Treating amyotrophic lateral sclerosis with allogeneic Schwann cell–derived exosomal vesicles: a case report

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From the Contents

Abstract

Schwann cells are essential for the maintenance and function of motor neurons, axonal networks, and the neuromuscular junction. In amyotrophic lateral sclerosis, where motor neuron function is progressively lost, Schwann cell function may also be impaired. Recently, important signaling and potential trophic activities of Schwann cell-derived exosomal vesicles have been reported. This case report describes the treatment of a patient with advanced amyotrophic lateral sclerosis using serial intravenous infusions of allogeneic Schwann cell-derived exosomal vesicles, marking, to our knowledge, the first instance of such treatment. An 81-year-old male patient presented with a 1.5-year history of rapidly progressive amyotrophic lateral sclerosis. After initial diagnosis, the patient underwent a combination of generic riluzole, sodium phenylbutyrate for the treatment of amyotrophic lateral sclerosis, and taurursodiol. The patient volunteered to participate in an FDA-approved single-patient expanded access treatment and received weekly intravenous infusions of allogeneic Schwann cell-derived exosomal vesicles to potentially restore impaired Schwann cell and motor neuron function. We confirmed that cultured Schwann cells obtained from the amyotrophic lateral sclerosis patient via sural nerve biopsy appeared impaired (senescent) and that exposure of the patient's Schwann cells to allogeneic Schwann cell-derived exosomal vesicles, cultured expanded from a cadaver donor improved their growth capacity in vitro. After a period of observation lasting 10 weeks, during which amyotrophic lateral sclerosis Functional Rating Scale-Revised and pulmonary function were regularly monitored, the patient received weekly consecutive infusions of 1.54×10^{12} (×2), and then consecutive infusions of 7.5×10^{12} (×6) allogeneic Schwann cell-derived exosomal vesicles diluted in 40 mL of Dulbecco's phosphate-buffered saline. None of the infusions were associated with adverse events such as infusion reactions (allergic or otherwise) or changes in vital signs. Clinical lab serum neurofilament and cytokine levels measured prior to each infusion varied somewhat without a clear trend. A more sensitive in-house assay suggested possible inflammasome activation during the disease course. A trend for clinical stabilization was observed during the infusion period. Our study provides a novel approach to address impaired Schwann cells and possibly motor neuron function in patients with amyotrophic lateral sclerosis using allogeneic Schwann cell-derived exosomal vesicles. Initial findings suggest that this approach is safe.

Key Words: allogeneic; amyotrophic lateral sclerosis; exosomes; infusion; neuromuscular junction; Schwann cell

Introduction

Amyotrophic lateral sclerosis (ALS) is an incurable neuromuscular condition with accelerated loss of motor neurons (MN), resulting in the progressive paralysis of affected patients. Most patients die within 3 years of their diagnosis (Mitchell and Borasio, 2007) and remain aware of their decline as cognitive functions are relatively preserved (Irwin et al., 2007). ALS is either sporadic (90%) or genetically transmitted (10%) (Ghasemi and Brown, 2018) and affects cortical (pyramidal) and spinal cord (lower) MN, axons, and respective muscle endplates. Many treatments have been tested, but none have yet arrested or reversed the disease, with current US Food and Drug Administration (FDA)-approved therapies providing only limited survival benefits (Wong et al.,

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2021). The leading hypothesis to explain disease progression has been neurodegenerative excitotoxicity (Van Den Bosch et al., 2006), and thus approved systemic drugs, riluzole, edaravone, and sodium phenylbutyrate-taurursodiol (Relyvrio) target excitotoxic cell stress and cell death (Bensimon et al., 1994; Okada et al., 2018; Paganoni et al., 2020).

Treatments have mainly focused on central nervous system (CNS) MN survival while considering changes in muscle and neuromuscular junctions (NMJ) to be downstream consequences of "dying-forward" pathophysiology (Eisen, 2021). Although the primary theories of ALS are based on cortical and spinal cord motoneuronal injury mechanisms (Saberi et al., 2015), ALS results in severe effects on muscle and progressive damage at neuromuscular endplates (Tsujihata et al., 1984; Alhindi et al., 2022; Verma et al., 2022), with dysfunction and loss of "terminal" Schwann cells (Santos et al., 2000; Alhindi et al., 2023), which has led to a counter proposed dying-back hypothesis (Fischer et al., 2004; Baker, 2014; Verma et al., 2022).

Schwann cells (SCs) are essential partners to MN in carrying the electrical signals required to activate skeletal muscles to generate voluntary or reflex motion. SCs are intimately associated with motor neuron axons and aid axonal development and regeneration (Lasiene and Yamanaka, 2011). Amongst the non-myelinating SC, terminal Schwann cells (TSCs) support the NMJ and modulate neuromuscular transmission (Santosa et al., 2018). TSCs can normally respond to markers of severe neuronal stress such as locally released hydrogen peroxide, mitochondrial DNA, and cytochrome c with mitogen-activated protein kinases and extracellular signal-regulated kinases activation triggering local regenerative process growth (Duregotti et al., 2015). However, TSCs exhibit abnormal morphology in human ALS muscle samples with invasion of the synaptic cleft (Bruneteau et al., 2015), and in rodent ALS models, TSCs have abnormally elevated intracellular calcium levels (Arbour et al., 2015). Thus, SCs are implicated in contributing to ALS-related pathological changes and disease progression (Yang et al., 2001; Lobsiger et al., 2009).

Over the course of several human studies, the authors have established the safety of transplanted autologous SC and developed robust, highly characterized production methods in FDA-approved studies in both spinal cord (Anderson et al., 2017; Gant et al., 2022) and severe peripheral nerve injuries (Gersey et al., 2017). Our human SC culture and manufacturing methods are reliable and have produced therapeutic cells that have been transplanted in three clinical trials (Levi et al., 2016; Anderson et al., 2017; Gersey et al., 2017; Santamaria et al., 2020; Khan et al., 2021, 2022; Gant et al., 2022). These trials have utilized autologous cell preparations which require a nerve biopsy and several cell culture steps. The manufactured SCs were then delivered by injection directly to focal injury sites in the spinal cord or nerves. Systemic or intrathecal delivery has not been considered. However, it has long been apparent in cell culture studies that SC release trophic factors that condition their media (Bosch et al., 1988), but only recently has the secretion of extracellular vesicles (EVs-intended as exosomes in this report) been appreciated as an important cell signaling factor (Maggio et al., 2019). Cultured SCs have a non-myelinating dedifferentiated phenotype akin to the phenotype of "repair" SCs after nerve injury (Jessen and Mirsky, 2016) and produce EVs.

