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Original article

Exercised blood plasma promotes hippocampal neurogenesis in the Alzheimer's disease rat brain

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Abstract

Background: Exercise training promotes brain plasticity and is associated with protection against cognitive impairment and Alzheimer's disease (AD). These beneficial effects may be partly mediated by blood-borne factors. Here we used an *in vitro* model of AD to investigate effects of blood plasma from exercise-trained donors on neuronal viability, and an *in vivo* rat model of AD to test whether such plasma impacts cognitive function, amyloid pathology, and neurogenesis.

Methods: Mouse hippocampal neuronal cells were exposed to AD-like stress using amyloid-β and treated with plasma collected from human male donors 3 h after a single bout of high-intensity exercise. For *in vivo* studies, blood was collected from exercise-trained young male Wistar rats (high-intensity intervals 5 days/week for 6 weeks). Transgenic AD rats (McGill-R-Thy1-APP) were injected 5 times/fortnight for 6 weeks at 2 months or 5 months of age with either (a) plasma from the exercise-trained rats, (b) plasma from sedentary rats, or (c) saline. Cognitive function, amyloid plaque pathology, and neurogenesis were assessed. The plasma used for the treatment was analyzed for 23 cytokines.

Results: Plasma from exercised donors enhanced cell viability by 44.1% ($p = 0.032$) and reduced atrophy by 50.0% ($p < 0.001$) in amyloid-β-treated cells. *In vivo* exercised plasma treatment did not alter cognitive function or amyloid plaque pathology but did increase hippocampal neurogenesis by ~3-fold, regardless of pathological stage, when compared to saline-treated rats. Concentrations of 7 cytokines were significantly reduced in exercised plasma compared to sedentary plasma.

Conclusion: Our proof-of-concept study demonstrates that plasma from exercise-trained donors can protect neuronal cells in culture and promote adult hippocampal neurogenesis in the AD rat brain. This effect may be partly due to reduced pro-inflammatory signaling molecules in exercised plasma.

Keywords: Cytokines; High-intensity interval training; Inflammation; Neurons; Plasma transfusion

1. Introduction

Development of Alzheimer's disease (AD), the most common cause of dementia, is a long process where neuropathological changes may occur up to 20 or more years prior to clinical manifestation.[1] Thus, the typical research approach

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of implementing treatments following clinical manifestation might be ineffective for AD as, by this stage, too much neural damage may have ensued. If correct, this scenario helps explain why AD drug candidates have one of the highest failure rates of any disease area.[1] This has led some researchers to shift their focus towards prevention by modifiable risk factors. A leading hypothesis is that the incidence of AD and dementia may be reduced substantially (30%−50%) through modifiable cardiovascular risk factors, such as by increased physical activity and increased age-specific cardiorespiratory fitness.[2−4]

Human and rodent studies have shown that exercise enhances blood delivery to the brain and induces molecular changes in blood which seem to mediate some of the neurological beneficial effects of exercise,[5−7] as reviewed.[4,8] Studies in rodents have shown exercise to decrease inflammation in the brain and increase hippocampal neurogenesis,[9−12] partly reversing the age-dependent reduction in cognition.[10,11,13] Neural stem cells that give rise to new neurons in the hippocampal dentate gyrus are located around blood vessels[14] and proliferate in response to vascular growth factors,[14,15] which indicates that exercise-induced increases in cerebral blood flow may contribute to improving the communication between the systemic environment and the neurogenic niche.[16] In line with this, studies in mice show that cognitive impairment and age-related decline in neurogenesis can partially be attributed to changes in blood-borne factors.[16,17] Notably, adult hippocampal neurogenesis also drops sharply in human subjects with AD compared to healthy controls,[18] which suggests that hippocampal neurogenesis is a potential therapeutic target.

Systemic administration of blood from young to old mice has been shown to have beneficial effects as far as counteracting age-related degeneration in the brains of old mice, while blood from old mice administered into young mice conversely has detrimental effects.[17,19] The beneficial effect of young blood administration into old animals appears to have a broad impact, increasing hippocampal spine density, synaptic plasticity, blood flow, and neurogenesis, which results in improved hippocampal-dependent learning and memory.[17,19−21] The first clinical trial involving transfusion of plasma from young donors to subjects with mild cognitive impairment or early AD found it to be safe and possibly beneficial as exploratory endpoint analysis indicated improvements in functional abilities.[22] Taken together, the abovementioned studies indicate that systemic manipulation may have wide-ranging rejuvenating and therapeutic effects, and they suggest that targeting the systemic environment may potentially be an effective strategy for preventing neurodegeneration and maintaining neurogenesis in the AD brain. Systemically delivering the exercise effects to individuals with limited ability to perform high-intensity exercise may thus present a novel approach for AD therapy.

A few studies have reported finding a causal link between exercise-induced circulating factors and exercise-like adaptations in organs not subjected to the exercise stimulus. These reports indicate that a protective exercise-induced effect may be induced by blood-borne molecules.[23] Such beneficial effects of exercise can be transferred through plasma injections causing increased hippocampal neurogenesis and improved cognitive function,[5] potentially through downregulation of inflammatory signaling.[6] Previously, only a single study has examined the effect of exercised blood in the context of an AD rodent brain, showing that blood plasma from exercise-trained young mice, when infused into the triple transgenic AD mouse model, increased hippocampal neurogenesis, improved mitochondrial function, and reduced expression of cell death markers, seemingly leading to improved spatial learning and memory performance.[24] As there is no ideal AD model to mimic human AD, it is important to expand upon previous research by examining the potential treatment effects of exercised plasma at different stages of AD development in several relevant AD models. Here, we expand upon this knowledge by studying the effects of exercised blood *in vitro* and *in vivo*.

