Original Article

Preconditioning with interleukin-1 alpha is required for the neuroprotective properties of mesenchymal stem cells after ischemic stroke in mice

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Abstract

Mesenchymal stem cell (MSC) pre-conditioning with interleukin-1 alpha (IL-1α) drives MSCs toward a potent anti-inflammatory phenotype. The aim of this study was to assess the therapeutic potential of intra-arterially administered IL-1α preconditioned MSCs, after experimental cerebral ischaemia in mice. After 3 h from the start of middle cerebral artery occlusion, animals were treated with vehicle, 9.1×10^4 non-conditioned or IL-1α preconditioned MSCs by intra-arterial administration. Animals were allowed to recover for 1.5 h after treatment to measure cerebral blood flow (CBF), and 3 days or 14 days post-stroke to evaluate lesion volume and functional outcomes. At 3-days post-stroke preconditioned MSCs reduced (by 67%) lesion volume and increased CBF (by 32%) compared to vehicle, while non-conditioned MSCs had no effect. A separate cohort of animals recovered to 14 days post-stroke also showed reduced infarct volume (by 51%) at 48 h (assessed by MRI) and better functional recovery at 14 days when treated with preconditioned MSCs when compared to vehicle. Preconditioning MSCs with IL-1α increases their neuroprotective capability and improves functional recovery after delayed intra-arterial administration. With increasing use of thrombectomy, the adjunct use of preconditioned MSCs therefore represents a highly relevant therapy to improve outcomes in ischemic stroke.

Keywords

Stroke, mesenchymal, therapy, translation, pre-clinical

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Introduction

With increasing use of effective intra-arterial thrombectomy treatment, both the Stroke Treatment Academic Round Industry Roundtable (STAIR) X consortium[4] and the 4th Stem Cell Therapies as an Emerging Paradigm in Stroke (STEPS)[1] meetings recommend greater investigation into the integration of endovascular thrombectomy with other therapeutics, such as cell therapy. Meta-analysis shows mesenchymal stem cells (MSCs) to be beneficial in terms of reducing infarct damage and improving functional outcomes in experimental stroke models,[2] and there are several ongoing or completed early phase feasibility, dose ranging or phase 2 clinical trials in stroke.[3] However, a recent study in comorbid animals showed that non-conditioned MSCs failed to exert neuroprotective actions after experimental stroke.[5] Our previous work has shown MSCs

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preconditioned with interleukin-1 alpha (IL-1α) adopt a potent anti-inflammatory and pro-trophic phenotype, which may translate into a more effective therapy for stroke.[6] Here we show for the first time that IL-1α preconditioning is required to reveal neuroprotective properties of MSCs when administered intra-arterially. Therefore, administration of IL-1α preconditioned MSCs alongside thrombectomy could potentially be a highly effective therapeutic approach for ischemic stroke.

Material and methods

Experimental design

Animal procedures were carried out in accordance with the Animal Scientific Procedures Act (1986) and the European Council Directive 2010/63/EU and were approved by the Animal Welfare and Ethical Review Body of the University of Manchester, UK. Experiments followed ARRIVE[7] and IMPROVE guidelines.[8] See methods section in supplementary information for details of blinding, randomisation and exclusions and Figure 1 for experimental timeline.

Blinding and randomisation

An experimenter independent of the main study assigned, coded and matched each individual animal and treatment doses, using a spreadsheet randomiser. The experimenter was only aware of the assigned codes to animals/treatment doses, but not to treatment group and so was blinded to treatment during all surgical procedures, functional tests and analyses. Functional tests were undertaken by a single trained scorer and blinded to treatment allocation.

Exclusions

Animals were excluded from cerebral blood flow (CBF) analysis for unsuccessful CBF capture (2 animals treated with vehicle, 1 animal in the unconditioned MSC group). There were exclusions in the 14 day experimental cohort. One IL-1α conditioned MSCs-treated animal was excluded from all analyses (weight, neurological deficit, cylinder, nest and burrowing) in the preconditioned MSC group, due to inadequate occlusion and a failure to achieve a 50% drop in cerebral blood flow from baseline (inclusion criteria was pre-set at least 70% drop from baseline) and showed no lesion on MRI scan. Additional animals were excluded from cylinder test (3 animals treated with vehicle, 2 animals in the conditioned MSC group) and burrowing (3 animals treated with vehicle) due to exclusion based on the criteria described below in the respective tests. There was an attrition of animals in the 14 day non-conditioned experimental group after MCAo, with an animal culled on days 1, 2 and 3 relating to declining post-stroke health issues and an animal per day on days 4, 6 and 7 relating to severe weight loss.