Given the extensive history of SCs-supported repair in the peripheral nervous system, both endogenously and through cellular transplantation, we became interested in whether Schwann cell-derived exosomal vesicles (SCEVs) could extend the repertoire of repair through systemic distribution. Following intravenous (IV) delivery, SCEVs could have effects both in the CNS and in the periphery involving nerves and muscles. Exosomes and other nanovesicles normally circulate in the blood (Sverdlov, 2012) and can have activity at sites distant from where they are produced. EVs also cross the blood-brain-barrier (Alvarez-Erviti et al., 2011; Huang et al., 2022) and can deliver therapeutic molecules (Qu et al., 2018). After experimental traumatic brain (Wei et al., 2019), spinal cord, and peripheral nerve injuries, SCEVs have been shown to have therapeutic effects, with several studies reporting beneficial outcomes in peripheral nerve injury and regeneration models (Lopez-Verrilli et al., 2013; Pan et al., 2022; Yuan et al., 2023). SC exosomes were shown to alleviate Paclitaxel-induced peripheral neuropathy through miR-21 repression of PTEN (You et al., 2023). Moreover, in a CNS model, SCEVs had a neurotrophic effect after optic nerve crush by upregulating the cAMP Response Element-Binding Protein pathway (Zhu et al., 2023).

We are also interested in EV biodistribution to muscle (Kang et al., 2021) and the NMJ (Pikatza-Menoio et al., 2021) that are implicated in non-neuronal cell autonomous aspects of the ALS disease process (Fischer et al., 2004; Ferrara et al., 2018), including abnormal NMJ and satellite cell function. Muscle typically accounts for approximately 30%–40% of the body's tissue and receives 20% of cardiac output at rest.

Exosomal transfer from blood can influence muscle health in animal models, and muscles release EV-containing microRNAs that are essential for muscle and endocrine homeostasis (Aoi and Sakuma, 2014; Guescini et al., 2015; Aoi and Tanimura, 2021). Experimental denervation of muscle has been shown to increase miR-206 and decrease miR-133 with probable perturbation of muscle fiber survival and trophic functions (De Gasperi et al., 2017). Intravenously administered exosomes derived from mesenchymal stem cells (MSCs) can support myogenesis (Wan et al., 2022) and muscle repair after limb ischemia (Doan et al., 2023). In mdx mutation, muscular dystrophy mice, 2.0×10^9 IV-delivered cardiosphere exosomes improved cardiac muscle function (Rogers et al., 2019), and SCEVs reduced denervation-induced muscle atrophy (Lin et al., 2023).

Prior to our current understanding of exosomal signaling, it was discovered that both the sera and cerebrospinal fluid of ALS patients had neuro and myelotoxic effects in neural tissue cultures (Field and Hughes, 1965; Wolfgram and Myers, 1973; Couratier et al., 1993; Ng Kee Kwong et al., 2021). Modern culture studies with ALS plasma show adverse effects on vascular endothelial growth factor expression (Khosla et al., 2023) and calcium-mediated neurotoxicity (Polgár et al., 2021). These findings underscore the systemic nature of ALS.

SCEVs are thus a novel potential therapeutic derivative of SCs that could be delivered systemically (**Figure 1**). From this collective evidence, we hypothesized that SCs-derived exosomes might have muscle, nerve, and NMJ trophic effects in ALS, including upon perisynaptic SCs (Duregotti et al., 2015; Arbour et al., 2017; Alhindi et al., 2023), as well as protective effects on CNS inflammation.

Case Report

Clinical application of IV SCEVs

In this case report, we present the case of an 81-year-old male patient with a 1.5-year trajectory of progressive ALS. The patient's condition advanced rapidly; he was ambulatory at home for four months, became wheelchair-bound within 8 months, required automatic positive airway pressure support after a year, and progressed to full dependence on automatic positive airway pressure within 14 months. Given the advanced stage of the patient's ALS and having exhausted all FDA-approved treatments for the disease, we sought an alternative therapeutic route through the FDA's Expanded Access Program. The patient was thoroughly informed about the compassionate use process, expressing understanding and willingness to proceed. Subsequent to receiving FDA approval, University of Miami Institutional Review Board (IRB) authorization was secured (September 20, 2022), and the patient provided his written informed consent, thereby initiating the exploratory treatment.

Nerve biopsy of the patient

In an attempt to produce autologous EV, we obtained a left sural nerve biopsy (5 cm) under local anesthesia from the patient. We cultured the extracted nerve fascicles according to our SCs culture and expansion procedures following current good manufacturing practices (CGMP) (Khan et al., 2022), which have been used for our clinical trials. We placed the biopsy in culture in an optimized SCs medium to expand and study the growth characteristics of the patient's SCs (Khan et al., 2022). After one passage of SCs culture, we noticed a senescent morphology. When we determined that SCs were not expanding well, we treated them with allogeneic SCEVs from young disease-free donors as a source of exogenous bioactive molecules that could mimic the natural supportive role of SCs. By incorporating these allogeneic EVs into the culture, the aim was to deliver therapeutic molecules directly to the patient's cells, thereby possibly overcoming the inherent growth limitations and promoting a more favorable environment for cell survival and proliferation.

Selection of allogeneic donor and preparation of SCs

To generate the SCEVs for clinical use, SCs were extracted from a nerve that was obtained through the Life Alliance Organ Recovery Agency (LAORA) University of Miami, FL) from a non-ALS cadaveric young adult donor (male, aged 22 years). SCs were dissociated from the nerve and culture-expanded, as previously reported (Khan et al., 2022). The process of selecting allogeneic donors adheres to established transplant protocols, ensuring all donors satisfy the eligibility criteria specified in 21 United States Code of Federal Regulations Part 1271 (https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=1271). The SCs were isolated and subsequently culture-expanded following documented methodologies. The manufacturing process aligns with the regulations for cGMP and current Good Tissue Practice (CGTP), ensuring the highest standards of quality and safety for clinical application.