First, we tested the potential effect of exercised plasma in a hippocampal neuronal cells (HT22) culture model of AD. We further tested the potential preventive and therapeutic effects of exercised plasma administration in a transgenic rat model of AD at 2 different stages of AD development. Our hypotheses were that (a) plasma from exercised donors would protect the HT22 cell model against AD-like damage, and (b) exercised plasma transfusions would promote adult hippocampal neurogenesis and cognitive function in the AD rats at an early presymptomatic stage but not at a later symptomatic stage.

2. Methods

2.1. Human donors and cell culture

Plasma was collected from 4 healthy young men (maximal oxygen uptake: 63.2 ± 3.1 mL/min/kg, age: 27 ± 4 years; mean ± SD). Donors performed a single high-intensity interval treadmill exercise session consisting of a 10-min warm-up, 4 times 4-min intervals (reaching ∼90% of maximal heart rate) separated by 3 min of active recovery at 50%−70% peak heart rate, and a 5-min cool-down. Blood was collected before exercise (resting plasma) and at different time points after a bout of exhaustive high intensity exercise (0 h, 1 h, 3 h, 6 h, and 24 h) (exercised plasma).

Cultured HT22 mouse hippocampal neuronal cells were used to test whether exercised plasma induces any protective effects on the cells. The HT22 mouse neuronal cell line was provided by Richard Dargusch, Cellular Neurobiology Laboratory (CNB-S) at The Salk Institute (San Diego, CA, USA). The HT22 cells were cultured at +37˚C in 5% CO_2 in air in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 50 U/mL penicillin/streptomycin. Colorimetric MTS ((3-4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) cell proliferation assay (CellTiter 96; Promega, Fitchburg, WI, USA) was then performed in different experimental settings, including in HT22 cells that were (a) untreated controls, (b) treated with human plasma collected prior to exercise or 0 h, 1 h, 3 h, 6 h, or 24 h post-exercise, (c) treated with amyloid-β (Aβ), or (d) co-treated with Aβ and human plasma collected prior to exercise or 0 h, 1 h, 3h, 6 h, or 24 h post-exercise.

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In the experimental settings (c) and (d), a neurotoxic fragment of Aβ (Aβ 25-35, A4559; Sigma-Aldrich, St. Louis, MO, USA) was used to simulate "AD-like stress" in the cells. Aβ 25-35 (Sigma-Aldrich) was dissolved in water to a concentration of 50 mmol/L and incubated at 37˚C overnight to allow oligomerization before being diluted to 50 μmol/L in medium and used to treat the cells for 24 h. In the final hypothesis-testing setting (d), which tested the effects of exercised plasma collected at different time points in this "AD model", HT22 cells were co-treated with 50 μmol/L Aβ 25-35 (Sigma-Aldrich) and fetal bovine serum (Thermo Fisher Scientific) was replaced by pre- or post-exercise plasma at a concentration of 1% for 24 h.

For each condition, the MTS assay was performed according to the manufacturer's instructions in 2−5 biological replicates. Relative viability was measured as the quotient obtained by dividing the mean absorbance of treated technical replicate wells (3 for each) by the mean absorbance of the control wells. In addition to assessing cell viability, cell size was measured in the experimental setting (d) by taking an image from approximately the center of each well prior to the MTS assay and having a colleague blinded to treatment groups analyze the images using ImageJ (National Institutes of Health, Bethesda, MD, USA). In each replicate, the first 15 cells starting from a random corner within each image were selected and the perimeter was drawn around the individual cells to measure cell size. For more details, see the Supplementary Materials.

2.2. Animal model

We used the McGill-R-Thy1-APP transgenic rat model for AD (hAPP751 Swedish KM670/671NL and Indiana V717F mutations, controlled by mouse Thy1.2 promoter)[25] along with non-transgenic littermates (wild type). For the rat model characterization data, see the Supplementary Materials (Supplementary Fig. 1 and Supplementary Table 1). Male homozygous AD rats were assigned to transfusion treatment beginning at an early pre-plaque pathology stage (age = 2.20 ± 0.14 months, mean ± SD; $n = 20$) and a later stage closer to the time point when extracellular plaques emerge (age = 5.00 ± 0.20 months; $n = 23$). The rats were randomly divided into 3 treatment groups: 1 receiving 0.9% NaCl (saline), 1 receiving plasma from sedentary young Wistar rats (SedPlas), and 1 receiving exercised plasma from young exercise-trained Wistar rats (ExPlas). Male Wistar donor rats aged 1−2 months were either subjected to 6 weeks of exercise training ($n = 48$) or remained sedentary ($n = 48$) in their standard housing conditions, thus providing either ExPlas or SedPlas. For the study overview and details on timing for procedures (Supplementary Fig. 2 and Supplementary Table 2).

The study was carried out in accordance with the Norwegian regulations on animal experimentation. All experimental procedures were approved by the competent authority for animal research at the Norwegian Food Safety Authority (FOTS ID 11740/2017) and are in accordance with the Norwegian Animal Welfare Act §§1-28, Norwegian Regulations on Animal Research §§1-26, and European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes.

2.3. Tests of cognitive function

A novel object recognition test was used to assess recognition memory in the rats. The phases of the test, conducted in a circular arena, were habituation on Day 1, object familiarization on Days 2−4, and a test phase with a novel object on Day 5. A fear conditioning paradigm was used to assess associative fear learning and memory. The 4-day protocol utilized a combination of a tone as a conditioned stimulus and an electric shock as an unconditioned stimulus. The ANY-maze video system and software (ANY-Maze, Stoelting, Dublin, Ireland) was used to track the rats. For more details, see the Supplementary Materials.