Animals

Mice were male C57BL/6 (Charles River, UK), aged 12 to 14 weeks at the time of surgery. A total of 36 animals (12 mice per experimental group including vehicle-treated, IL-1α conditioned MSCs-treated and non-conditioned MSCs-treated groups) underwent surgery in the short term experiment (3 day end-point) and 30 animals (10 mice per group; vehicle-treated, non-conditioned MSC-treated and IL-1α conditioned MSCs-treated) for the longer term (14 day) experiment. In a biodistribution and CBF experiment, 33 animals (11 mice per experimental group including vehicle-treated, IL-1α preconditioned MSCs-treated and non-conditioned MSCs-treated groups) were allocated to receive labelled MSCs for histological biodistribution analysis and underwent laser speckle imaging at 1.5 h after treatment, prior to culling 30 mins later to retrieve brains for MSC biodistribution analysis (2 h after treatment).

Mesenchymal stem cell culture

Human bone marrow derived MSCs (fetal source 20–22 weeks old) were purchased from 3H Biomedical (Sweden) and used at culture passages 4–6. MSCs were cultured in tissue culture flasks (Corning, UK) as a monolayer in MesenPro RS medium (Invitrogen, UK) supplemented with 2 mM glutamine and 1% penicillin/streptomycin. The cell growth medium was changed every 4–5 days until 70–80% confluent. MSCs were preconditioned with either recombinant human IL-1α (10 ng/ml, R&D Systems, UK) in serum free MesenPro RS medium or MesenPro alone for non-conditioned MSCs for 5 min as previously described.[6] The preconditioning medium was then removed and MSCs were washed twice with PBS before being dissociated with 0,5% trypsin-EDTA (Sigma-Aldrich, UK) and counted. MSCs designated for biodistribution experiments had an additional stage of labelling with CellTracker™ Deep Red dye. After preconditioning with IL-1α or vehicle (serum-free MesenPro) and detached by trypsinisation, MSCs were labelled by incubation with CellTracker™ Deep Red dye for 15 mins in suspension (5 μM, serum-free MesenPro) before removing excess dye and washing. IL-1α conditioned MSCs (as well as non-conditioned MSCs) for administration in mice were prepared on the day of surgery, kept on ice for up to 3 h and then warmed to room temperature before administration.