Isolation of allogenic SCEVs

SCEVs were collected from culture media at passage 3, filtered and ultra-centrifuged, resuspended in Dulbecco's phosphate-buffered saline (DPBS), and characterized using nanoparticle tracking analysis. Sterility and mycoplasma tests were negative, with endotoxin levels below < 0.5 EU/kg body weight. The resulting SCEV concentration was 3.74×10^{11} and 9.28×10^{9} with a modal size of 137.0 nm. The protein content was 0.48 g/L, while CD81 and CD63 expression levels were above 90% as measured by flow cytometry (**Table 1**). SCEVs were frozen until they were ready to be used.

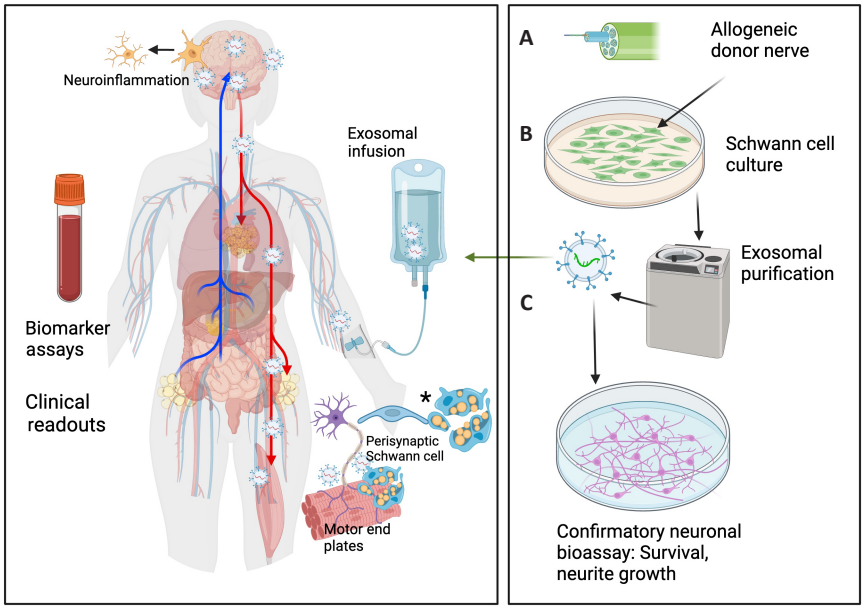

Figure 1 | Schematic of overall procedures.
(A) Nerve from a non-amyotrophic lateral sclerosis donor is used to derive a Schwann cell culture (B) and then generate allogeneic extracellular vesicles released by Schwann cells through media ultracentrifugation, which are then assayed for effects on neuronal culture (C). Exosomes are then infused and circulated with potential effects on central nervous system inflammation and trophic/supportive effects in muscles and nerves, including at the neuromuscular junction. Blood is sampled from the patient for biomarkers, and clinical readouts are performed.

Table 1 | **Release testing of SCEV**

Assay	Results
Sterility	Negative
Endotoxin	< 0.5 EU/kg body weight
Mycoplasma	Negative
Particle concentration per mL	$3.74 \times 10^{11} \pm 9.28 \times 10^{9}$
Particle mode size: between 50 and 200 nm	137.0
Protein content	0.48 mg/mL
Characterization by flow cytometry analysis	CD81 90.5%
	CD63 95.5%

EU: Endotoxin units, CD81 and CD63 are tetraspanins known to be enriched in exosomal membranes; SCEV: Schwann cell extracellular vesicles.

Bioassays

In the conducted bioassays, SCEVs were administered to both hippocampal and dorsal root ganglion neurons at a concentration of 6×10^{10}/mL particles. The assays aimed to measure the efficacy of SCEVs by quantifying neuron survival rates and the extent of neurite outgrowth. This process constitutes a potency assay, as it evaluates the ability of the SCEVs to exert a biological effect.

Clinical protocol for allogeneic SCEV infusions

Having determined that the patient's SCs were dysfunctional, exhibited correction with allogenic SCEVs, with positive neural bioassay effects with allogeneic SCEVs, as well as other preclinical data, we proceeded with our protocol of weekly allogeneic SCEV IV infusions for our patient. Each week before SCEV infusions, in addition to a complete physical examination, the patient was evaluated with the ALS Functional Rating Scale-Revised (ALSFRS-R) (Cedarbaum et al., 1999), and for pulmonary function with a spirometry device (Medical Technologies, EasyOne Air) administered by a trained technician under consistent conditions and schedule (**Tables 2** and **3**). Six standard measurements were performed over 10 weeks before infusions started, to document the ALS baseline progression, then nine times over the subsequent nine weeks to assess the impact of SCEV infusions.

The protocol provided to the FDA described the infusion and post-infusion procedures in detail. The first infusion was performed in the Intensive Care Unit at the University of Miami Hospital under close intensivist supervision. It contained 1.54×10^{12} SCEVs diluted in 40 mL of DPBS administered with an initial safety test of 5 mL over 10 minutes followed by a waiting period of 15 minutes, and then 2 mL/min. The infusion was accomplished without allergic reactions or adverse effects related to SCEVs. After that, for the patient's comfort, subsequent infusions were administered at his home with the FDA's approval according to a strict protocol. A dose escalation occurred for the third infusion (7.5×10^{12} SCEV diluted in 40 mL of DPBS). Subsequently, five more infusions (7.5×10^{12} SCEVs) were delivered without detectable adverse events. Thus, the patient received eight weekly infusions, two low doses (1.54×10^{12} SCEVs), and six medium doses (7.5×10^{12} SCEVs. Treatment administration was intermittently halted as the SCEV production process was still undergoing optimization, which led to an inability to produce the higher planned weekly doses of SCEVs.

Systemic biomarkers

Comprehensive blood analyses, detailed in the supplementary online materials, including a cytokine panel, were conducted the week before the first infusion and then each week just before initiating each infusion. In addition, as a posthoc test, we assayed two blood samples using a highly sensitive single molecule array (Simoa, Quanterix, Billerica, MA, USA) technology assay for neurofilament light (NF-L) and additional cytokines potentially associated with inflammasome activation.

