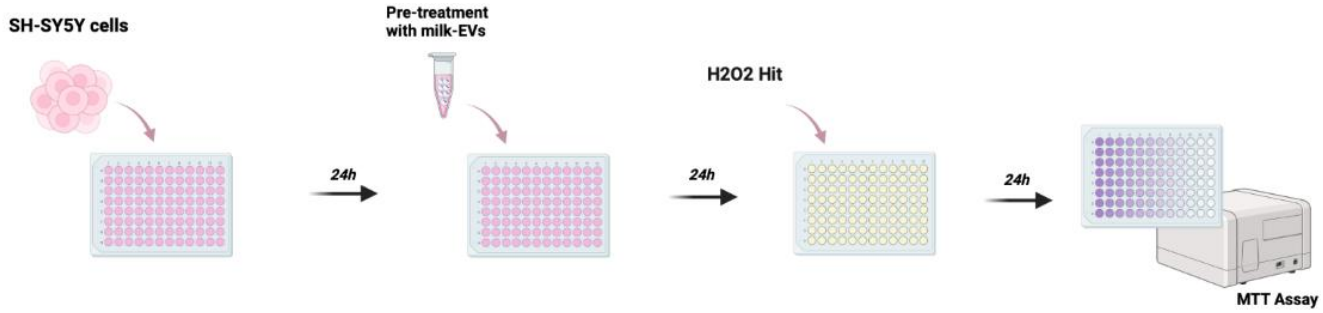


Figure 3. Characterization of milk EV concentration through nanoparticle tracking analysis (NTA) of total EVs isolated. A) Donor 43, EV sample. B) Donor 43, EV-depleted or procedural control.

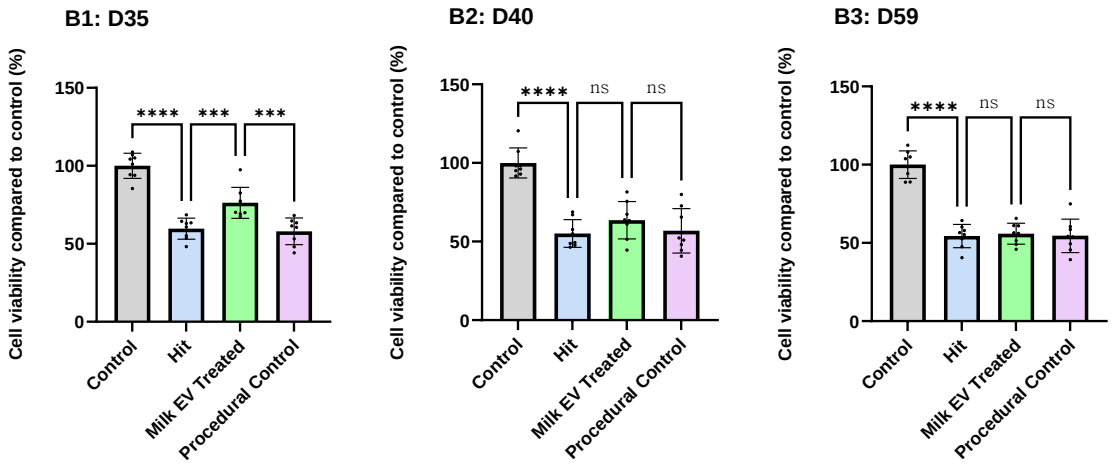
Pre-treatment with milk EVs does not prevent neuronal cell death after H2O2 induced oxidative stress *in vitro*

To evaluate the potential of milk EVs to prevent cytotoxicity in SH-SY5Y neurons, cells were exposed to milk EVs prior to H2O2-induced oxidative stress exposure. H2O2 is known to induce oxidative stress by generating ROS, leading to cellular damage and apoptosis. Administering milk EVs before the oxidative insult provides insights into how EV-neuron interactions influence subsequent cellular physiology. In figure 4B and 4C, milk EVs were applied at concentrations of 50% and 100% relative to the original EV-isolated samples. The cells solely exposed to the hit were significantly decreased compared to the control ($p<0.0001$). Notably, exposure to milk EVs derived from donor 35 (figure 4B1) significantly enhanced cell viability following H2O2, compared to the hit ($p=0.0009$, Fig 4B1) and procedural control ($p=0.0005$, Fig 4B1) at a concentration of 50% compared to the original isolated EV sample. Conversely, milk EVs from donors 40 and 59 did not significantly alter cell viability compared to the procedural controls (respectively, $p=0.4251$, Fig 4B2; $p=0.9872$, Fig 4B3) at concentration of 50%. At a concentration of 100%, milk EVs did not significantly enhance cell viability compared to the adjacent procedural controls across donors 35, 40, 59, and 60. ($p=0.8963$, $p=0.6401$, $p=0.8963$, $p=0.7989$; Fig 4C), which indicates that a pre-treatment of milk EVs does not rescue the neurons.

A: Schematic of Pre-treatment Set-up



B: Pre-treatment (50%)



C: Pre-treatment (90%)

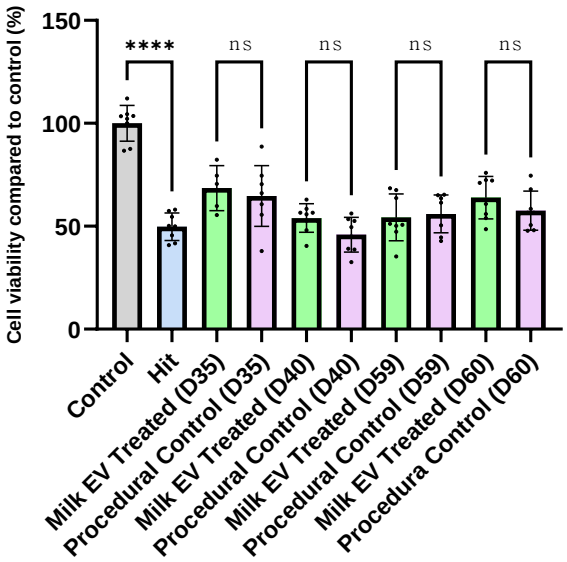


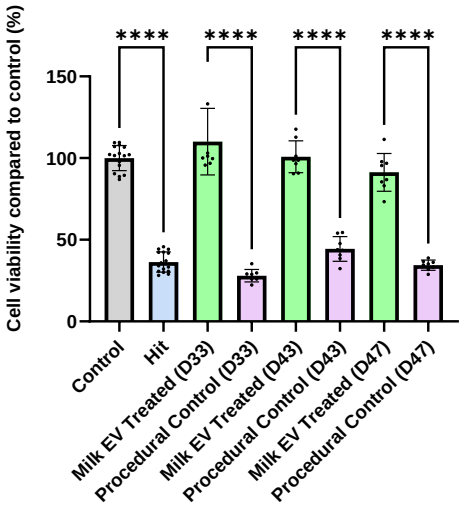
Figure 4. **A)** Schematic of pre-treatment set-up **B)** Neuronal cell viability after H2O2 induced oxidative stress preceded by milk EV pre-treatment at a concentration of 50% to the isolated EV sample. *B1)* Donor 35 with H2O2 hit (180 μM). *B2)* Donor 40 with H2O2 hit (60 μM). *B3)* Donor 59 with H2O2 hit (80 μM). **C)** Neuronal cell viability after H2O2 (80 μM) induced oxidative stress preceded by milk EV pre-treatment at a concentration of 90% compared to the original sample using donors 35, 40, 59 and 60. n=8 for all experimental groups. ***<0.001 ****p<0.0001. Data represents mean + SEM.

Co-treatment of milk EVs mitigates neuronal cell death during H2O2 induced oxidative stress *in vitro* in a dose-response relationship

Equivalently to the pre-treatment model, the co-treatment model aims to evaluate the therapeutic efficacy of milk EVs in SH-SY5Y neurons, but now during H2O2-induced stress. The cells solely exposed to the hit were significantly decreased compared to the control ($p<0.0001$). Neurons treated with milk EVs from donors 33, 43 and 47 at a concentration of 80% exhibited significantly higher cell viability compared to the respective procedural controls (D33, $p<0.0001$; D43, $p<0.0001$; D47, $p<0.0001$; Fig 5B). Furthermore, cell viability was fully recovered in neurons treated with milk EVs, as evidenced by non-significant differences compared to the control group that was not exposed to the H2O2 hit (D33, $p=0.0977$; D43, $p=0.8693$; D47, $p=0.1749$; Fig 5B). Moreover, milk EVs dose-dependently increased cell viability at concentrations of 80% ($p<0.0001$, Fig 5C), 60% ($p<0.0001$, Fig 5C), and 40% ($p<0.0001$, Fig 5C), as well as 20% ($p=0.0091$, Fig 5C), compared to the isolated EV sample. However, the treatment effect was lost at concentrations of 10% ($p=0.1792$, Fig X4) and 5% ($p=0.1792$, Fig X4). In summary, milk EVs effectively restore neuronal cell viability in a dose-dependent manner in a co-treatment oxidative stress model with H2O2.