2.4. Exercise training protocol for Wistar donor rats

Wistar rats were exercised according to a 6-week high-intensity interval training (HIIT) protocol on treadmills (Columbus Instruments, Columbus, OH, USA) with a 25˚ incline.[26] Prior to the first exercise session, animals were habituated to the treadmills. The habituation consisted of 5 min on the treadmill while turned off, followed by 8.0 m/min for 10 min. The HIIT sessions were performed 5 times/week and started with a 10-min warm-up at 6 m/min, immediately followed by 10 high-intensity intervals, each lasting 4 min, with 2-min active resting periods. The active rest speed was increased gradually from 6 m/min to 8 m/min during the 6-week exercise training period. According to our research group's extensive experience training rats, the 4-min intervals required the rats to run at a speed that corresponds to an exercise intensity of approximately 80%−90% of their peak oxygen uptake (VO_{2peak}).[27] To maintain a similar workload throughout the training period, the treadmill speed was progressively increased from the initial 12 m/min to the final 18 m/min. For more details, see the Supplementary Materials.

2.5. Cardiorespiratory fitness testing

A graded maximal exercise protocol in an enclosed treadmill with an indirect open circuit calorimeter system (Oxymax; Columbus Instruments) was used to determine VO_{2peak} of the donor rats before and after the 6-week HIIT protocol. All rats were habituated to the enclosed treadmill before testing. This consisted of 5 min on the treadmill while turned off, after which the speed of the treadmill was set to 8.0 meters per min for 10 min. Both the habituation and the VO_{2peak} test were performed at a treadmill incline of 25˚. The rat being tested was weighed and then put in the treadmill chamber, and the test was started with 10 min of warm-up at 8 m/min, after which the speed was increased by 1.8 m/min every 2 min, based on our previously published protocol.[27] The test was terminated when we observed a VO_2 plateauing despite increased workload. The absolute VO_2 values were scaled for size differences by dividing by body weight to the

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power of 0.75, VO_{2peak} hence being expressed as mL/kg$^{0.75}$/min. For more details, see the Supplementary Materials.

2.6. Rat donor plasma collection

Based on the findings from cell culture experiments, blood collection for the exercise-trained rats was performed 3 h after the last exercise session. Donor rats were deeply anesthetized (5% isoflurane) and their entire blood volumes were obtained by cardiac puncture of the left ventricle. The blood was collected in citrate tubes, which were centrifuged (2200 relative centrifugal force (RCF) for 10 min at +4˚C) before plasma was pooled and stored at −80˚C until use. Approximately 6 mL of plasma were collected from each rat.

2.7. Enzyme-linked immunosorbent assay in rat donor plasma

Plasma from exercised and sedentary donors was analyzed for brain-derived neurotrophic factor (BDNF) using a BDNF Rat enzyme-linked immunosorbent assay Kit (Invitrogen, Carlsbad, CA, USA) on a fully automated enzyme-linked immunosorbent assay system (Dynex DS2; Dynex Technologies, Chantilly, VA, USA) according to the manufacturers' instructions. For more details, see the Supplementary Materials.

2.8. Cytokine assay in rat donor plasma

Duplicate samples of pooled plasma from exercised and sedentary donors (3 pools from 16 donors each) were analyzed using a Bio-Plex Pro Rat Cytokine 23-Plex Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. The plate was read using a Luminex 200 System (Luminex Corp., Austin, TX, USA) and data was analyzed using the Bio-Plex Manager software (Version 6.1; Bio-Rad). For more details, see the Supplementary Materials.

2.9. Plasma injections

The AD rats were treated with either saline, ExPlas, or SedPlas via intravenous tail vein injections over the course of 6 weeks. The number of injections done in the study by Villeda et al.[17] was adapted to fit our longer treatment period. There were 14 injections in total, and they were administered in alternating fashion between 2 and 3 times per week. The injection volume used was the maximal volume recommended for intravenous injections for rats, which is 2 mL/kg body weight.[28]

To obtain a robust marker of cell proliferation, half of the AD rats (ExPlas = 7, SedPlas = 8, Saline = 7, all male) were given intraperitoneal injections of 5-bromo-2′-deoxyuridine (BrdU; ab142567; Abcam, Cambridge, UK, diluted in 0.9% sterile saline and 0.02% dimethyl sulfoxide) calculated to give a dose of 50 mg/kg. The other half of the AD rats (ExPlas = 7, SedPlas = 6, Saline = 9, all male) were given intraperitoneal injections of a weight-adjusted volume of saline. The intravenous injection was immediately followed by the intraperitoneal injection, and the last of the 14 treatments was administered 48 h before euthanasia. All injections were given under anesthesia (5% induction, 2.5% maintenance isoflurane).

2.10. Echocardiography

Echocardiography was performed using the Vevo 3100 Imaging System (FUJIFILM VisualSonics, Toronto, ON, Canada). For more details, see the Supplementary Materials.

2.11. Brain tissue collection and sectioning

The rats given intraperitoneal injections of BrdU were transcardially perfused with Ringer's solution (0.85% NaCl, 0.025% KCl, 0.02% $NaHCO_3$; pH 6.9) and 4% paraformaldehyde (Merck kGaA, Darmstadt, Germany) using a Unified Masterflex Drive peristaltic pump (Thermo Fisher). Brains were post-fixated in 4% paraformaldehyde for 24 h at +4˚C and then transferred to 2% dimethyl sulfoxide in 0.1 M phosphate buffer with 20% glycerol (Sigma-Aldrich) and stored at +4˚C until sectioning. The rats given intraperitoneal injections of saline were similarly anesthetized with 5% isoflurane in a chamber and weighed before exsanguination and heart excision for collecting and snap freezing the tissues.

The paraformaldehyde-fixed brains were sectioned serially at 40 μm in a coronal plane using a freezing microtome (Microm HM430; Thermo Fisher Scientific). The brains were cut in 6 equally spaced series, where 5 of the series were stored at +4 ˚C in tubes with 2% dimethyl sulfoxide in 0.1 mol/L phosphate buffer with 20% glycerol, and 1 series was mounted on SuperFrost Plus Adhesion Slides (Thermo Fisher Scientific) for histological orientation. For more details, see Supplementary Materials.