Patient's SC cultures and partial rescue with allogeneic SCEVs

The patient's SCs appeared dysfunctional (they exhibited a rounded morphology with stunted processes) and proliferated poorly (**Figure 2A**), they could not be expanded beyond passage 2. The cultured SCs were interspersed with numerous fibroblasts (**Figure 2A**), affecting the purity of the culture.

When cultured with allogeneic control SCEVs generated from normal (younger, non-ALS) donor SCs (**Figure 2B**), an enhancement in the patient's SC vitality was observed, evidenced by an increased expression of the SC marker (CD 271) at 38.51% (**Figure 1D**), as compared to 20.43% (**Figure 2C**) without the non-ALS SCEVs. In addition, the presence of fibroblasts (CD90) was reduced to 29.10% (**Figure 2D**) as compared to 50.65% (**Figure 2C**). This experiment suggests that the peripheral nerve SCs of the patient were poorly functional, possibly contributing to declining MN function.

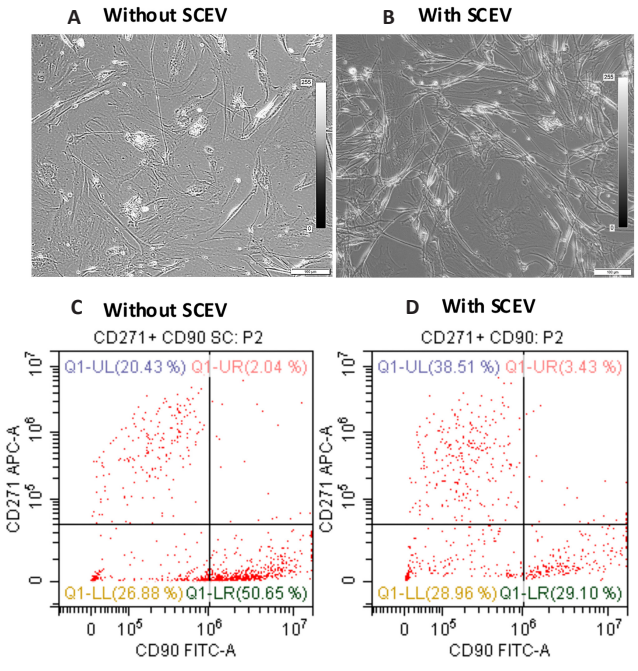

Figure 2 | **Patient Schwann cell culture analyses.**
Schwann cell cultures from the ALS subject with and without added allogenic extracellular vesicles released by Schwann cells (SCEV) from a donor without ALS. Phase contrast microscope, original magnification, 10×. Without (A) or with allogeneic SCEV (B) was added. (C, D) Flow cytometric analysis for Schwann cell marker CD271, and fibroblast marker CD90, same conditions as A and B. In D, with allogeneic SCEV, the percent of CD271 is increased and that of CD90 is decreased.

Table 2 | **Time and event schedule for two low (1.54 × 10^{12} SCEVs) and two medium doses of SCEVs (7.5 × 10^{12} SCEVs).**

	Baseline	Day 0	Day 1	Day 2	Day 7 (±2 days)	Day 8	Day 9	Day 14 (±2 days)	Day 15	Day 16	Day 21 (± 2 days)	Day 22	Day 23
Demographics	X												
Medical history	X												
Physical exam	X	X			X			X			X		
Vitals[a]	X	X			X			X			X		
Telephone call			X	X		X	X		X	X	X	X	X
CBC and CMP	X	X			X			X			X		
ALSFRS-R	X				X			X			X		
Spirometry	X				X			X			X		
SCEVs IV Infusion		X			X			X			X		
Biomarkers	X	X			X			X			X		
CRP													
Serum NFL													
IL6, IL8, IL5 & IL2													
Adverse event monitoring							X						

[a]Vital signs will be done at specific intervals pre, during and post infusion. ALSFRS-R: Amyotrophic Lateral Sclerosis Revised Functional Rating Scale; IL: interleukin; NFL: neurofilament light chain; SCEVs: extracellular vesicles released by Schwann cells.

Table 3 | **Time and events schedule for 4 medium doses of SCEVs (7.5 × 10^{12} SCEVs)**

	Day 28 (±2 days)	Day 29	Day 30	Day 35 (±2 days)	Day 36	Day 37	Day 42 (±2 days)	Day 43	Day 44	Day 49 (±2 days)	Day 50	Day 51
Demographics												
Medical history												
Physical exam	X			X			X			X		
Vitals[a]	X			X			X			X		
Telephone call		X	X		X	X		X	X	X	X	X
CBC and CMP	X			X			X			X		
ALSFRS-R	X			X			X			X		
Spirometry	X			X			X			X		
SCEVs IV Infusion	X			X			X			X		
Biomarkers	X			X			X			X		
CRP												
Serum NFL												
IL6, IL8, IL5 & IL2												
Adverse event monitoring							X					

[a]Vitals will be done at specific intervals pre, during and post infusion. ALSFRS-R: Amyotrophic Lateral Sclerosis Revised Functional Rating Scale; IL: interleukin; NFL: neurofilament light chain; SCEVs: extracellular vesicles released by Schwann cells.

Clinical outcome

None of the infusions were associated with adverse effects such as infusion reactions or changes in vital signs. Unfortunately, the highest planned dose (35 × 10^{12}) of SCEV was not administered as during the weeks-long pause in infusions to collect additional SCEVs; the patient deteriorated to a point below the inclusion criteria. Hence, this therapy, if supported by future studies, may require sufficient supplies of SCEVs for long-term administration.

The only significant patient complaint that had started prior to infusions, besides his rapid loss of strength and breathlessness, which was corrected most of the time by his automated positive airway pressure, was difficulty in expectorating his phlegm. This issue would become acute about once a day and is a known ALS complication that continued after the initiation of infusions despite all preventive maneuvers with nebulizers, mucolytics, and coughing-support devices.

The pre-infusion rate of functional decline (ALSFRS-R) (Mandrioli et al., 2015) shown in **Figure 3** averaged 1.71 points/month, followed by seven measurements during the infusion period with an average rate of decrease of 1.22 points per month (difference 0.49 points per month). The last two measurements were obtained after the discontinuation of the infusions. The shaded boxes (**Figure 3**) illustrate an apparent stabilization of the disease during the infusion period.