A: Schematic of Co-treatment Set-up

B: Co-treatment (80%)



C: Dose-response Co-treatment (D43)

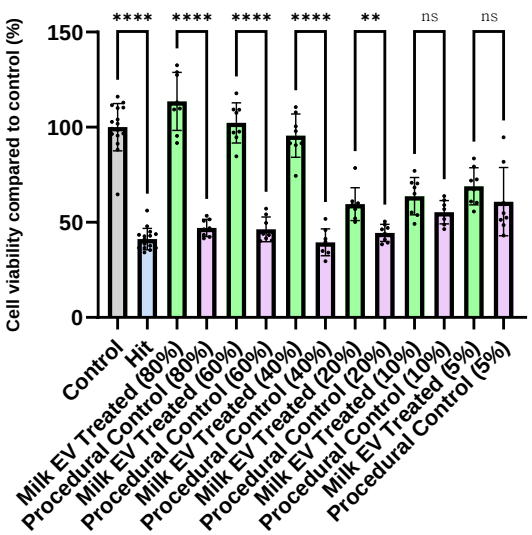
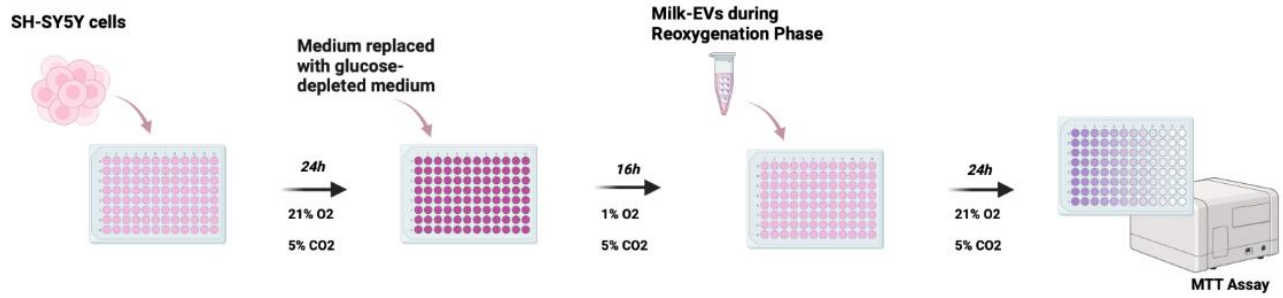


Figure 5. **A)** Schematic of co-treatment set-up. **B)** Neuronal cell viability after H2O2 (80 µM) induced oxidative stress and milk EV co-treatment at a concentration of 80% of the original EV isolation. **C)** Neuronal cell viability after H2O2 (80 µM) induced oxidative stress and milk EV co-treatment at concentrations of 80%, 60%, 40%, 20%, 10% and 5% of the original EV isolation sample of donor 43. Control and hit groups: n=16; Milk EV Treated and Procedural Control groups: n=8. *p<0.05. **p<0.01. ****p<0.0001. Data represents mean + SEM.

Milk EV treatment during reoxygenation in an oxygen-glucose deprivation hit model did not mitigate neuronal cell death

The model of oxidative stress in SH-SY5Y cells induced by oxygen-glucose deprivation (OGD) is utilized to assess the therapeutic potential of milk EVs on oxidative injury in neurons. During OGD, the hypoxic phase leads to energy depletion and cellular stress, while the reoxygenation phase intensifies neuronal injury through oxidative stress and inflammation, collectively resulting in neuronal injury. In figure 7B, the cells solely exposed to the hit were significantly decreased compared to the control ($p<0.0001$). Notably, donor 47 demonstrated a significant increase in neuronal survival compared to the respective procedural control ($p<0.001$), whereas milk EVs from donors 33 and 43 did not exhibit such a difference ($p=0.7354$, $p=0.7354$, respectively). Consequently, the collective findings regarding the therapeutic effects of milk EVs in an *in vitro* OGD model of oxidative stress are inconclusive.

A: Schematic of OGD Set-up



B: Treatment during reoxygenation (90%)

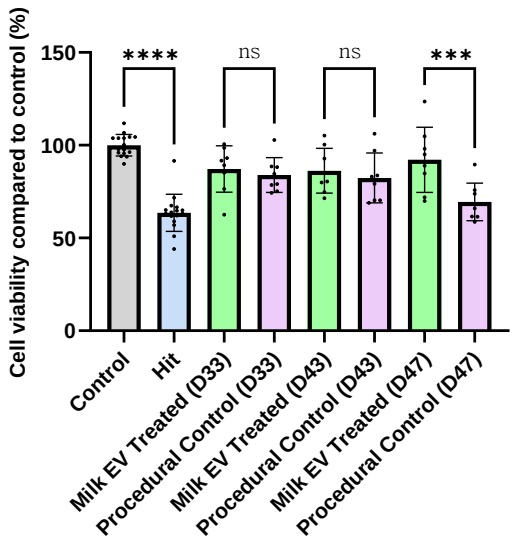


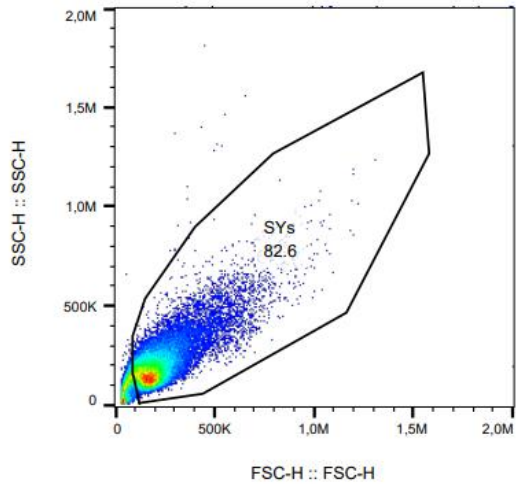
Figure 6. **A)** Schematic of OGD set-up. **B)** Neuronal cell viability under OGD and after reoxygenation with simultaneous EV co-treatment at a concentration of 90% compared to the original sample Control, n=16; Hit, n=16; Milk EV Treated groups, n=8; Procedural Control groups, n=8. p***<0.001. ****p<0.0001. Data represents mean + SEM.

Flow Cytometry and Identification of EV - SHSY-5Y association

Figure 7.A1 and Figure 7.B1 shows a dot-plot portraying the FSC-H (Forward Scatter Height) against the SSC-H (Side Scatter Height) to identify the living-cells after exposure to the EV sample of donor 43 and its procedural control, respectively. Subsequently, the living cells area were plotted in figures 7.A2 and 7.B2, in which the single-cell were defined for both the EV sample its procedural control. Then, the remaining normally sized, single-cells were plotted in figures 7.A3 and 7.B3, where the C5-Maleimide positive cells were defined for a fluorescence intensity that was approximately 10^4 or more. Lastly, in figures 7.A4 and 7.A5 the distribution of the fluorescence intensities from figures 7.A3 and 7.B3 are illustrated. Gating strategies, C5-maleimide positivity analysis, and fluorescence distribution of the EV samples and procedural controls from donors 33 and 47 are shown in the supplementary section (S4). The attention was focused on showing milk EV-SHSY-5Y association, as milk EVs impact neuronal survival in the *in vitro* oxidative stress model of EoP. In figure 7.C the fluorescence intensity of 3 EV samples and 3 adjacent procedural controls from donors 33, 43 and 47 are demonstrated together. It is evident the three EV samples (light green, dark green and pink) have an intensity of approximately 10^4 (C5-maleimide positive), while the procedural controls (red, orange and blue) have an intensity of approximately 10^3, thus indicating EV-SHSY5Y association after incubation in the hypoxic incubator. In figure 7.D a paired t-test comparison of C5-maleimide positivity between EV samples and procedural samples across biological replicates showed an increased trend in the EV samples; however, this trend was not statistically significant (p=0.2500).