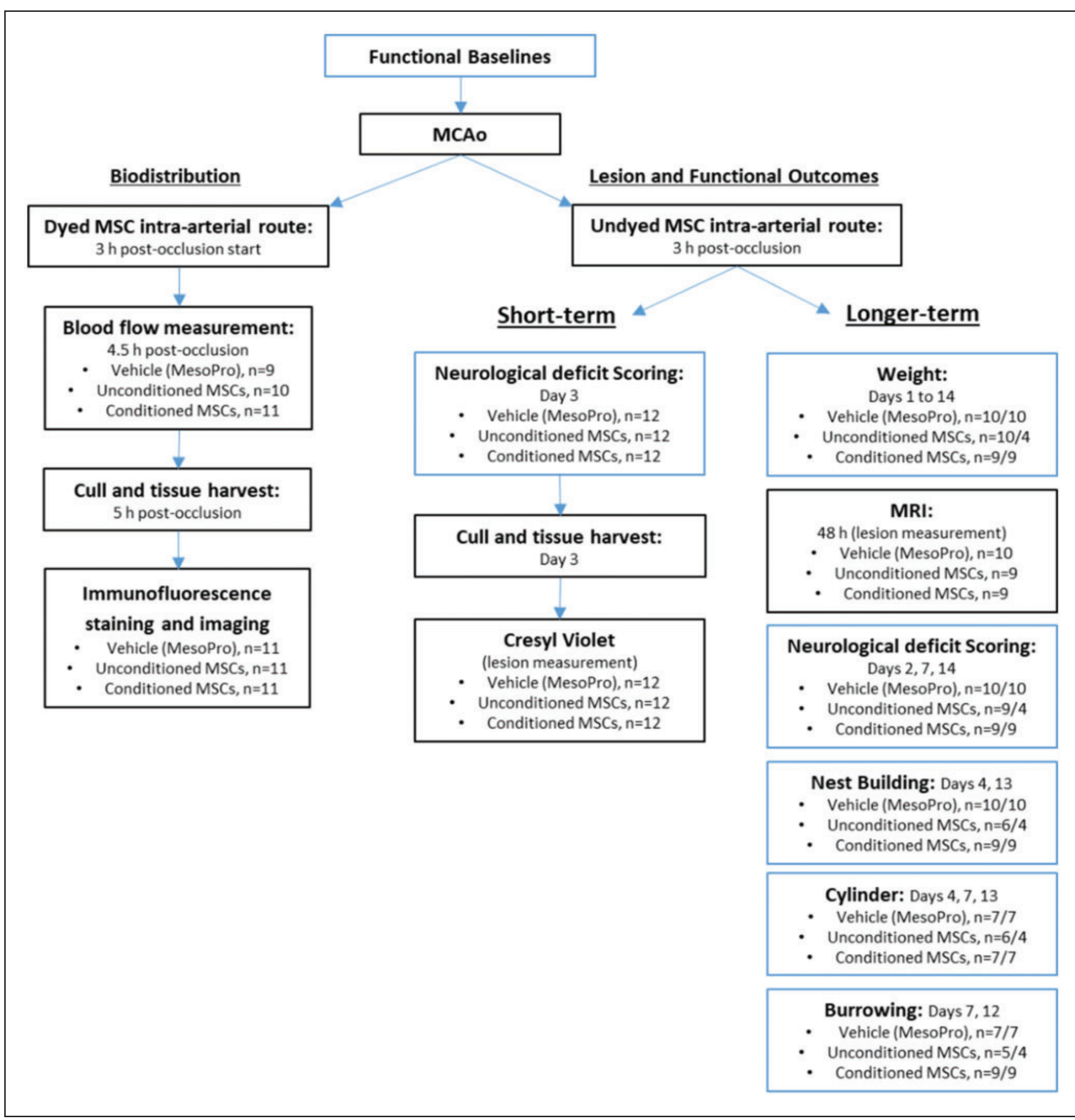

Figure 1. Experimental timeline of interventions and experimental groups. Baselines for functional outcomes weight, nest building and burrowing was conducted before MCAo surgery. After experimental stroke, mice were allocated to the dyed MSC experimental group to measure blood flow and biodistribution, or undyed cells for lesion volume and functional outcome experiments (mice were vehicle, non-conditioned or conditioned MSC treated). Animals in the undyed MSC experimental group were either allocated to short-term or longer-term experiments for lesion and functional outcomes. The short-term group was scored for neurological deficits at 3 days post-stroke before culling and lesion volume measurement, while the long-term group was assessed for lesion volume at 2 days post-stroke and functional outcomes up to 14 days.

Focal cerebral ischaemia and MSC treatment

Transient focal cerebral ischaemia was induced by middle cerebral artery occlusion (MCAo) for 30 mins, based on our previously published protocol using the intraluminal filament model.[9] Briefly, a midline incision was made on the ventral surface of the neck and the left common carotid artery isolated and ligated. Topical anaesthetic (EMLA, 5% prilocaine and lidocaine, AstraZeneca, UK) was applied to skin incision sites prior to incision. The internal carotid artery and the pterygopalatine artery were temporarily ligated. A 6-0 monofilament (Doccol, Sharon, MA, USA) was introduced into the internal carotid artery via an incision in the common carotid artery. The filament was advanced approximately 10 mm into the common carotid with the filament making its way distal to the carotid bifurcation, beyond the origin of the middle cerebral artery. A 10 mm mark was made on the filament to visualise the required length to be inserted

beyond the carotid bifurcation. At 3 h from start of occlusion, animals were treated under isoflurane anaesthesia with vehicle (serum-free Mesenpro media), 9.1×10^4 IL-1α conditioned MSCs or 9.1×10^4 non-conditioned MSCs by intra-arterial infusion with a syringe driver (20 µl cell suspension, 0.5 µl/sec), via the filament incision site. Animals were recovered and returned to normal housing prior to behavioural testing.

Laser speckle imaging

Animals receiving labelled MSCs underwent laser speckle imaging at 1.5 h post-treatment (4.5 h post-stroke) to measure CBF. Mice were anaesthetised with isoflurane (4% in 30% O^2 and 70% N_2O) and fixed in a stereotactic frame with ear bars and mouth bar to prevent movement. The scalp was exposed by a mid-line skin cut and the blood flow recorded for 5 mins.