Pre-infusion pulmonary function declined as expected in ALS (Schiffman and Belsh, 1993; forced vital capacity in liters measured by spirometry), as shown in **Figure 3** upper panel. The following seven measurements correspond to the infusions period, and the last two measurements were obtained after the discontinuation of the infusions.

Comprehensive blood analysis (detailed in **Additional Table 1**), including a cytokine panel, was obtained the week

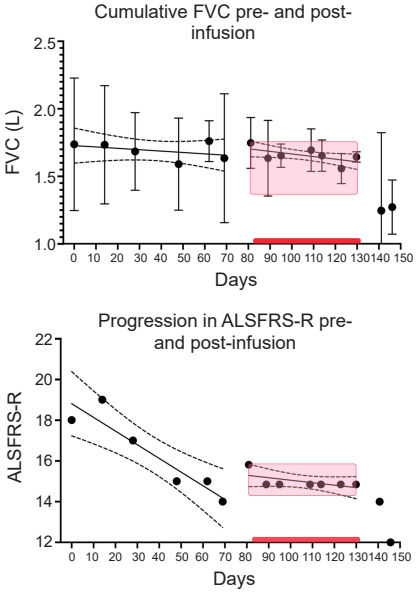

Figure 3 | **Patient's forced vital capacity and ALSFRS-R.**
Values of FVC and ALSFRS-R during the study. The pre-infusion period was 70 days. The period in which the seven infusions occurred is indicated on the x-axis with the red bar, and with the pink boxes. There were two values after the infusions were paused. The stabilization of values is indicated by the pink boxes. Overall slopes are shown for the pre-infusion and infusion periods, including all values. ALSFRS-R: Amyotrophic Lateral Sclerosis Revised Functional Rating Scale; FVC: forced vital capacity.

before the first infusion and then each week just before initiating each infusion. Over a wide range of laboratory tests capturing hematology, blood chemistry, and liver function, no perturbations related to exosomal infusions are detectable. No notable changes in the cytokines interleukin (IL)-6, IL-10, or the ALS biomarker NF-L were observed during the infusion period compared to baseline. However, markers of possible inflammasome activation (elevated IL-18 and apoptosis-associated speck-like protein) were discovered in a separate assay on 2 weekly blood samples, which were analyzed to assess if the clinical lab NF-L reports were valid.

One notable biomarker associated with ALS progression is serum creatinine (Cr) (Sun et al., 2020). **Additional Figure 1** shows the patient's Cr trajectory over 673 days. His Cr of 0.54 mg/dL at the beginning of infusions indicates that he is late in the disease course at therapy onset, Cr is stable during the infusional period but drops markedly afterwards. This parallels the other findings such as ALSFRS-R.

While no significant adverse events related to infusions were noted during the entire infusion period, two days after the last infusion, the patient complained of diarrhea temporally associated with an elevation of C-reactive protein (from 20 mg/L at baseline to 200 mg/L). Then a week later, 2 days after repeated blood work that showed a decrease in his C-reactive protein and no elevation of his white blood cells and neutrophils, the patient presented with acute respiratory destabilization and was admitted to the hospital and needed emergent endotracheal intubation and ventilation (the patient had requested to remain full code at that point). In addition, systemic hematological stress indices were highly elevated, with elevated WBC and a neutrophil-lymphocyte ratio (Choi et al., 2020; Murdock et al., 2021) of 39.8.

Diagnostic imaging revealed right upper lobe pneumonia on the patient's X-ray. The patient requested the removal of his endotracheal tube and rejected the offer of further advanced respiratory care, including a tracheostomy. Subsequently, the focus of care shifted to ensuring his comfort, and he peacefully deceased thereafter.

Discussion

Dysfunction of the ALS patient's SCs

The patient's nerve and SCs were prepared per a validated protocol that is generally effective for the generation of a large number of SCs with high purity (Bunge et al., 2017) and has been used for our clinical trials. We have substantial experience with monitoring such cultures, with expected expansion rates and ranges published (Casella et al., 2000). The patient's sural nerve biopsy was handled in accordance with our protocols with no deviations. In our experience with culturing nerve donors, it is uncommon to have culture failure. However, it is worth noting that this patient was older than what is typically observed and had ALS.

Initially, all SC cultures contain extensive fibroblasts as these are components of nerve structure. The initial process to isolate SCs from nerve fascicles results in a mixture of both fibroblasts and SCs. The clinical cGMP methods we have developed favor a greater rate of SC expansion as compared to fibroblasts due to added mitogens, including heregulin and forskolin (Bunge et al., 2017).

This patient's early culture contained SCs with a senescent morphology. The lack of SC proliferation resulted in an inability to select and purify the cells. In our experience, human SCs are prone to senescence (Fuentes-Flores et al., 2023) after three culture passages, while fibroblasts will continue to divide. It is thus essential that the initial passages yield strongly dividing SCs.

Thus, ALS nerve donors may have compromised SC populations, and the process of obtaining autologous SC cultures can be fraught with significant delays and risk of failure. One notable advantage of exploring allogeneic exosomes, as demonstrated in our first patient, is the feasibility of clinical lot production and administration, with no apparent adverse responses detected. The use of SC exosomes could represent a significant advance in the application of cellular therapeutic programs for ALS.

Additionally, it is important to acknowledge that the utilization of nerve fascicles from older individuals, as seen in this case where the patient was 81 years of age, can potentially lead to reduced repair capacity (Kerezoudi and Thomas, 1999; Sardella-Silva et al., 2021; Wagstaff et al., 2021). Aged SCs are known to exhibit diminished repair capabilities, which may have contributed to the challenges we faced.

The patient is one of the oldest individuals from whom we have attempted to expand SCs. It is thus possible that the poor expansion was also related to his age rather than ALS. The inability to expand the culture would greatly limit the

quantity of exosomes that could be harvested. The ability to deliver exosomes from younger allogeneic donor sources may thus be an advantage in this situation.

The rationale for treating ALS patient-derived SCs, which was exhibiting growth arrest, with allogeneic cadaveric SCEVs is founded on the premise that these vesicles may contain pro-survival and regeneration-promoting molecules that can rejuvenate or stimulate the patient's cells in culture. SCs play a critical role in the support and regeneration of neurons in the peripheral nervous system, and their EVs are known to carry a multitude of growth factors, cytokines, and genetic materials that can influence cell growth and survival (Ghosh and Pearse, 2023). The ability to improve SC cultures using added EVs may also have implications to improve the efficacy of SC transplant paradigms by further optimizing SC function (Haertinger et al., 2020; Liu et al., 2020).