A: EV D43

A1



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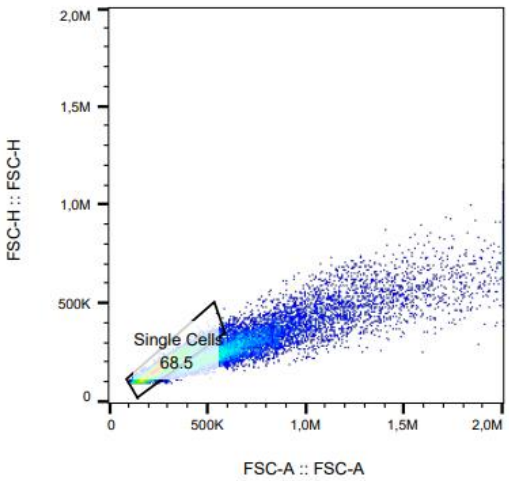

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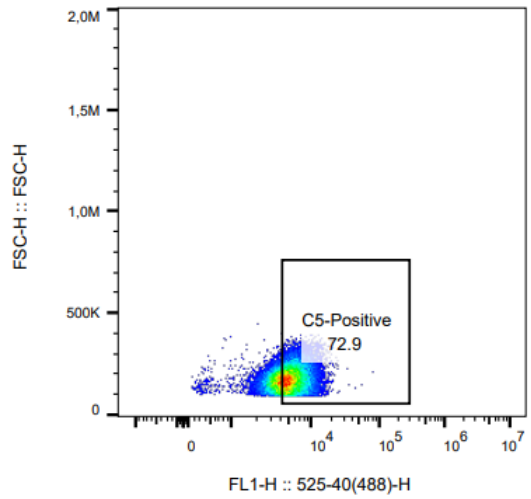

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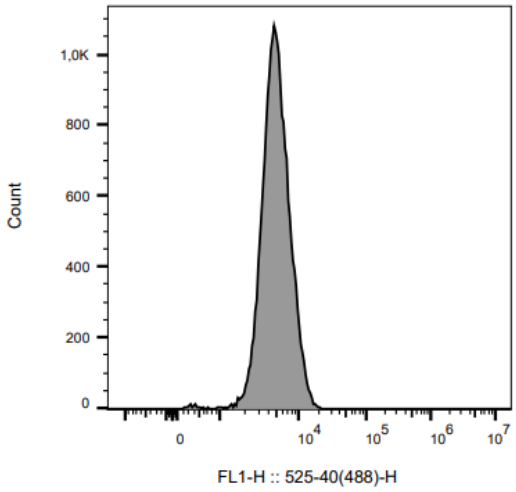
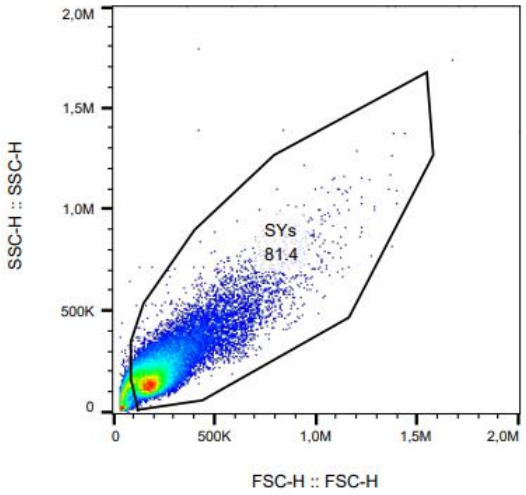
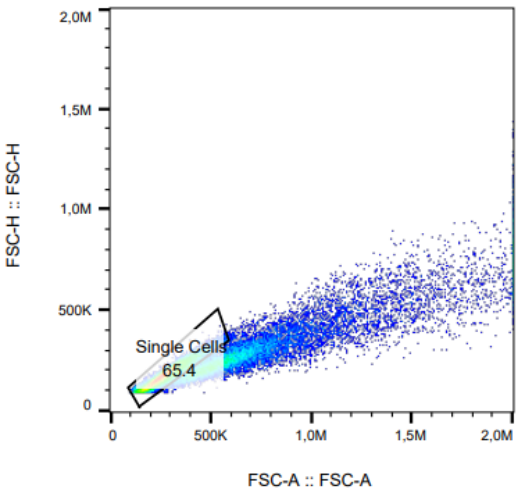


B: D43 EV-depleted

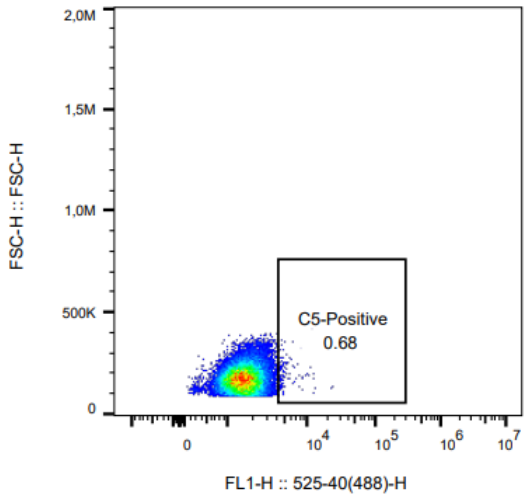
B1



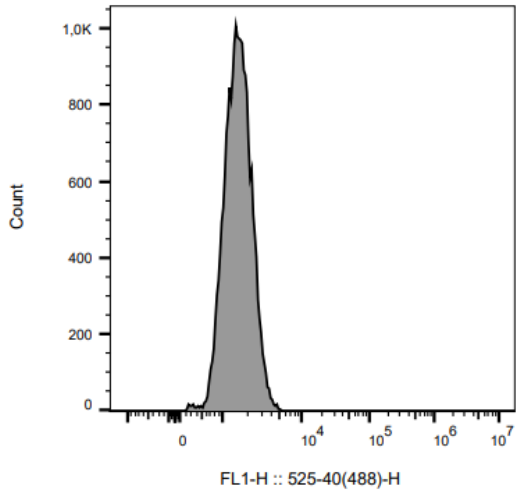
B2



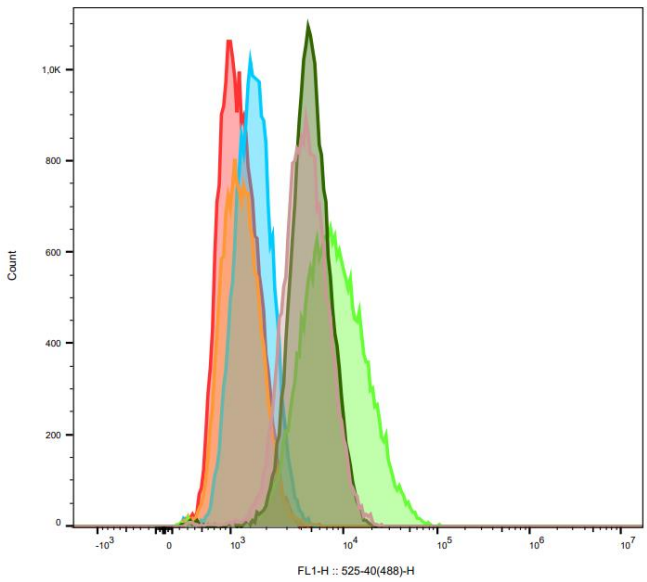
B3



B4



C



Color	Sample	Count
	EV Donor 47	22305
	EV Donor 43	22657
	EV Donor 33	23911
	EV-D Donor 33	17943
	EV-D Donor 43	21937
	EV-D Donor 47	23406

D

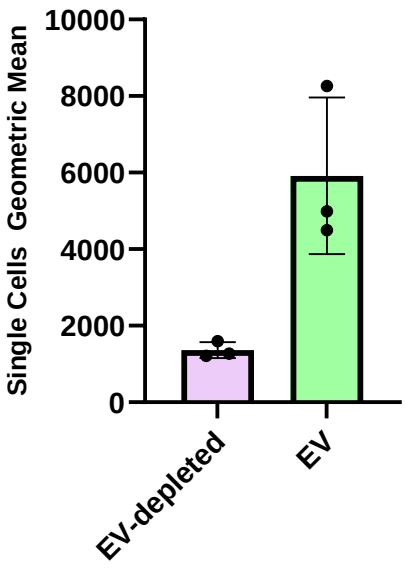


Figure 7. A) Gating strategy for milk EV identification and obtaining geometric mean for EV sample Donor 43. **B**) Gating strategy for milk EV identification and obtaining geometric mean of procedural control donor 43. **C**) Cell count of EV Treated SH-SY5Y neurons and SH-SY5Ys that received the respective procedural controls. **D**) Paired T-test of geometric means of EV Treated groups (n=3) compared to EV-depleted groups (n=3).

Chapter 4: Discussion

Preterm infants are at risk for the development of EoP, which is associated with long-term neurodevelopmental consequences. Human milk feeding lowers the risk of neurodevelopmental impairments. Since milk EVs contain anti-inflammatory and pro-regenerative cargo, it is probable that milk EVs contribute, at least partially, to the neuroprotective effects of milk.. This study used *in vitro* neuronal injury models with SH-SY5Y cells to investigate the neuroprotective potential of milk EVs. The results demonstrated for the first time that milk EVs associate with neurons and that milk EV exposure increased neuronal survival in a dose-response relationship. The results suggest that milk EV may have a potential protective effect on the developing brain in preterm infants. Before clinical translation, several preliminary steps in milk EV research are necessary. First, milk EVs could be exposed in *vitro* to injured primary neuronal cultures derived from an EoP mouse model (35). If *in vitro* treatment is successful, milk EVs should be administered intranasally *in vivo* to EoP mice to determine if the EVs reach the brain and to evaluate milk EV treatment's safety and efficacy.