Magnetic resonance imaging

In the longer-term experiment (14 days post-stroke), mice underwent magnetic resonance imaging (MRI) scans at 48 h post-stroke. Animals were anaesthetised with 4% isoflurane and T2-weighted scans were conducted on Bruker Advance III console (Bruker Biospin Ltd, UK) using a 7 Tesla magnet. A total of 14 serial slices with a thickness of 1 mm were acquired. Lesion volumes were measured using ImageJ and corrected for oedema.

Tissue processing and histology

Mice were either perfused transcardially with 0.9% saline followed by paraformaldehyde (PFA) under terminal 4% isoflurane (4% in 30% O^2 and 70% N_2O) for the short-term (3 days post-stroke) and longer-term experiment (14 days post-stroke) or by terminal rising CO_2 concentrations for animals allocated for blood flow and biodistribution (5.5 h post-stroke) studies. Tissue processing and cresyl violet staining was performed to measure lesion volume, as previously described.[5] For each brain, infarct volumes were measured on defined coronal sections (using image J), spaced at approximately 360 µm apart. Each defined coronal section, with its brain co-ordinates and lesion was integrated to estimate total lesion volume for each brain and corrected for oedema. MSCs were identified and counted by colocalising DAPI and CellTracker™ Deep Red positively stained cells across sections by QuPath 0.3.2.

Immunofluorescence

Immunofluorescence staining was performed by incubating brain sections with blocking buffer consisting of 5% normal donkey serum (Jackson laboratories, Bar Harbor, ME, USA), 1% bovine serum albumin (BSA), 0.1% Triton X-100, 0.05% Tween 20 (Sigma-Aldrich), 0.2 M Glycine (Fisher Scientific) in PBS for 1 h. Blocking buffer was then removed and brain sections were incubated with goat anti-CD31 (1:200, R&D Systems, #AF3628) primary antibody, diluted in primary antibody buffer (1% BSA, 0.3% Triton X-100 in PBS) at 4 °C overnight. . Sections were washed in 0.1% Tween in PBS, and incubated with goat derived Alexa-Fluor 594 (1:500, ThermoFisher, UK) secondary antibody diluted in 0.05% tween in PBS. After 2 h incubation, the secondary antibody was removed, and sections were then incubated with DAPI solution for 5 minutes prior to washing with 0.1% Tween in PBS, mounted on glass slides and left to dry in the dark, before being cover slipped with mounting medium (ProLong Gold without DAPI, ThermoFisher, UK).

Weight recovery

Animals were weighed every day, for 7 days, and at day 14 post-stroke, as an indication of their general well-being. Body weight is presented as a percentage change compared to initial body weight on the day of surgery.

Neurological deficit scoring

Mice were neurologically scored for focal deficits with the use of a 28-point neurological scoring system based from Clark et al.1998.[10] This is a cumulative score of the seven subcategories; body symmetry, gait, climbing, circling, front limb symmetry, compulsory circling and whisker response, with animals ranked from 0 (normal) to 4 (extreme deficit) for each subcategory.

Cylinder test

The cylinder test was used to assess forelimb and rotational asymmetry and set up as described in Roome et al. 2015.[11] Video editing software was used to accurately replay forelimb touches frame by frame for analysis (Adobe Premier Pro 13.1, Adobe Systems). The first left, right or both forelimb cylinder touches from a total of 20 independent rears were recorded per session for each animal and used to calculate an asymmetry score used in Li et al. 2003.[12] The greater the score, the greater the non-impaired forelimb preference. Animals with 70% or greater non-impaired forelimb touches at baseline were excluded from analysis (2 animals treated with vehicle).

Nest building

Nest building is a spontaneous home-cage behaviour and was used as an assessment of wellbeing.

Animals were placed in individual cages containing 20 g of Sizzle-Nest nesting material (Datesand Ltd, UK) at least 1 h before the onset of the dark cycle. Mice were left over night and nests were scored in the morning after onset of the light cycle. Nests were scored using the following, 1 = no manipulation of nesting material, 2 = no obvious nest site present (majority of nesting material not contained to one quadrant of cage), 3 = nest present but flat, 4 = nest has raised walls ≤30 mm in height, 4 = nest walls 31–49 mm in height, 5 = nest walls ≥50 mm in height.

Burrowing

Sickness behaviour was assessed with the burrowing task post-stroke. Burrowing tubes were made from 200 mm lengths of 68 mm diameter PVC downpipe as described previously by Deacon et al. 2012.[13] Animals were placed in individual cages with a burrowing tube containing 150 g standard rodent diet food pellets (SDS, UK). After 2 h, the food remaining in the tubes was weighed and subtracted from the initial weight (150 g) to calculate the mass burrowed. Two baseline sessions were conducted at least 48 h apart prior to MCAO. Mice which burrowed <30 g at baseline, were excluded from analysis (3 animals treated with vehicle).