2.12. Immunohistochemical staining and quantification for amyloid plaque analysis

Amyloid plaques were visualized in untreated and treated later-stage AD rats using the anti-Aβ mouse monoclonal antibody, McSA1 (Supplementary Table 3) specific for aggregates of 39- to 43-amino-acid-long Aβ peptides. Sections were incubated with the primary antibody, McSA1, overnight before incubation with the secondary antibody, biotinylated goat anti-mouse. The sections were finally incubated in the avidin-biotin complex (ABC; Vectastain ABC kit; Vector Laboratories, Newark, CA, USA), mounted on SuperFrost Plus Adhesion Slides, and left to dry overnight on a heating plate. The mounted sections were then de-fatted in xylene and coverslipped using Entellan mounting medium (Sigma-Aldrich).

Glass slides were digitalized using Slide Scanner Axio Scan.Z1 and processed using the software Zen 2.6 (Carl Zeiss Microscopy GmbH, Jena, Germany). A total of 7 brain sections from 1 of the 6 series (intersection distance 240 μm), approximately within the bregma range −3.14 to −4.52,[29] were analyzed for plaque pathology in the hippocampal and cortical areas utilizing Fiji software (Version 1.53j; National Institutes of Health, Bethesda, MD, USA)

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and a tailored script. For more details, see Supplementary Materials.

2.13. Immunofluorescent staining and quantification for neurogenesis analysis

For quantification of neurogenesis, 7 brain sections with intersection distance 240 μm were co-labeled with immunofluorescence markers of cellular proliferation (BrdU) and mature neurons (hexaribonucleotide binding protein-3 (NeuN)). Sections were incubated with primary antibodies (BrdU mouse monoclonal antibody and NeuN rabbit monoclonal antibody) at 4˚C overnight. The following day, the sections were incubated for 60 min in a solution of Tris-buffered saline containing fluorescently conjugated secondary antibodies (Goat anti-Mouse IgG H&L Alexa Fluor 488 and Goat anti-Rabbit IgG H&L Alexa Fluor 594) and mounted on SuperFrost Plus Adhesion Slides (Thermo Fisher Scientific).

For all sections, Z-stack images of the dorsal dentate gyrus (bregma range −3.14 to −4.52[29]) were bilaterally imaged with Zeiss 880 Airyscan Confocal Microscope (Carl Zeiss AG, Oberkochen, Germany). The Zen image-acquisition software (Carl Zeiss AG) enabled a reusable imaging routine setup with the experiment designer module. The image analyses for quantification of neurons and neurogenesis were done using Fiji software.[30] For more details, see Supplementary Materials.

2.14. Statistical analyses

Due to exploratory nature of this study (no prior studies have been published using a similar approach), conventional power calculations could not be undertaken. This study should therefore be regarded as a proof-of-concept study that builds a foundation for future studies. All tests and analyses were conducted while blinded to the treatment given. Data are expressed as mean ± standard error of the mean (mean ± standard error of the mean). A 2-tailed unpaired *t* test was used for all analyses between 2 groups. Otherwise, 1-way analyses of variance followed by Bonferroni *post hoc* comparisons were used to analyze between-group differences. Only p values < 0.05 were considered significant. All statistical analyses were performed using IBM SPSS Statistics software (Version 28.0.1; Armonk, NY, USA).

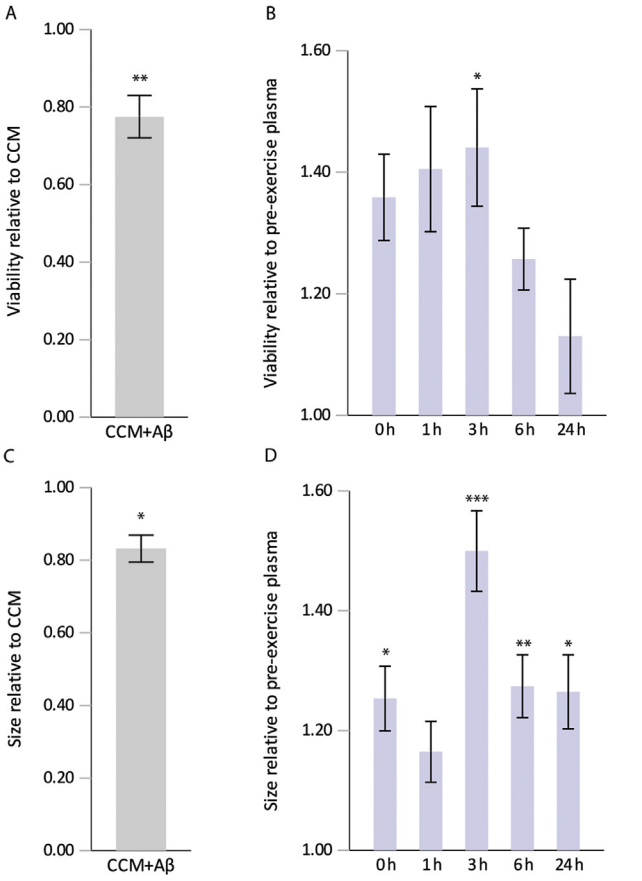

Fig. 1. Effects of exercised plasma collected at different timepoints post-exercise on viability and size of HT22 mouse hippocampal neuronal cells exposed to oligomerized Aβ fragment 25-35. (A) Including Aβ 25-35 in regular CCM reduced HT22 cell viability ($n = 5$ each). ** $p = 0.006$. (B) Treatment effect of plasma collected at different time points post-exercise on viability of cells cultured in CCM+Aβ ($n = 5$ each). 3 h * $p = 0.032$. The Y-axis displays cell viability normalized to pre-exercise (resting) plasma. (C) Including Aβ 25-35 in CCM reduced HT22 cell size. * $p = 0.014$. The Y-axis shows cell size relative to CCM. (D) Effect of plasma collection time on size of cells cultured in CCM+Aβ. 0 h: * $p = 0.021$, 3 h: *** $p < 0.001$, 6 h: ** $p = 0.008$, 24 h: * $p = 0.013$. The Y-axis displays cell size normalized to pre-exercise (resting) plasma. All data are presented as mean ± SEM. Aβ = amyloid-β; CCM = complete culture medium; SEM = standard error of the mean.