In the context of ALS—a condition characterized by the degeneration of motor neurons leading to progressive muscle weakness and atrophy—the introduction of SCEVs may potentially provide trophic support to the ailing motor neurons or the surrounding glial cells. The growth arrest observed in the patient's cells *in vitro* suggests a dysfunctional cellular environment, possibly due to deficits in autocrine or paracrine signaling, which are essential for maintaining cell proliferation and function. Allogeneic cadaveric SCEVs represent a source of exogenous bioactive molecules that could mimic the natural supportive role of SCs.

EV dose selection

Limited current guidance exists for the therapeutic dosing of exosomes and scale-up from animal models. The dose selected was an extrapolation from simplified allometric scaling (primarily body weight (Health and Services, 2001)), *in vitro* DRG SC exosome assays, ongoing rodent traumatic brain and spinal cord injury SC exosome studies, and other considerations from human studies such as allometric rodent-to-human estimates for IV cell delivery (Brown et al., 2021). In a meta-analysis of pre-clinical exosome studies, most rodent doses were in the (10^8–10^{10})/kg range (Gupta et al., 2021). A dose-dependent biodistribution is reported with lung and liver saturation present in mice at (1.0–1.5) × 10^{10} particles per gram 24 hours after IV administration (Wiklander et al., 2015).

Our selected doses are also within the approximate range of reported MSC EV doses, which are currently the most prominent in clinical trial protocols. These dosing reports vary from using exosomal mass such as 100 µg/kg (Nassar et al., 2016) to particles per volume. ExoFlo, an EV product, 15 mL (10^9/mL) was delivered in a COVID ARDS clinical trial (Sengupta et al., 2020) with a dose similar to that used in our patient ([1.54–7.5] × 10^{12} total particles). Eventual dose optimization in the exosome field will benefit from more detailed response biomarker studies and reports of observed toxicity in the literature.

Clinical changes

Our patient started to receive SCEV late in the course of his ALS while continuing his previous regimen of two FDA-approved drugs, riluzole, 50 mg twice daily (Miller et al., 2003), and Relyvrio (a combination of sodium phenylbutyrate, 6 g/d, and tauroursodeoxycholic acid 1 g/d) (Paganoni et al., 2020). The reduction in ALSFRS-R during SCEV infusions compared to before infusions (0.49 points/month) is similar to the average benefit reported in the CENTAUR study of RelyvrioTM (0.42 points/month; Benatar and McDermott, 2020; Paganoni et al., 2020). It is also interesting to note the similarity between Figure 3 of ALSFRS "stabilization" and Figure 2A in Petrou et al. (2021), a study of repeated intrathecal MSC delivery in ALS patients. However, the patient's rapid decline during a pause in infusions suggests that a "reset" of his disease had not occurred.

Biomarkers

Systemic response biomarkers for SC exosomes have yet to be identified. We obtained serial standard laboratory NF-L assays that did not show clear changes during the study. To increase sensitivity, we also submitted two blood samples for an in-house multiplex Simoa assay, in which we also found substantial differences in the reported NF-L concentrations between the standard lab test reports and the in-house assays (higher NF-L). The in-house multiplex Simoa assay also included inflammasome activation markers. IL-18 was highly elevated, as was the apoptosis-associated speck-like protein speck protein (Hulse and Bhaskar, 2022). Since inflammasome markers are implicated in ALS (Italiani et al., 2014; Johann et al., 2015; Moreno-García et al., 2021), these should be assessed in follow-up studies. We also obtained a clinical cytokine panel before each infusion that included IL-2, IL-2R, IL-4, IL-5, IL-6, IL-1B, IL-10, IL-12, IL-13, IL-13, IL-17, interferon gamma, and tumor necrosis factor-α. These cytokines were below the limit of detection during the study except for IL-6 and IL-10, which showed varying slightly elevated levels across the time points without a clear trend. As with NF-L, more sensitive assays may be needed to capture changes in the cytokine markers. If effects are peripheral and at muscle and the NMJ, biomarkers other than NF-L might be more important, such as from muscle biopsies (Si et al., 2021) or neurophysiology (Benatar et al., 2016). We are conducting a detailed study of microRNA contents of SC exosomes that will be reported in another publication.

Mechanisms of action

The specific mechanism of action of SC exosomes, which contain numerous potentially bioactive molecules circulating systemically, with uptake to many organs, will be challenging to determine precisely, as several effects are possible. Like their cell of origin, SC exosomes have been shown to have neurotrophic, neuroprotective, and pro-regenerative effects (Ghosh and Pearse, 2023). We know they contain neurotrophic factors and microRNAs (unpublished data) and increase neuronal survival and neurite growth *in vitro*. The cell culture conditions of SCEV production influence their cargo (Jia et al., 2018), adding potential complexity regarding specific contents. In TBI models (data not yet published), IV SCEV delivery was associated with reduced microglial activation and CNS tissue sparing. The injury region tissues of IV-infused animals showed reduced inflammation, and there has been no evidence of infusion reactions or increased mortality in

controlled rodent studies. A logical next step to examine NMJ effects for SCEV in ALS would be in ALS neuron-muscle-coculture systems (Hörner et al., 2021).

Conclusion

This study reports the IV delivery of serial infusions of allogenic SCEVs in an advanced-stage ALS patient. The patient had transient clinical stabilization during treatment but deteriorated rapidly once the infusions were paused. Infusions were temporarily halted due to difficulties in consistently producing the required weekly quantities of SCEVs, as the SCEV isolation technique was still being optimized. Throughout the course of treatment, no adverse effects related to the IV SCEV infusions were noted, suggesting that allogeneic SCEVs were tolerable for IV use in this instance.

A notable finding, if reproducible with multiple ALS patients, is that the peripheral nerve SCs of the participant were poorly functional in culture, possibly contributing to the decline of MN function, but showed partial culture rescue with allogeneic non-ALS SCEVs. Our study provides a novel approach to address impaired SC and MN function in ALS using allogenic SCEVs, which appeared to be safe and could have contributed to a period of disease stabilization of the disease process. Other effects of the SCEVs, not explored here, may also influence disease progression. Further exploratory studies are warranted to examine the potential beneficial effects of SCEVs in ALS.