Exposure of SH-SY5Y neurons to the H2O2 oxidative hit did not protect the cells from injury. At a concentration of 50% of the original EV isolation sample, neuronal survival was improved in one of out of three donors. In addition, at a concentration of 90% of the original EV isolation sample, three donors were all non-significant. However, this does not necessarily mean that pre-treatment with milk EVs would be futile. One possible explanation for the non-significant effect could be that the protective effect of milk EV exposure prior to injury is transient. A pre-treatment exposure duration of 24 hours may lead to a loss of the neuronal protective effect of milk EVs. Therefore, a future experiment could be conducted with a pre-treatment exposure duration of 4 to 6 hours prior to the H2O2 oxidative hit. Another explanation for the non-significant effect could be that the MTT assay measurement of cell metabolic activity is not sufficiently accurate. A future experiment could therefore be conducted using a Seahorse assay, which measures the metabolic activity of SH-SY5Y cells in real-time (36). Lastly, a limitation of the pre-treatment set-up could be the use of SH-SY5Y neurons. The SH-SY5Y cell line is derived from a tumor, which introduces genetic peculiarities that could potentially alter the expected response of cells to milk EV treatment (37).

However, when EVs are administered during the H2O2 hit, a full rescue effect is observed using three different donors. Additionally, a dose-response experiment showed an increase in neuronal cell viability relative to increasing doses of milk EVs. This result validates the hypothesis that milk EVs have protective effect in an *in vitro* neuronal injury model. A similar result was found with MSC-EVs that had neuroprotective and immunomodulatory effects on hippocampal neuronal cultures (38). Another study confirmed the protective effect of MSC-EVs which was attributed to the antioxidant miRNA cargo associated with the Nrf2 defense system in response to H2O2 injury (39). This miRNA cargo consisted of has-miR-215-5p, hsa-miR-424-5p, hsa-miR-31-3p, hsa-miR-31-3p, hsa-miR-193b-3p, hsa-mi-200b-3p (39). Another study found that MSC-EV-mediated protection of hippocampal neuroglial cultures led to prevention of mitochondrial calcium overload, thereby preventing apoptosis (40).

Therefore, the protective effect by milk EVs could be attributed to the regulatory role of the mentioned miRNA cargo which is also present within milk EVs (40–42). Future experiments could focus on measuring calcium concentrations or measuring Nrf2-mediated signaling activity in SHSY-5Y neurons to validate these new hypotheses. On the other hand, the treatment effect might not be attributed to EV-neuron interaction, but rather to the ability of EVs to scavenge ROS (43). This effect was also found in a study by Ikemoto et al. (2023), which found that hNSC-EVs propagate an H2O2-induced radical scavenging effect with SH-SY5Y cells (44). A future experiment to validate the scavenging activity of EVs for H2O2 could be validated conducted with an Amplex assay, which quantifies the H2O2 degradation in the presence of milk EVs (45).

Furthermore, treatment during reoxygenation in an OGD model of SH-SY5Y neurons revealed a rescue effect with milk EVs in one donor, but non-significant effects with treatment with milk EVs derived from two other donors. This variability in results may be attributed to interdonor heterogeneity of milk EVs in quantity and cargo compositions, thereby influencing EV-cell interactions and cargo-mediated effects across biological replicates (46). A study by Liu *et al.* (2020) showed that MSC-EVs in an *in vitro* OGD model can be internalized by neurons and internalize ROS, thereby decreasing neuronal apoptosis during the reoxygenation phase (47). Another study by Pisano et al. (2020) demonstrated that human milk EVs have both anti-apoptotic and pro-proliferative effects in intestinal cells in an vitro OGD model (48). Therefore, the anti-apoptotic effect might also be the hypothetical underlying mechanism for milk EVs in the *in vitro* OGD model. To investigate this anti-apoptotic property, future experiments might check for relative apoptotic reduction in milk EV-treated neurons through western blot analysis of the expression of apoptotic markers such as Bcl-2, Bax and Caspase-3 (49).

Flow cytometry using C5-maleimide-labeled milk EVs revealed that milk EVs interact with SH-SY5Y neurons, suggesting either EV docking at the cell membrane or endocytosis of the milk EVs. A similar result was demonstrated in a study by Näslund *et al.* (2014), in which PKH67-labeled milk exosomes were used to validate binding to monocyte-derived dendritic cells (50). Taken together, these findings suggest that human milk EVs might have an extensive binding affinity. However, a major limitation of this flow cytometry experiment is that it cannot establish whether milk EVs enter the neurons and if their cargo is released into the recipient cells, thereby modulating cellular physiology. To establish this, a future experiment could be conducted using fluorescence resonance energy transfer to test whether membrane fusion between milk EVs and the cell membrane or internal organelles has taken place (51).

All in all, this study confirms the promising potential of milk EVs in protecting neuronal cells, as evidenced by increased neuronal survival and interactions with SH-SY5Y neurons in an oxidative stress model with hydrogen peroxide. In the context of reducing the risk for the development of EoP, future experiments should focus on elucidating the protective effects on other cell lines as well, including neural stem cells, oligodendrocytes, astrocytes and microglia. The data suggest that milk EVs could play a significant role in mitigating oxidative stress and apoptosis. Future research should focus on refining the understanding of milk EVs'

mechanisms of action and their application in treatment strategies to prevent neurodevelopmental impairments in preterm infants. Further research is still required to enhance comprehension of factors and pathways involved, ensuring the safety and effectiveness of these optimization strategies both *in vitro* and *in vivo*.

Acknowledgements

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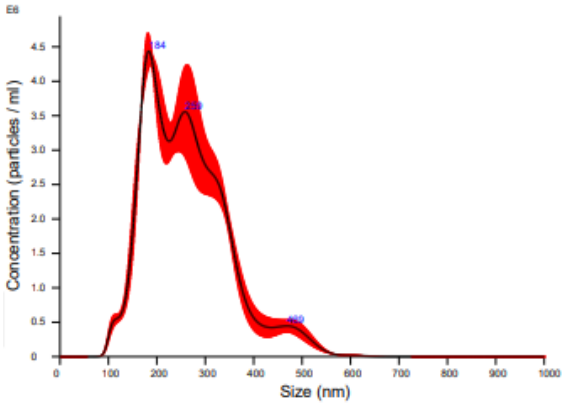
Supplementary

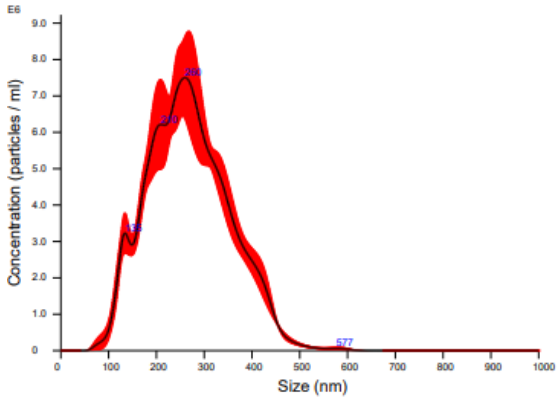
Quantification of EV concentration with NTA

Donor 35's EV sample exhibited a concentration of 7.85 x 10^8 ± 5.20 x 10^7 particles/mL (A). For Donor 40's EV sample, the concentration was 1.55 x 109 ± 3.40 x 10^7 particles/mL (B). Donor 59's EV sample showed a concentration of 9.51 x 10^8 ± 1.02 x 10^8 particles/mL (C). The EV sample from Donor 60 had a concentration of 8.21 x 10^8 ± 6.27 x 10^7 particles/mL (D). In the case of Donor 33, the EV sample concentration was 2.14 x 10^9 ± 8.67 x 10^7 particles/mL (E), while the EV-depleted sample showed a lower concentration of 1.52 x 10^8 ± 2.19 x 10^7 particles/mL (F). For Donor 47, the EV sample concentration was 1.26 x 10^9 particles/mL (I), whereas the EV-depleted sample had a concentration of 1.42 x 10^8 particles/ml (J). Donor 48's EV sample showed a concentration of 1.40 x 10^9 particles/mL (K), with the EV-depleted sample having 1.30 x 10^8 particles/mL (L). Donor 54's EV sample concentration was measured at 9.48 x 10^8 ± 7.54 x 10^7 particles/mL (M), while the EV-depleted sample was 1.79 x 10^8 ± 9.34 x 10^6 particles/mL (N). Another sample from Donor 59 had an EV concentration of 1.05 x 10^9 ± 1.79 x 10^8 particles/mL (O), with the corresponding EV-depleted sample showing a concentration of 2.99 x 10^8 ± 9.70 x 10^7 particles/mL (P).