Statistical analysis

Sample size for experiments was determined by a priori power calculation using G*Power 3.1.9.4 with mean differences and standard deviations based on pilot studies and previous experiments on lesion volume as the primary outcome (power 80%, α 0.05).[9,14] A sample size of n = 12 per group was determined for the short-term experiment and the subsequent lesion result was used to calculate the sample size for the long-term experiment as n = 10 per group (power 80%, α 0.05). However, due to limited MRI availability, animals excluded or lost from attrition could not be replaced to reach the pre-determined power calculated sample size in the longer-term experimental group (see exclusions). Data were assessed for normal distribution using the Shapiro-Wilk test to determine parametric or non-parametric analysis. Analysis of non-repeated data was by one-way ANOVA followed by post-hoc Tukey multiple comparisons for parametric (cerebral blood flow) and Kruskal-Wallis test combined with Dunn's multiple comparisons for non-parametric data (lesion volumes, MSC counts and neurological score at day 3 end point). Mixed-effects analysis was used for repeated parametric/non-parametric data with missing values followed with Tukey's multiple comparison for neurological scores, burrowing, nest building and cylinder test (GraphPad Prism version 9.5.1 for windows, GraphPad Software). Weights was analysed by linear mixed model (SPSS version 25.0, IBM Corp).

Results

All animals survived to the end time point of 3 days post-stroke in the short-term experiment and were included in analysis. After delayed IA infusion, preconditioned MSCs reduced lesion volume (67%, 95% CI 4.29 to 19.93, $p = 0.002$, Figure 2(a)) and neurological deficits (52%, 95% CI 5.32 to 10.02, $p = 0.002$, Figure 2(b)) compared to vehicle at 3 days post-stroke. In the biodistribution and CBF experiment, preconditioned MSCs increased CBF in the ipsilateral hemisphere when compared to vehicle (32%, 95% CI −31.75 to −20.52, $p = 0.0344$), Figure 2(c)) but not unconditioned MSCs. There was no difference in the number of MSCs found in the ipsilateral side of the brain between unconditioned and preconditioned MSC treatment (Figure 2(f)).

To investigate longer-term effects of IL-1α preconditioning on MSCs, animals were recovered to 14 days post-stroke. MRI at 48 h confirmed our finding from the short-term experiment that preconditioned MSCs were neuroprotective, reducing lesion volume by 51% compared to vehicle (95% CI 6.71 to 24.37, $p = 0.042$) and 61% compared to unconditioned MSCs (95% CI 28.41 to 50.61, $p = 0.003$) at 48 post-stroke (Figure 3(a)). In addition, animals treated with preconditioned MSCs showed improved neurological score (Figure 3(b)) at 7 days (41%, 95% CI 1.36 to 7.32, $p = 0.007$) and 14 days (41%, 95% CI 0.40 to 7.80, $p = 0.029$) and more rapid recovery of body weight (95% CI 87.81 to 89.50, $p = 0.001$) compared to vehicle treated mice. Secondary outcomes of general wellbeing (burrowing, nest building, Figure 3(c) and (d)) and the cylinder test showed no differences between treatment groups (Figure 3(e)).

Discussion

Thrombectomy has provided a considerable opportunity for adjunct treatments, for which cell therapy is uniquely suited to, by allowing a more precise route to the area of infarct with a more optimal dose. IA administration allows a greater number of cells to reach the ischemic penumbra, bypassing filtering organs, such as the lung, liver and spleen, allowing cells to traverse into the intraparenchymal space from the intravasculature.[15] Numerous studies have utilised this route of administration and have found it to be beneficial in delivering cell-based therapies in many species, ranging from rodent models to human.[15–17]