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Data availability statement: *All relevant data are within the paper and its Additional files.*

Additional files:

Additional Table 1: *The laboratory data of the patient.*

Additional Figure 1: *Change in serum creatinine level.*

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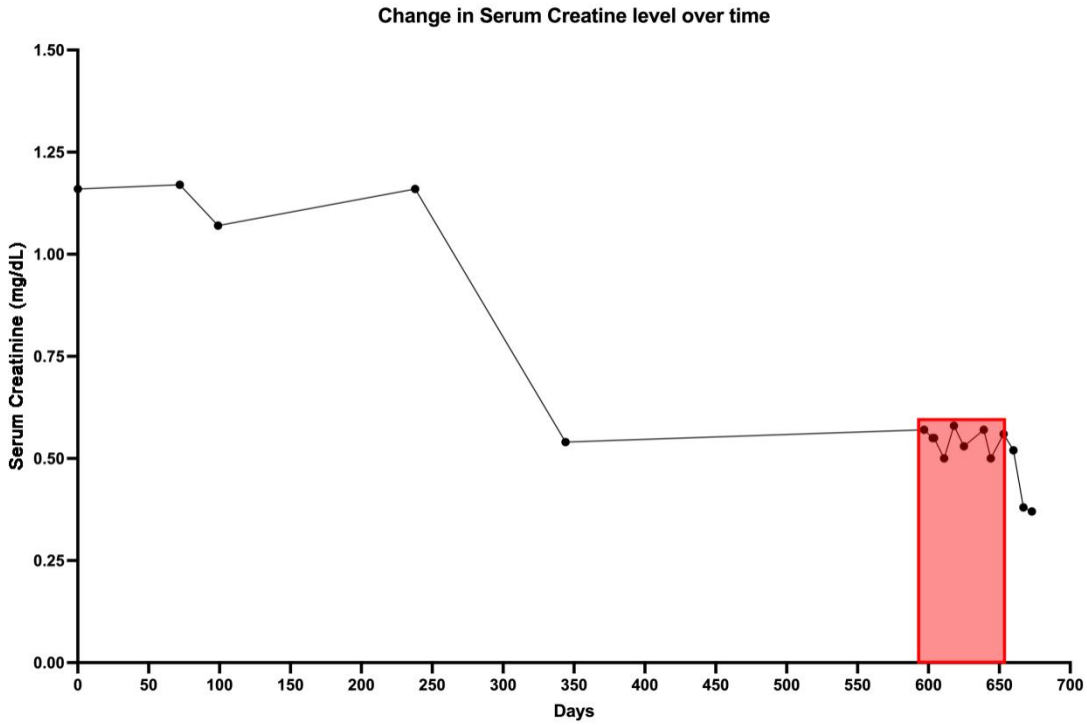

Additional Figure 1 Change in serum creatinine level.

Reported serum creatinine levels are shown before, during (red box), and after the Schwann cell extracellular vesicle infusions.