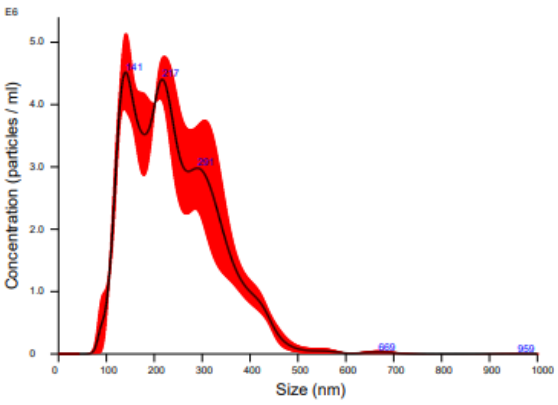
A: Donor 35 EV



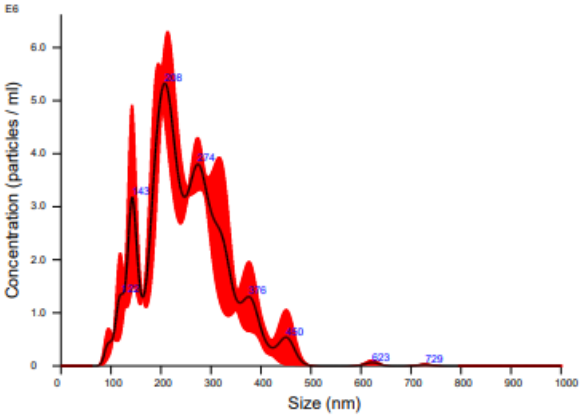
B: Donor 40 EV



C: Donor 59 EV

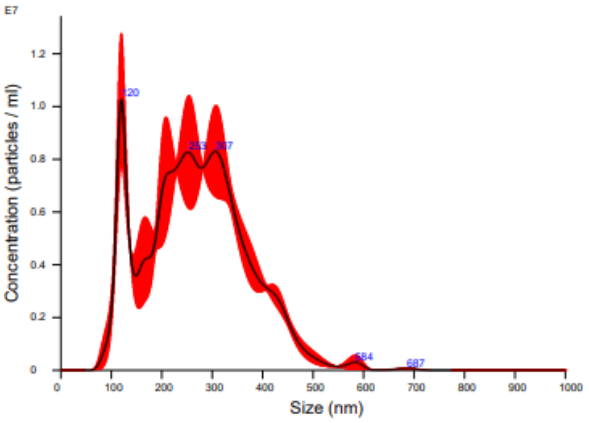


D: Donor 60 EV

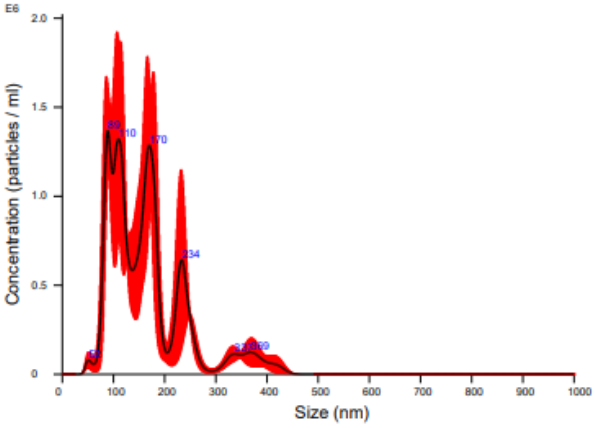


E: Donor 33 EV

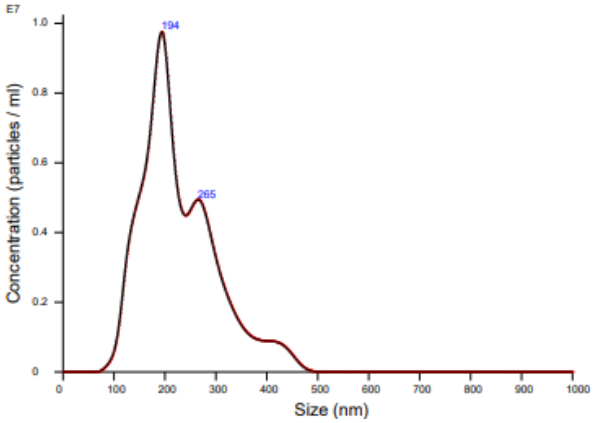
F: Donor 33 EV-D



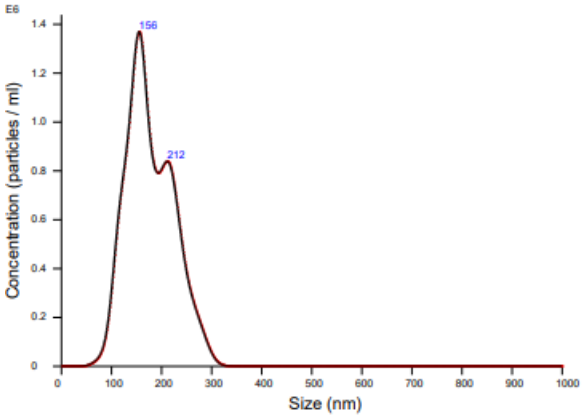
G: Donor 43 EV



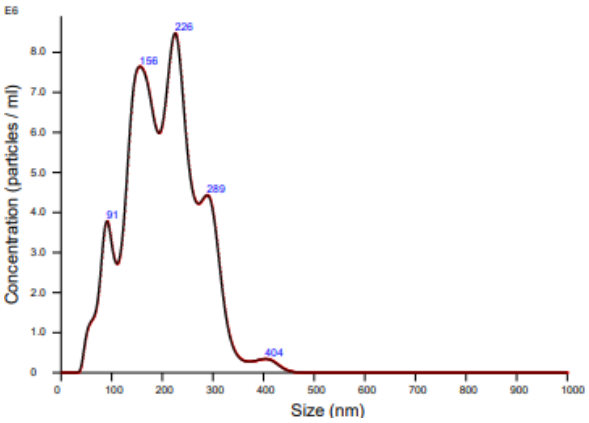
H: Donor 43 EV-D



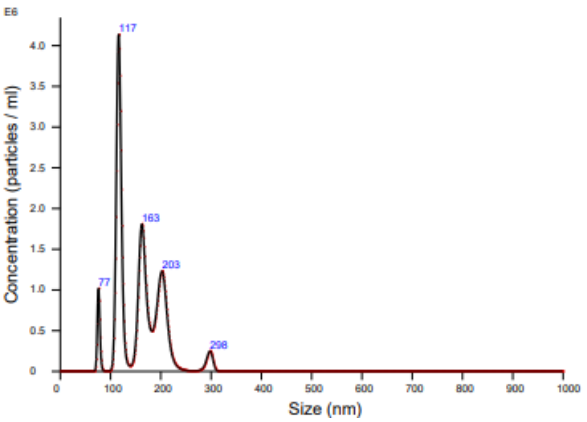
I: Donor 48 EV



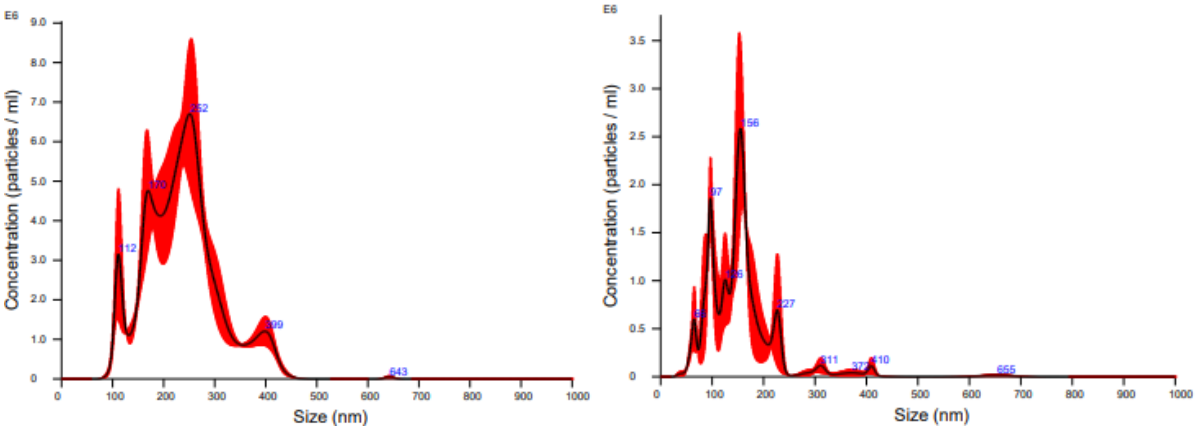
J: Donor 48 EV-D



K: Donor 54 EV



L: Donor 54 EV-D



M: Donor 59 EV **N: Donor 59 EV-D**

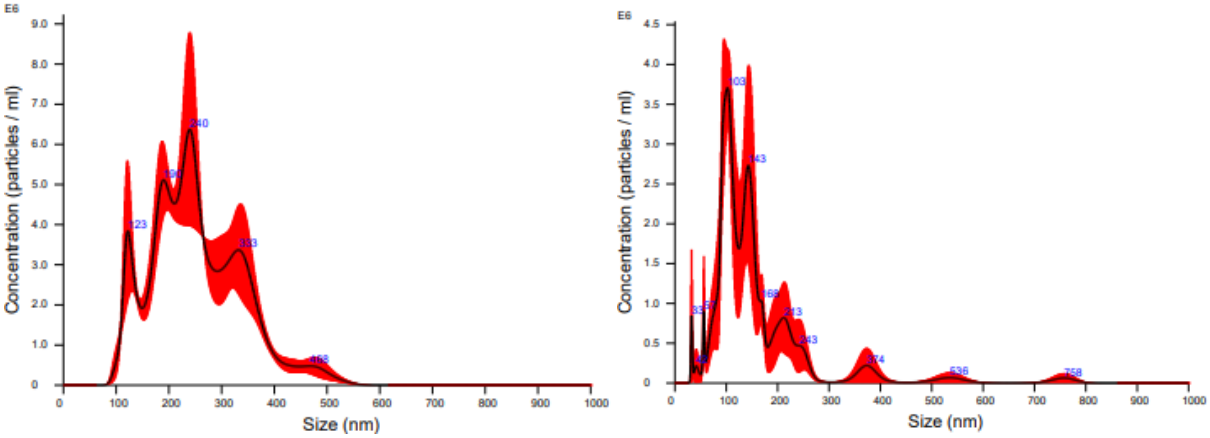


Figure X. Characterization of milk EV concentration through nanoparticle tracking analysis (NTA) of total EVs isolated using NTA 3.4 Build 3.4.4. A) Donor 35, EV sample. B) Donor 40, EV sample. C) Donor 59, EV sample. D) Donor 60, EV sample. E) Donor 33, EV sample. F) Donor 33, EV-depleted sample. G) Donor 43, EV sample. H) Donor 43, EV-depleted sample. G) Donor 47, EV sample. H) Donor 47, EV-depleted sample. I) Donor 48, EV sample. J) Donor 48, EV-depleted sample. K) Donor 54, EV sample. L) Donor 54, EV-depleted sample. M) Donor 59, EV sample. N) Donor 59, EV-depleted sample.

A H2O2 hit (80 µM) creates a rescue window for an *in vitro* oxidative stress model

Optimization experiments were executed on the *in vitro* H2O2-induced oxidative stress model to determine the concentration threshold at which a relative cell viability of approximately 50% was attained in comparison to the control. Noteworthy findings revealed significant perturbations in the H2O2 dose-response curve specifically at a concentration of 80 µM ($p<0.0001$). As a result, subsequent co-treatment experiments were conducted at these concentration to assess the therapeutic potential of milk EVs.

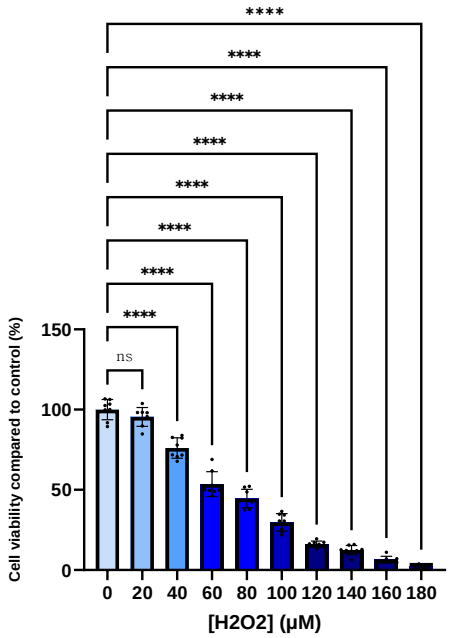