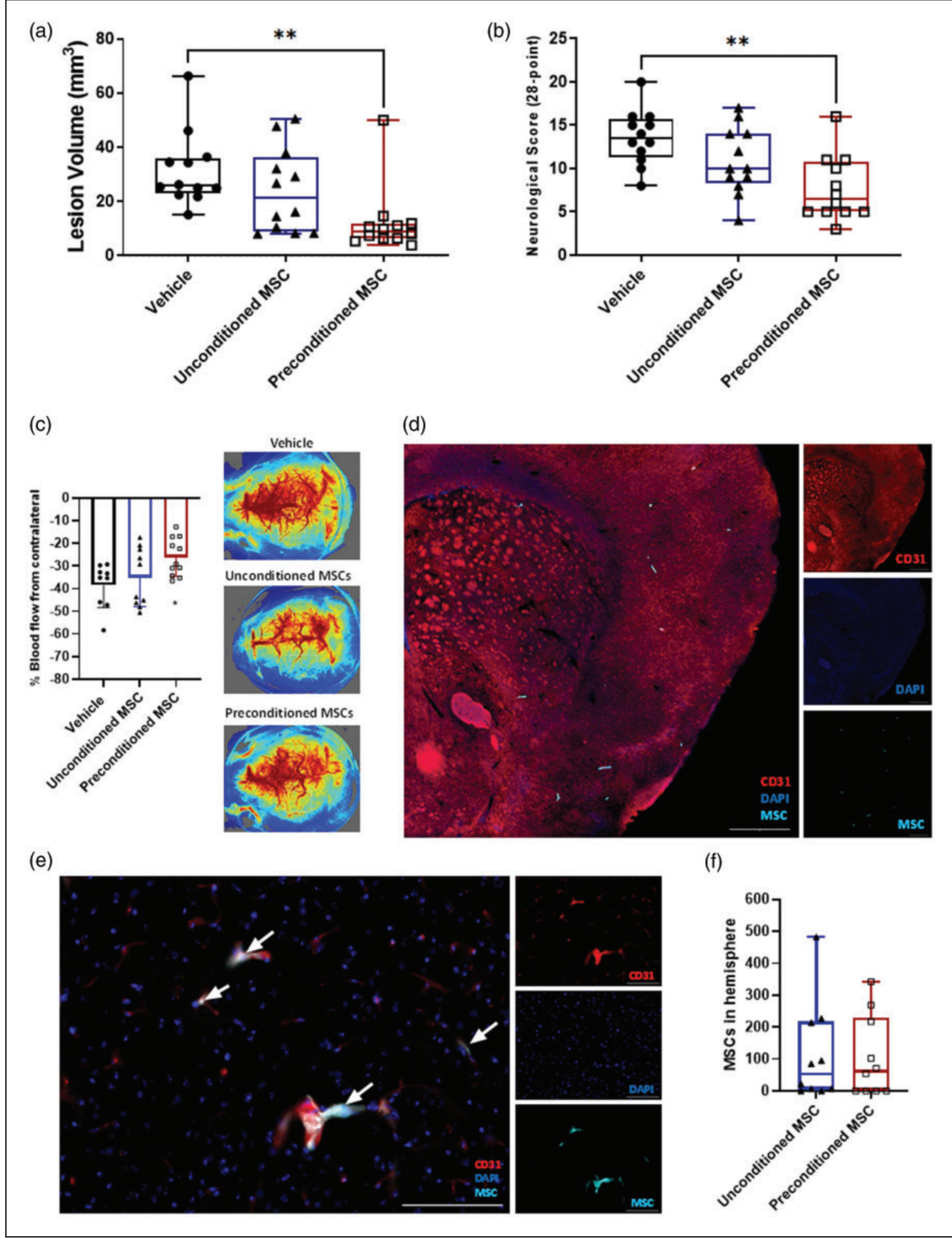

Figure 2. Effect of IL-1α preconditioned and non-preconditioned MSCs on short-term outcomes. A short-term experiment with an end point of 3 days post-stroke showed a reduction in (a) Lesion volume, $^{**}p = 0.002$ (Cresyl Violet staining) and (b) Neurological deficits (neurological deficit scoring), $^{**}p = 0.002$ when compared to vehicle. In a separate experiment, MSCs were labelled with CellTracker™ Deep Red dye for biodistribution analysis and laser speckle imaging was used to measure the effects of MSC preconditioning on cerebral blood flow (CBF). (c) Preconditioned MSCs increased CBF in the infarct region (represented as % blood flow to the contralateral region, measured at 1.5 h after dosing) when compared to vehicle $^{*}p = 0.0344$, while non-preconditioned MSCs did not. At 30 mins after laser speckle imaging (2 h after dosing), brains were harvested and labelled MSCs were counted after immunofluorescence imaging. MSCs were found in the area of the lesion (d) and cerebral vasculature of the ipsilateral side of the brain (e) (arrows indicate MSCs in vessels, scale bar is 500 μm [d] and 100 μm [e]), but IL-1α preconditioning did not alter biodistribution. Anti-CD31 and secondary Alexa-Flour 594 antibody (excitation and emission spectra 590/618 nm respectively) was used to visualise vessels (labelled in red), while MSCs stained with CellTracker™ Deep Red dye (excitation and emission spectra 630/650 nm respectively) are visualised in cyan. DAPI was used to stain cell nuclei and labelled as blue. Vehicle was MesenPro media, MSCs were injected intra-arterially at 9.1×10^4 3 h after the start or occlusion. Data expressed as median ± IQR (a, b and f) or mean ± SD (c).