3. Results

3.1. Exercise-conditioned medium protected neuronal cells in vitro

The results showed that HT22 cells grown in a complete culture medium with 50 μmol/L oligomerized Aβ protein fragment 25-35 had a 22.5% lower viability (p= 0.006) (Fig. 1A) and a 16.8% reduction in size (p = 0.014) (Fig. 1C) relative to control cells grown in a regular complete culture medium, suggesting detrimental effects associated with adding Aβ to the culture medium. The viability of HT22 cells was not significantly reduced in cultures where control fetal bovine serum was replaced with 1% human plasma collected from fit young adults at resting state (pre-exercise plasma, without clotting factors) (Supplementary Fig. 3A). There were no significant differences in the viability of HT22 cells treated with media containing exercised or resting plasma at a concentration of 1% for 24 h (Supplementary Fig. 3B). When grown in a medium with both oligomerized Aβ and human plasma collected at different time points after a single bout of high-intensity exercise, both viability (Fig. 1B) and size of HT22 cells (Fig. 1D and Supplementary Fig. 4) increased relative to cells grown in a medium supplemented with Aβ and plasma collected prior to exercise (resting plasma). This increase was dependent on the time point of the plasma collection post-exercise. The greatest effect was seen when applying plasma collected 3 h post-exercise, where mean cell viability was 44.1% ($p = 0.032$) greater and cell size was 50.0% ($p < 0.001$) greater when compared to cells grown with resting plasma.

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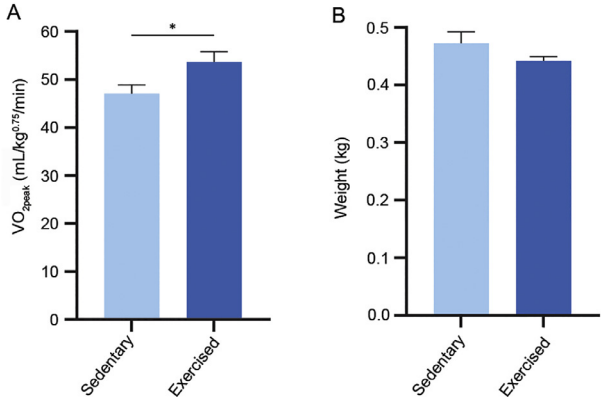

Fig. 2. Cardiorespiratory fitness and weight in the wild-type donor rats. The exercised-trained donors ran on treadmills following a 10 × 4 min HIIT protocol, 5 times per week for 6 weeks, while the sedentary donors were not subjected to exercise. After the 6-week period, all donors were weighed and cardiorespiratory fitness was measured as VO_{2peak} (mL/kg$^{0.75}$/min). (A) VO_{2peak} was significantly higher in the exercise-trained donor rats ($n = 15$, all male) compared to the sedentary donor rats ($n = 14$, all male, $^*p = 0.028$). (B) There was no significant difference in the weight of the exercise-trained and sedentary donor rats. All data are presented as mean ± SEM. HIIT = high-intensity interval training; SEM = standard error of the mean; VO_{2peak} = peak oxygen uptake.

3.2. Six weeks of HIIT increased cardiorespiratory fitness in donor rats

Following the 6-week exercise paradigm, trained donor rats had significantly higher VO_{2peak} values compared to sedentary donor rats (Fig. 2A, $p = 0.028$). There was no significant difference in the weight of the 2 groups of donor rats measured right before the VO_{2peak} test (Fig. 2B).

3.3. HIIT had no effect on BDNF levels in plasma of donor rats

There were no significant differences in BDNF levels between the ExPlas and SedPlas donor plasma (Supplementary Fig. 5).

3.4. Exercised donor plasma had reduced levels of several cytokines

The multiplex immunoassay of 23 cytokines revealed that 7 cytokines were significantly altered by exercise training in the donor rat plasma (Fig. 3). Specifically, the cytokines granulocyte macrophage colony stimulating factor ($p = 0.018$), growth-regulated oncogene/keratinocyte chemoattractant (GRO/KC) ($p = 0.026$), Interleukin-1β (IL-1β) ($p = 0.017$), Interleukin-7 (IL-7) ($p = 0.028$), Monocyte chemoattractant protein-1 (MCP-1) ($p = 0.004$), Macrophage inflammatory protein-1α (MIP-1α) ($p = 0.008$), and Macrophage inflammatory protein-3α (MIP-3α) ($p = 0.045$) all had significantly lower concentrations in ExPlas compared to SedPlas. Levels of granulocyte colony-stimulating factor, interferon-γ, Interleukin-1α (IL-1α), Interleukin-2 (IL-2), Interleukin-4 (IL-4), Interleukin-5 (IL-5), Interleukin-6 (IL-6), Interleukin-10 (IL-10), Interleukin-12 (IL-12) (p70), Interleukin-13 (IL-13), Interleukin-17A (IL-17A), Interleukin-18 (IL-18), macrophage stimulating factor, RANTES, tumor necrosis factor-α, and vascular endothelial growth factor were not significantly altered by exercise training in the donor rat plasma (Supplementary Table 4).

3.5. Exercised plasma showed no effect on recognition memory or contextual memory in AD rats

A novel object recognition test and a 4-day fear conditioning paradigm were used to evaluate the potential effects of plasma injections on the cognitive function of AD rats following the 6-week treatment period when started at either an early, pre-plaque stage or at a later stage. There were no significant differences between the early-stage treatment groups (Supplementary Fig. 6A) and later-stage treatment groups (Supplementary Fig. 6B) in the preference for the novel object over the familiar object and, hence, in recognition memory. Likewise, there were no significant differences in the freezing behavior of the rats at any phase of the fear conditioning test (Supplementary Fig. 7).