Figure S2. H2O2 dose response curve in DMEM:F12 medium. 0 µM, n=8; 20 µM, n=8; 40 µM, n=8; 60 µM, n=8; 80 µM, n=8; 100 µM, n=8; 120 µM, n=8; 140 µM, n=8; 160 µM, n=8; 180 µM, n=8. ****p<0.0001. Data represents mean + SEM.

A 16 hour window of oxygen-glucose deprivation (1% O2, 5% CO2) creates a rescue window for an *in vitro* OGD model

Optimization trials were conducted on the OGD *in vitro* model to ascertain the optimal concentration yielding a relative cell viability of approximately 50% relative to the control condition. Notably, a significant hit was observed in the OGD dose-response curve following exposure to 16 hours of OGD (1% O2, 5% CO2) ($p<0.0001$). Consequently, subsequent milk EV experiments were conducted under OGD conditions for 16 hours (1% O2, 5% CO2).

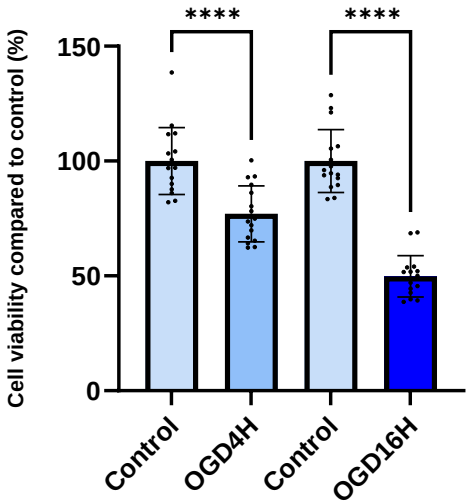


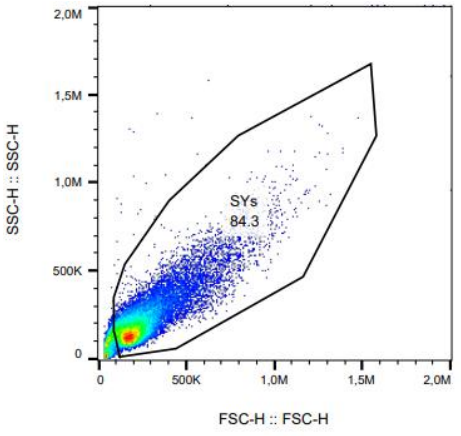
Figure S3. Relative cell viability after oxygen-glucose deprivation (1% O2, 5% CO2) for 4 h and 16 h. Control, n=16; OGD4H, n=16; Control, n=16; OGD16h, n=16;. ****p<0.0001. Data represents mean + SEM

Flow Cytometry

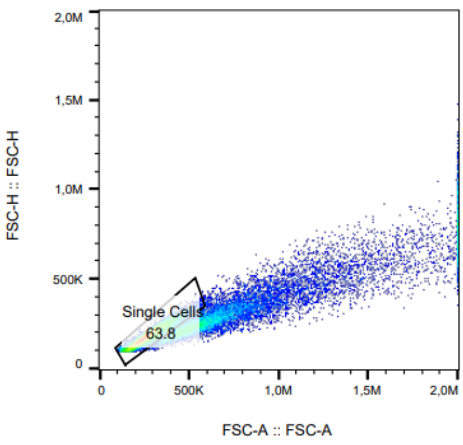
Figure S4.A1, S4.B1, S4.C1, S4.D1, S4.E1 and S4.F1 shows a dot-plot portraying the FSC-H (Forward Scatter Height) against the SSC-H (Side Scatter Height) to identify the living-cells after exposure to the C5 control, SH-SY5Y control and EV donors 43 and 47 and its procedural controls respectively. Subsequently, the living cells area were plotted in figures S4.A2, S4.B2, S4.C2, S4.D2, S4.E2 and S4.F2, in which the single-cell were defined for both the EV sample its procedural control. Then, the remaining normally sized, single-cells were plotted in figures S4.A3, S4.B3, S4.C3, S4.D3, S4.E3 and S4.F3, where the C5-Maleimide positive cells were defined for a fluorescence intensity that was approximately 10^4 or more. Lastly, in figures S4.A4, S4.B4, S4.C4, S4.D4, S4.E4 and S4.F4 the distribution of the fluorescence intensities from figures S4.A3, S4.B3, S4.C3, S4.D3, S4.E3 and S4.F3 respectively, are illustrated. Gating strategies.