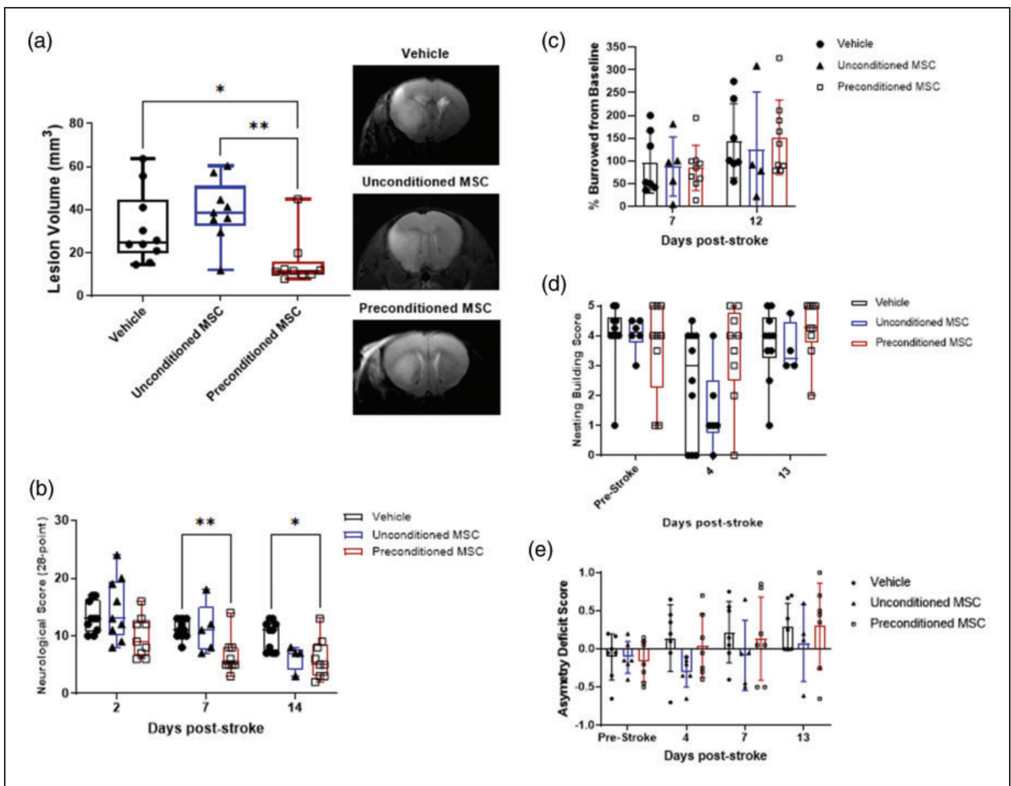

Figure 3. Effect of IL-1α preconditioned and non-conditioned MSCs up to 14 days post-stroke. IL-1α preconditioning on MSCs reduced (a) Lesion volume (at 48 h using MRI imaging), $*p = 0.042$ compared to vehicle and non-conditioned MSCs $**p = 0.003$. Also, IL-1α preconditioning on MSCs reduced (b) Neurological deficits (neurological deficit scoring), $**p = 0.007$ (Day 7), $*p = 0.029$ (Day 14) (c) Body weight loss, $***p = 0.004$, while (d) Burrowing (e) Nesting (f) Cylinder test remained neutral between treatment groups. Vehicle was MesenPro media, MSCs were injected intra-arterially at 9.1×10^4 3 h after the start of occlusion. Data expressed as median ± IQR (a, b + d) or mean ± SD (c + e).