3.6. Plasma injections showed no harmful effects on the heart

Echocardiography measured from images taken in parasternal short axis view showed no volume overload or other harmful effects on the heart following plasma injections.

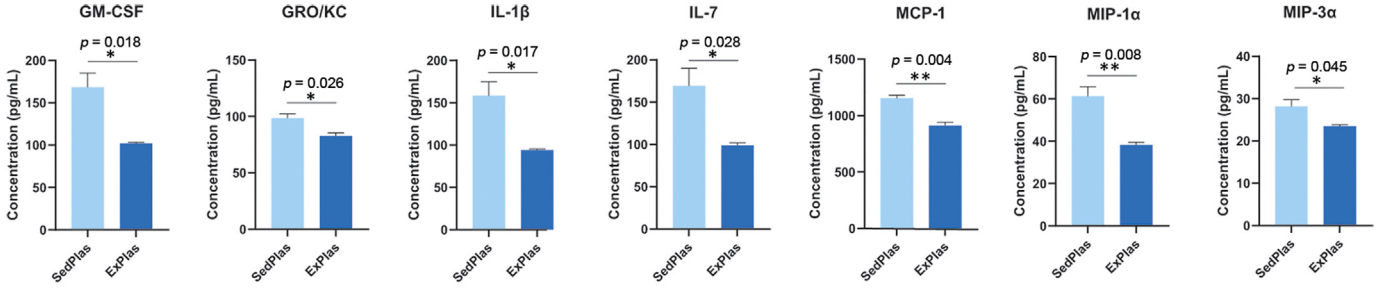

Fig. 3. Cytokines in exercised and sedentary donor plasma. Out of 23 cytokines, 7 were found at significantly lower concentration in ExPlas ($n = 3$, pooled plasma from 16 donors) compared to SedPlas ($n = 3$, pooled plasma from 16 donors). The significantly altered cytokines included GM-CSF ($p = 0.018$), GRO/KC ($p = 0.026$), IL-1β ($p = 0.017$), IL-7 ($p = 0.028$), MCP-1 ($p = 0.004$), MIP-1α ($p = 0.008$), and MIP-3α ($p = 0.045$). All data are presented as mean ± SEM. ExPlas = exercised plasma; GM-CSF = granulocyte macrophage colony stimulating factor; GRO/KC = growth-regulated oncogene/keratinocyte chemoattractant; SedPlas = sedentary plasma; IL-1β = Interleukin 1β; IL-7 = interleukin 7; MCP-1 = Monocyte Chemoattractant Protein-1; MIP-1α = Macrophage inflammatory protein-1α; MIP-3α = Macrophage inflammatory protein-3α; SEM = standard error of the mean.

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There were also no differences between any of the treatment groups in terms of fitness (Supplementary Fig. 8).

3.7. Exercised plasma showed no significant effects on amyloid plaque load

All rats in the ExPlas, SedPlas, and saline-only treatment groups showed either no plaques at all or only a few plaques, and there were no significant differences between the different treatment groups in the number of plaques (Supplementary Fig. 9A). Similarly, the size of plaques (Supplementary Fig. 9B) and the total percentage area covered by the plaques (Supplementary Fig. 9C) were low for all groups and showed no statistically significant difference between groups.

3.8. Exercised plasma promoted adult hippocampal neurogenesis

The mean total number of newborn neurons in the brains of AD rats with ExPlas treatment was 3.2-fold higher in early-stage rats ($p = 0.059$, Fig. 4A) and 3.4-fold higher in later-stage rats ($p = 0.008$, Fig. 4D) when compared to the respective saline-treated rats, although the difference was found to be statistically significant only for the rats treated at the later-stage. There were no statistically significant differences in the total number of newborn neurons between the ExPlas- and SedPlas-treated rats at either timing of treatment (Figs. 4A and 4B).

Because there are indications of structural and functional differences between the left and right hemispheres of the brain,[31] including at the level of the dentate gyrus in spatial and contextual differentiation,[32] we also performed the analyses separately for each hemisphere. The results showed a significantly greater number of newborn neurons in the right ($p = 0.045$) but not the left ($p = 0.090$) hemisphere of the early-stage AD rats treated with ExPlas when compared to those treated with saline (Fig. 4B and C).

For the later-stage AD rats, there were significant effects from both ExPlas ($p = 0.001$) and SedPlas ($p = 0.021$) treatments in the left hemisphere compared to saline treatment (Fig. 4K). The ExPlas-treated rats also had a significantly greater number of newborn neurons in the right hemisphere compared with the saline-treated rats ($p = 0.046$, Fig. 4L).

There were no significant differences in the total number of neurons (NeuN+) in the dorsal dentate gyrus (whether hemispheres were looked at separately or combined) between the treatment groups of either age.

4. Discussion

Here we showed that administration of plasma collected from fit men after a single bout of exercise improved cell viability and hindered neuronal cell atrophy in an AD cell model. We also showed that intravenous injections of blood plasma from exercise-trained donor rats promoted hippocampal neurogenesis in a rat model of AD, whether administered at an early pre-plaque pathology stage or at a later stage after expected extracellular plaque formation. Increased hippocampal neurogenesis did not translate into improved memory test performance. Downregulation of pro-inflammatory cytokines in plasma from exercise-trained donor rats was detected as a potential mediator of the neurogenesis-promoting effect of ExPlas.