Additional Table 1 The laboratory data of the patient

	2021/2/18	2021/4/30	2021/5/27	2021/10/13	2022/1/27	2022/10/7	2022/10/13	2022/10/14	2022/10/21	2022/10/28	2022/11/4	2022/11/18	2022/11/23	2022/12/2	2022/12/9	2022/12/16	2022/12/22
Hematology blood																	
White blood cells (× 10^9/L)				3.4	4.6	4.2	4.2	4.6	3.4	4.2	4.9	4	3.9	3.6	4	4.1	4.3
red blood cells (× 1012/L)				2.65	3.49	2.74	2.58	2.49	2.78	2.73	2.72	2.62	2.8	2.72	2.7	2.64	2.36
Hemoglobin (g/dL)				9.5	12	9.7	9	8.9	10	9.8	9.6	9.3	9.6	9.6	9.7	9.1	8.1
Hematocrit (%)				30.3	37.2	29.6	28.3	27.5	29.4	29.3	29.2	27.7	31	29.5	29.2	29.8	25.7
Mean corpuscular volume (fL)				114.3	108.6	108	109.7	110.4	106	107	107	106	118	109	108	113	109
Mean corpuscular hemoglobin (pg)				35.8	34.4	35.4	34.9	35.7	36	35.9	35.3	35.5	36.6	35.3	35.9	34.5	24.3
Mean corpuscular hemoglobin concentration (g/dL)				31.4	32.3	32.8	31.8	32.4	34	33.4	32.9	33.6	31	32.5	33.2	30.5	31.5
Red cell distribution width (%)				14.4	13.7	12.5	14.7	14.6	12.6	12.7	12.9	12.2	13.1	12.9	12.3	12.7	12.8
Nucleated RBC (%)				0	0		0	0									
Platelet count (× 10^9/L)				114	124	126	106	109	106	129	140	122	129	128	120	145	150
Mean platelet volume (fL)				12.3	12.2		11.5	11.4									
Neutrophils (%)				74.8	73.8	73	85.9	74.8	69	76	74	75	73.5	75	77	75	77
Immature granulocytes (%)				0.6	0.7		0.5	0.7		0	0	1		0	1	1	1
Lymphocytes (%)				14.5	14.3	17	10.5	16.9	20	15	16	16	19.7	18	14	15	14
Lymphocytes, Absolute (× 10^9/L)										0.6	0.8	0.6	0.8	0.6	0.6		
Monocytes (%)				8.6	9.6	7	2.9	6.8	8	7	8	6	5.4	5	6	7	6
Eosinophils (%)				0.9	0.7	1	0	0.4	1	1	1	1	1.1	1	1	1	1
Basophils (%)				0.6	0.9	1	0.2	0.4	1	1	1	1	0.3	1	1	1	1
Neutrophils, Absolute (× 10^9/L)				2.54	3.37	3.1	3.62	3.4	2.4	3.2	3.6	3	2.9	2.7	3.1	3.1	3.3
Lymphocytes, Absolute (× 10^9/L)				0.02	0.03	0.7	0.44	0.77	0.7	0.6	0.8	0.6	0.8	0.6	0.6	0.6	0.6
Immature granulocytes, Absolute						1	0.02	0.03		0		0		0	0		
Monocytes, Absolute (× 10^9/L)						0.3			0.3	0.3	0.4	0.2	0.2	0.2	0.2	0.3	0.3
Eosinophils Absolute (× 10^9/L)						0			0	0	0	0	0	0	0	0	0
Basophils, Absolute (× 10^9/L)						0			0	0	0	0	0	0	0	0	0
Routine coagulation																	
Prothrombin time (s)					13.5												
Point-of-care international normalized ratio					1.1												
Activated partial thromboplastin time (s)					31.8												
Chemistry blood																	
Glucose (mg/dL)	90	110	112	109	94	101	129	160	86	94	97	105	100	116	116	83	69
Sodium (mM)	140	134	137	139	137	138	141	137	140	135	139	135	137	137	137	139	140
Potassium. Plasma (mM)	4.6	4.6	4.4	4.2	4.3	4	4.3	3.7	4.3	4.8	4.2	6.2	4.2	4.7	C	4.1	4
Chloride (mM)	105	101	102	107	102	103	106	104	105	101	103	99	106	101	101	103	105
CO2 (mM)	29	30	30	25	24	25	29	26	19	22	26	25	22	25	26	28	23
Anion gap (mM)	6	3	5	7	11		6	7	16	12							
Osmolality calculation	280	272	279	286	278		288	283									
Blood urea nitrogen (mg/dL)	17	21	26	36	25	32	26	28	20	22	23	22	22	14	14	11	12
Creatinine (mg/dL)	1.16	1.17	1.07	1.16	0.82	0.54	0.57	0.55	0.5	0.58	0.53	0.57	0.5	0.56	0.52	0.38	0.37
Blood urea nitrogen/creatinine ratio						59		8.2	40	38	42	39	44	25	27	29	
Calcium (mg/dL)	8.3	9.4	9.2	9	8.9	8.9	8.8	8.2	8.5	9.2	9.1	9	8.5	9	8.8	9	8.2
Magnesium (mM)	1.7			1.8			1.8										
Protein, total serum	7.7	8.6	8.9	8.4	8.9	8.4	7.6	7.5	8	8.1	8.1	8.1	7.3	7.9	8.2	8	6.7
Albumin (g/dL)	4.1	4.8	4.8	4.8	4.7	4.5	4.2	4.2	4.2	4.5	4.4	4.7	3.7	4.6	4.5	4.4	3.7
Globulin, Total (g/dL)						3.8			3.8	3.6							
A/G ratio						1.2			1.1	1.3	1.2	1.4	1.03	1.4	1.2	1.2	1.2
Bilirubin, total (mg/dL)	0.5	0.4	0.6	0.8	0.5	0.6	0.6	0.5	0.7	0.8	0.8	0.9	0.8	0.9	0.9	0.8	0.5
Aspartate aminotransferase (IU/dL)	25	23	25	19	16	48	10	12	22	28	11	61	16	24	40	9	10
Alanine aminotransferase (IU/dL)	11	16	21	12	9	16	7	7	9	7	7	11	6	7	7	5	4
Alkaline phosphatase (IU/dL)	50	76	67	51	45	9	45	47	44	44	43	37	30	36	39	42	31
C-reactive protein (mg/dL)			4.64			3	<0.1	<0.1	2	2	1	2	0	2	1	20	16
eGFR (mL/min/1.73)	60	59	65	59	84	100			102	98							
eGFR CKD-EPI																	
eGFR-EKFC																	
Hemoglobin A1c (%)								4.3									
Interleukin 2 (pg/mL)						<2.1		<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	
Interleukin 2 receptor (CD25), Soluble (pg/mL)						732.9		443.2	554.1	696.1.1	696.1.1	696.1.1	1190.2	730.7	732.2	659	
Interleukin 4 (pg/mL)						<2.2		<2.2	<2.2	<2.2	<2.2	<2.2	<2.2	<2.2	<2.2	<2.2	
Interleukin 5 (pg/mL)						<2.1		<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	<2.1	
Interleukin 6 (pg/mL)						6.1		9.4	5.8	7.1	7.1	7.3	11.5	8.4	9.3	11.2	
Interleukin 8 (pg/mL)						<3.0		<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	<3.0	
Interleukin 1 beta (pg/mL)						<6.5		<6.5	<6.5	<6.5	<6.5	<6.5	<6.5	<6.5	<6.5	<6.5	
Interleukin 10 (pg/mL)						5.3		<2.8	3.8	5.3	5.3	3.5	9.3	4.3	4.7	4.4	
Interleukin 12 (pg/mL)						<1.9		<1.9	<1.9	<1.9	<1.9	<1.9	<1.9	<1.9	<1.9	<1.9	
Interleukin 13 (pg/mL)						<1.7		<1.7	<1.7	<1.7	<1.7	<1.7	<1.7	<1.7	<1.7	<1.7	
Interleukin 17 (pg/mL)						<1.4		<1.4	<1.4	<1.4	<1.4	<1.4	<1.4	<1.4	<1.4	<1.4	
Interferon gamma (pg/mL)						<4.2		<4.2	<4.2	<4.2	<4.2	<4.2	<4.2	<4.2	<4.2	<4.2	
Tumor necrosis factor alpha (pg/mL)						< 1.7		<1.7	<1.7	<1.7	<1.7	<1.7	<1.7	<1.7	<1.7	<1.7	

Yellow highlight- Pre-infusion; Green highlight- infusion period; Orange highlight- Post-infusion period. eGFR: Estimated glomerular filtration rate

ALS 1	IFN-γ	IL-10	IL-12p70	IL-13	IL-2	IL-6	IL-8	TNF-α	NfL	ASC	Caspase-1 2nd gen	IL-18
2023/10/21	0.050774498	0.608972898	0.072794876	0.401676763	0.40508969	0.2654268	2.74558234	1.78338178	100.747556	357	2.28	388
2023/10/28	0.426300539	0.289409105	0.080677053	0.658807227	0.088193344	0.21050619	4.75491628	1.3882768	106.78945	397	2.54	333
Percent change	739.5957659	-52.4758646	10.82792841	39.02969684	-359.320025	-26.089787	73.1842536	-22.154817	5.99706303	11.2044818	11.40350877	-14.175258

Unit = pg/mL. IFN-γ: Interferon gamma; IL: interleukin; TNF-α: tumor necrosis factor alpha.