Previous studies have explored *in vitro* approaches to enhance the therapeutic potential of MSCs, such as pharmacological preconditioning, molecular priming, hypoxic preconditioning, tissue engineering and growth medium.[16,18] The present study demonstrates for the first time that preconditioning of MSCs with IL-1α is required to induce their neuroprotective properties in a MCAO model of stroke. Importantly, to confirm clinical translatability in the context of intra-arterial thrombectomy, the preconditioned MSCs were administered 3 h from start of occlusion and given intra-arterially, which is more efficient in targeting delivery of cells to the site of injury compared to intravenous (IV) administration and requires lower doses.[17,19] Stem cell delivery by IA administration is already well characterised clinically in terms of safety and efficacy,[18,20] with several ongoing trials.[3] Although most published studies show unconditioned MSCs to be beneficial in preclinical ischemic stroke, not all favour MSC treatment and a meta-analysis shows publication bias, indicating the possibility of more neutral studies.[17] MSCs are heterogeneous in their function which can be attributed to being sourced from different donors and *in vitro* conditions,[21] which may in part explain why in our hands non-conditioned MSCs do not exert a strong therapeutic effect. Preconditioning methods, such as using IL-1α in this study, can promote MSC therapeutic functions to help to push them toward a more beneficial therapeutic phenotype and reduce MSC functional heterogeneity.[22,23]

In this study we found that MSCs preconditioning with IL-1α does not influence their ability to enter the cerebrovasculature in comparison to non-conditioned cells when examining brain tissues from mice culled at 2 h after MSC administration (5 h post start of occlusion, see Figure 1). Although MSCs were visualised in some animals, they appear not to be present in other animals (Figure 2(f)), despite exerting an effect on CBF (Figure 2(c)). IA transplanted MSCs are known to temporarily attach to the walls of cerebral vessels before either migrating through the blood brain barrier or return to the blood stream whilst still being able to exert their therapeutic effects through a paracrine mechanism,[24] which could be the case here.

The therapeutic effects of MSCs have been mainly attributed to paracrine actions of their secretome;

consisting of multiple cytokines, morphogens, small molecules and exosomes.[16] We have previously shown that the MSC secretome can be polarised toward a more anti-inflammatory and pro-trophic phenotype by IL-1α[6] and have shown IL-1α-primed MSC-derived conditioned medium treatment after ischemic stroke resulted in improved outcomes.[14] MSCs stimulated by IL-1α secrete G-CSF, involved in the inhibition of apoptosis and the immune regulatory cytokine IL-10 are just some examples of trophic and anti-inflammatory factors,[6,25] which could be beneficial after stroke, but their contribution will need to be further investigated *in vivo*.

This study shows that early neuroprotection conveyed by preconditioned MSCs could be in part attributed to increasing blood flow to the infarct area. Furthermore, the long-term study indicates that beneficial actions of preconditioned MSCs occur primarily in the acute phase post stroke, which is subsequently maintained longer term to 14 days post-stroke in terms of reduced neurological deficits. MSC treatments did not appear to affect outcomes of well-being, such as nest building, burrowing and fore-limb asymmetry, indicating they are independent from MSC therapeutic effects in our hands or are not sufficiently powered. A possible mechanism of MSCs altering CBF may be due to their ability to secrete vascular endothelial growth factor (VEGF),[19,26] which is a potent vasodilator.[20,27] Beneficial effects from MSCs could be from other mechanisms, such as mitochondria cell-to-cell transfer and exosomes. MSCs can transfer mitochondria through cell-to-cell contact leading to increased survival and energy metabolism to damaged cells of the neurovascular unit.[28,29] Exosomes derived from MSCs can comprise cytokines, growth factors, signalling lipids, mRNAs, and regulatory miRNAs, all capable of modulating stroke through various mechanisms.[30] MSC derived exosomes that contain selective non-coding MSC-RNAs, such as MiR-17-92, can enhance neurofunctional recovery by axon-myelin remodelling.[31] This study demonstrates however that the neuroprotective mechanisms triggered by IL-1α preconditioning of MSCs are likely to be multi-factorial.

This study was limited to experiments on young male mice and future studies must consider the use of female animals and co-morbidities. Sex dimorphism of cerebral vessels and CBF have been observed in both stroke patients[32–34] and in rodent stroke models.[35–37] Stroke is also an age-related disease and strongly affects patients with co-morbidities.[38] Therefore, it will be beneficial to further investigate the effects of IL-1α MSC preconditioning in aged and/or relevant co-morbid models.

In summary, our results demonstrate preconditioning MSCs with IL-1α increases their therapeutic neuroprotective properties after delayed IA administration in transient focal cerebral ischaemia and could be an effective adjunctive strategy alongside intra-arterial thrombectomy.

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Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Authors' contributions

RW designed and performed experiments, data analysis and drafted the manuscript. CS, SA and EP contributed to the conception and revising the manuscript.

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