4.1. HT22 mouse hippocampal neuronal cell culture

The effects of exercised plasma treatment on the neuronal cell culture differed with the timing of blood collection post-exercise. This suggests that temporal changes in blood plasma composition occur after a single bout of exhaustive exercise in humans. Plasma collected 3 h after exercise was found to most potently enhance both cell viability and survival, suggesting that relatively slow, transient changes in plasma-born factors induced by exercise could help the neuronal culture counteract the detrimental effects of Aβ. Further investigations into temporal changes in the molecular composition of human plasma following exercise are needed to detect the mediators of the observed effects. One study into molecular changes in human blood following a bout of exhaustive exercise identified great variation in the pattern of temporal concentration changes of plasma molecules post-exercise, but also found that most molecules returned to baseline levels within 1 h.[33] After an initial inflammatory response, anti-inflammatory signaling is increased; pathways involved in immune cell regulation can remain upregulated for a prolonged duration of more than 1 h.[33,34] Anti-inflammatory IL-3 and IL-10 are examples of cytokines released by activated immune cells that have been found to promote neuronal viability in Aβ-induced neurotoxicity.[35] Although acute changes in blood biomolecules following exhaustive exercise are similar in rats and humans,[36] it is possible that the optimal timing for blood collection could be different between the 2 groups. Nevertheless, our results demonstrate the beneficial effects of both human and rat blood plasma when collected 3 h after exercise. To the best of our knowledge, this is the first study to apply the HT22 model to study the potential protective effect of plasma from exercise-trained donors.

4.2. Cognitive function

We assessed the cognitive function of AD rats following the treatment period, as exercise has been shown to promote both neurogenesis and cognitive function in aged animals and in AD models.[10,37] While we found that ExPlas treatment significantly increased hippocampal neurogenesis, we did not see any significant differences in recognition memory or fear associative contextual memory between the treatment groups, regardless of the timing of treatment. Previous studies suggest neurogenesis to be important for specific cognitive functions. For example, ablating adult hippocampal neurogenesis has been shown to particularly impair pattern separation.[37,38] It is possible that the objects used in the present novel object recognition task were not similar enough to detect differences between treatment groups despite multiple-fold differences in the number of newborn neurons.[39] Notably, an increase in hippocampal neurogenesis alone may not be sufficient to enhance cognitive function, which may depend on additional

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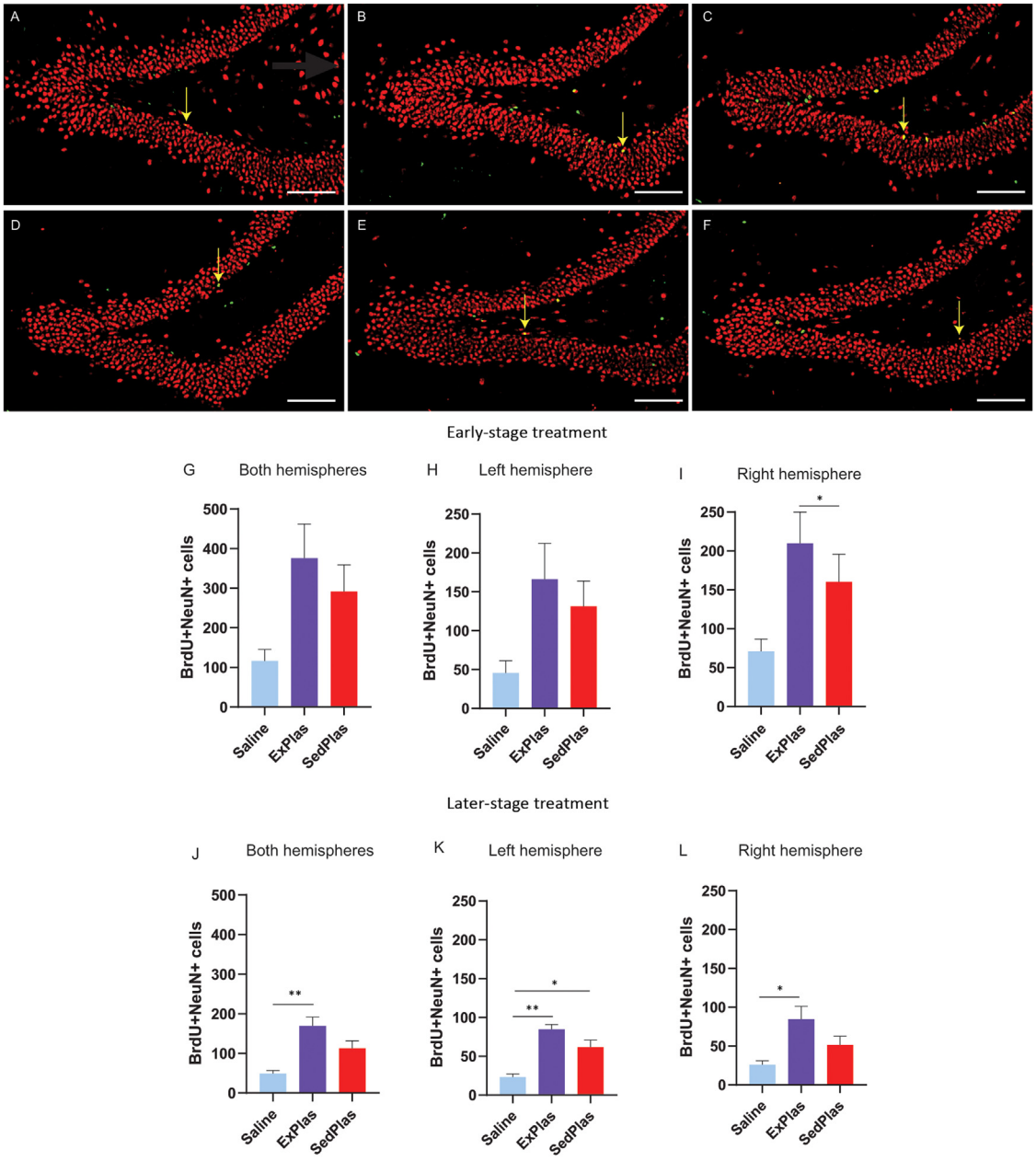