A: C5 Control

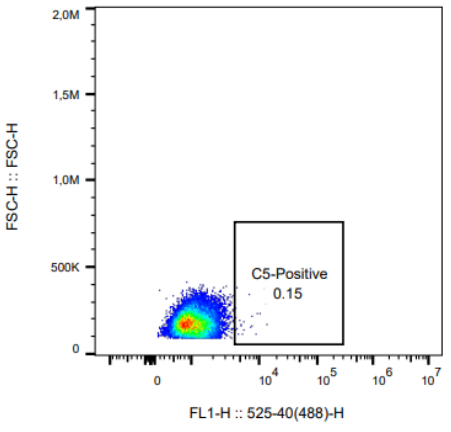
A1



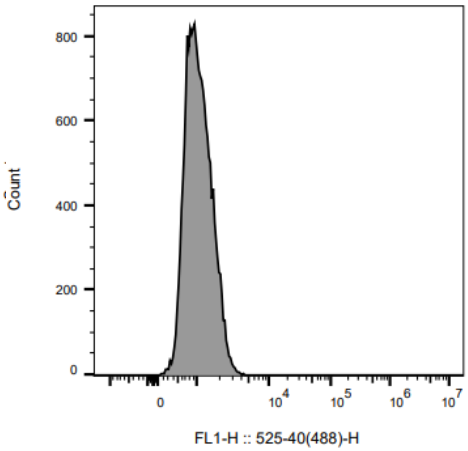
A2



A3



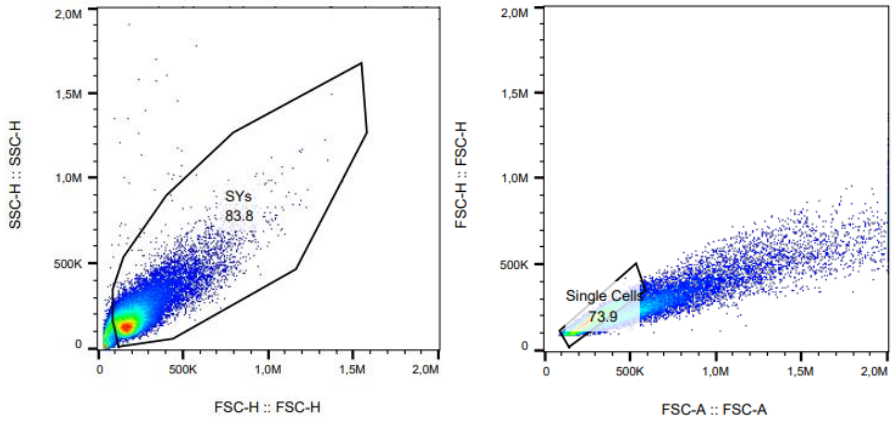
A4



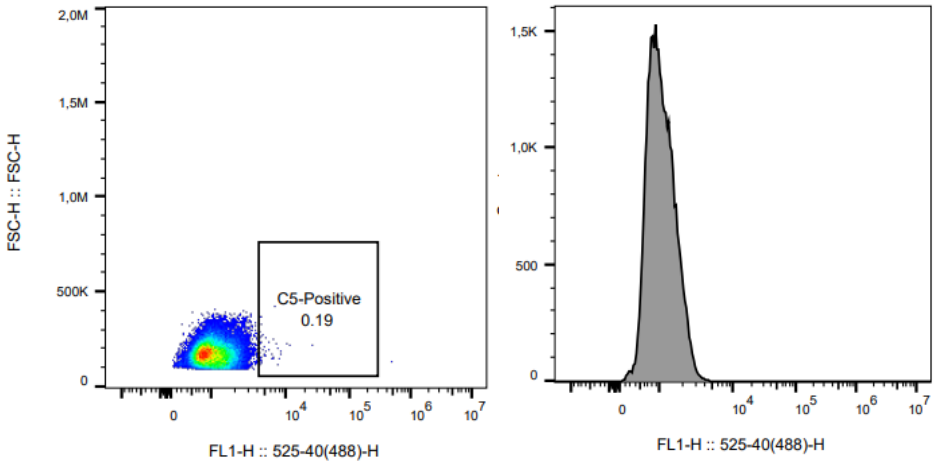
B: SH-SY5Y Control

B1

B2

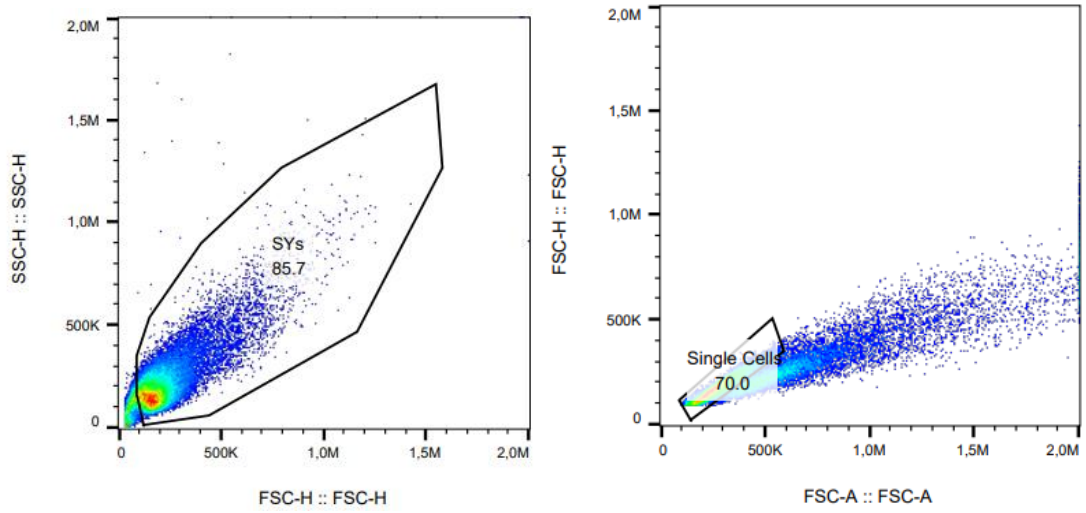


B3 **B4**

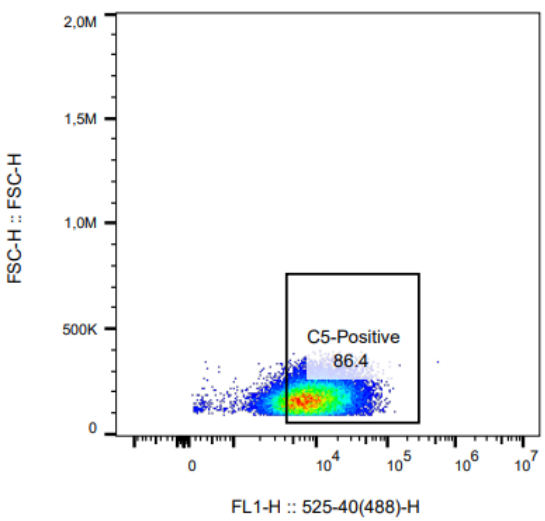


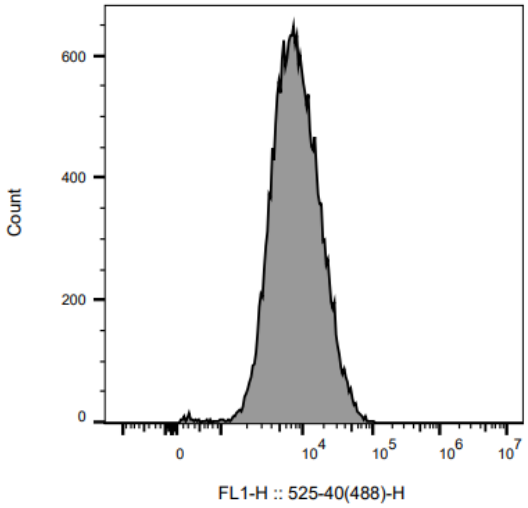
C: EV D33

C1 **C2**



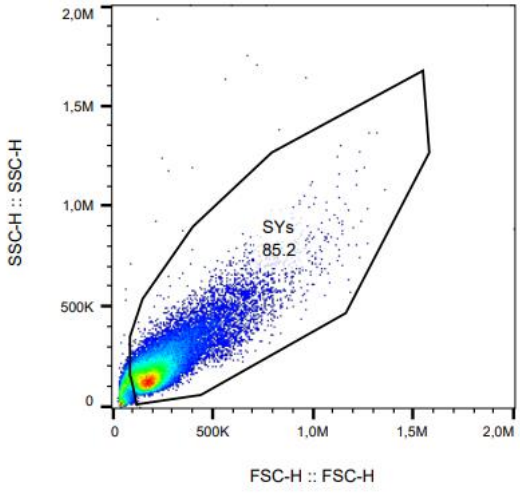
C3 **C4**



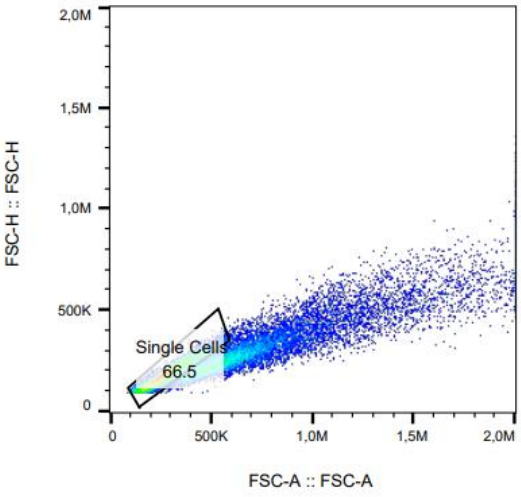


D: EV-Depleted D33

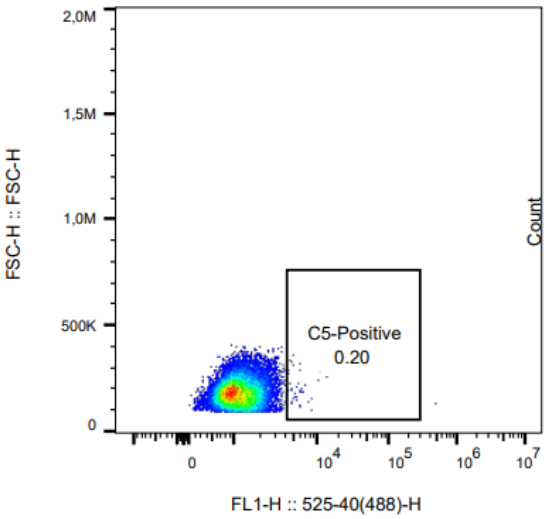
D1



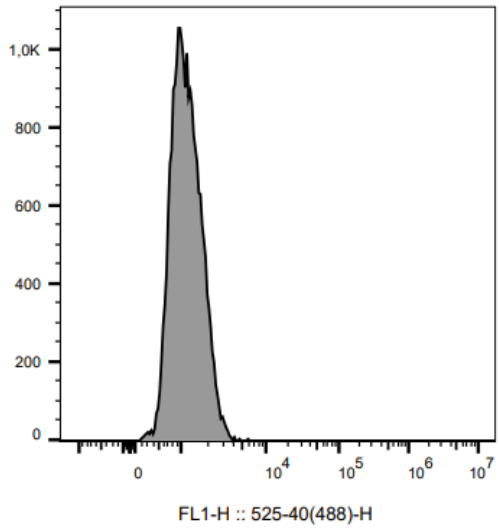
D2



D3

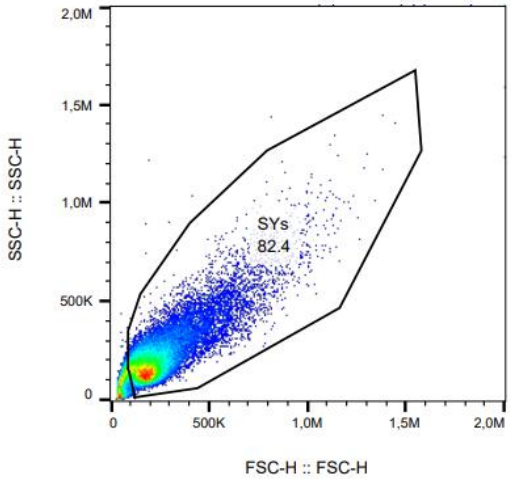


D4

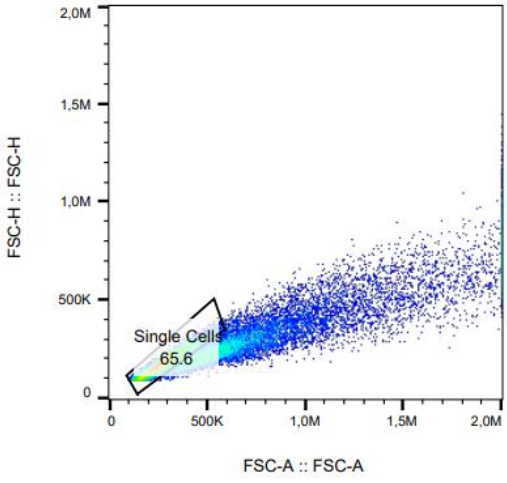


E: EV D47

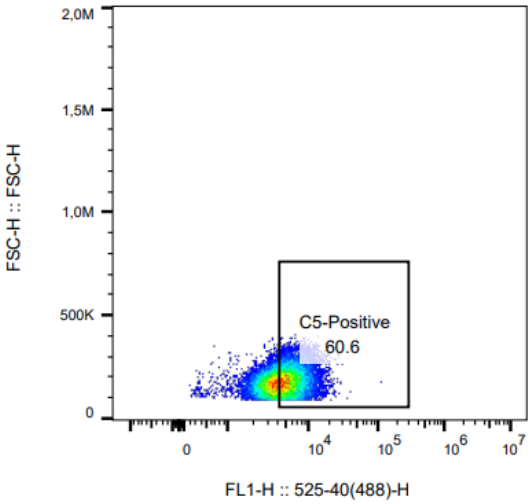
E1



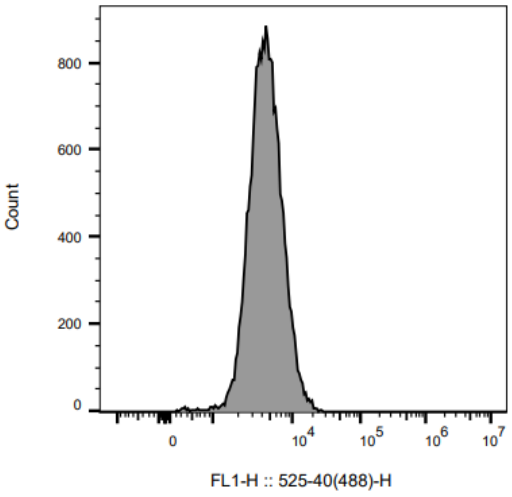
E2



E3

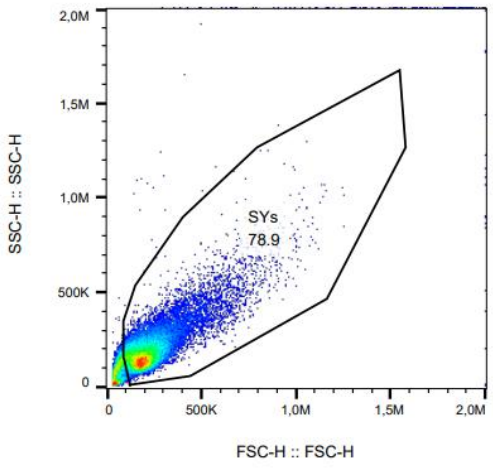


E4

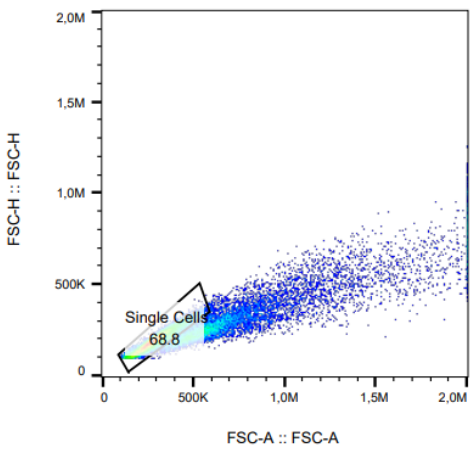


F: EV-Depleted 47

F1



F2



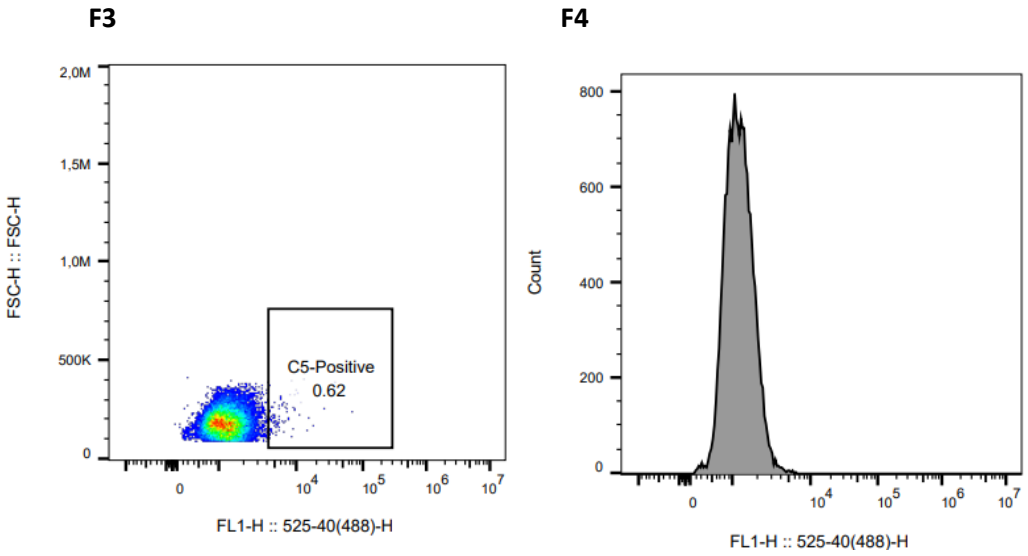


Figure S4. Gating strategy for milk EV identification and obtaining geometric mean. A) C5 Control; B) SH-SY5Y control; C) EV Sample Donor 33; D) Procedural Control Donor 33; E) EV Sample Donor 47; F) Procedural Control Donor 47