Fig. 4. Adult hippocampal neurogenesis in the treatment recipient AD rats. Neurons born during the treatment period were assessed in early-stage (3 months old) and later-stage (6 months old) male AD rats treated with intravenous injections of saline (A and D), ExPlas (B and E), and SedPlas (C and F). The 6-week treatment period consisted of 14 intravenous injections, each followed by an intraperitoneal injection of BrdU to label proliferating cells. Single 3.98 μm images showing immunofluorescent labeling of BrdU (green) and NeuN (red) in a part of the dorsal dentate gyrus that was analyzed to assess the degree of neurogenesis. BrdU/NeuN-double-labelled cells, seen here in yellow, were quantified as new neurons born during the study period. The yellow arrows point to an example of a BrdU+NeuN+ -double-labelled cell per image. Scale bar = 100 μm. The total number of newborn neurons ($BrdU^{+}NeuN^{+}$ cells) in the dentate gyrus of the early-stage (G) and later-stage (J) saline-, ExPlas-, and SedPlas-treated AD rats ($n = 3–4$ in each group, all male). Later-stage treatment with ExPlas significantly increased the total number of newborn neurons (** $p = 0.008$) when compared with the saline treatment. Number of newborn neurons in the left (H) and right (I; * $p = 0.045$) hemisphere of the early-stage AD rats ($n = 3–4$ in each group). Number of newborn neurons in the left (K; * $p = 0.021$, ** $p = 0.001$) and right (L; $p = 0.046$) hemisphere of the later stage AD rats ($n = 3–4$ in each group). All data are presented as mean ± SEM. AD = Alzheimer's disease; BrdU = 5-bromo-2′-deoxyuridine; ExPlas = exercised plasma; NeuN = hexaribonucleotide binding protein-3; SedPlas = sedentary plasma; SEM = standard error of the mean.

neurotrophic support.[37] As it takes several weeks for newborn neurons to fully develop and integrate into existing neural circuits,[40] it is also possible that a longer treatment period and/or a later implementation of the task could have affected the outcome.

4.3. Amyloid pathology

We found no effects of plasma injections on early amyloid plaque deposition in later-stage AD rats. Whereas the effects of exercised plasma transfusions on amyloid pathology have

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not been previously assessed, evidence from studies employing direct exercise training in transgenic animal models of AD suggests that exercise reduces amyloid plaque pathology.[41] However, the duration of training in studies reporting an effect was at least 4 weeks, with 5 exercise sessions per week.[41] The number of injections per week and in total might therefore have been too low to result in a significant effect in our study. Two studies examining young blood transfusion effects on amyloid pathology in AD mouse models have also reported reduction in amyloid plaques following 4 or 8 weeks of treatment.[20,21] In these studies, the recipients of young blood were aged mice with advanced plaque pathology. Therefore, the greater age difference between blood donors and recipients might be reflected in the molecular composition of the blood, resulting in more profound effects. On the other hand, our results might simply reflect individual differences, in which case a larger number of samples could better account for the variation in the emergence of plaques. Even so, a study with heterochronic parabiosis of old AD mice joined to young wild type mice found no effect on amyloid pathology from young blood exposure.[42]

4.4. Neurogenesis

Our results suggest a positive effect of ExPlas treatment on hippocampal neurogenesis, independent of the treatment timing in relation to the stage of AD pathology, hence strengthening existing evidence that exercise-induced changes in blood can serve as potent mediators of hippocampal neurogenesis.[5,6] A study in triple-transgenic AD mice found similarly that exercised plasma treatment promoted hippocampal neurogenesis in the aged AD mice compared to untreated controls.[24] Together these findings indicate that there are exercise-induced changes in plasma that confer beneficial effects on hippocampal neurogenesis in AD models.

Previous studies have suggested the upregulation of neuroprotective molecules such as BDNF may mediate the effects of exercise and exercised plasma transfusions on neurogenesis.[5,6,24] However, our analysis of ExPlas and SedPlas showed no differences in BDNF levels, suggesting that donor plasma BDNF content plays no role in the observed neurogenesis effects. On the other hand, exercise and exercised plasma transfusion may counteract the adverse effects of inflammation and AD pathology on the hippocampal neurogenic niche and neural stem cells.[6,43] Our results are in line with these findings, showing significantly reduced levels of several pro-inflammatory cytokines in ExPlas compared to SedPlas. For example, MCP-1 (CCL2), which was significantly downregulated (21%) in ExPlas, has previously been found to be correlated with aging-associated decline in neurogenesis.[16] As both aging and AD are associated with upregulation of inflammatory molecules in plasma and in cerebrospinal fluid,[44] treatment of the later-stage AD rats with young donor plasma might have been expected to result in greater effects than treatment of the early-stage rats of similar age as the donors. Still, we found remarkably similar magnitude effects for both ExPlas and SedPlas treatments on neurogenesis in early-stage and later-stage AD rats as compared to those who received the corresponding control saline treatments.

4.5. Limitations and strengths

A major strength of this study is that all experiments and analyses were conducted while blinded to the treatment allocation of AD rats. This study has several limitations. First, we only analyzed cytokine levels in the rat donor plasma, and not in the human plasma used to treat the HT22 cells. Second, although we included approximately the same number of animals as were included in a comparable pre-clinical study,[24] our sample is small, which is why we suggest this be regarded as a proof-of-concept study that may help form the basis for future studies with larger sample sizes. Another potential limitation is the length of the treatment period. Further studies are needed to explore whether a longer treatment period could result in an even stronger treatment effect with respect to neurogenesis, and potentially cognitive function and amyloid pathology as well. One should also consider whether a plasma exchange protocol (removing a given amount of plasma equivalent to the volume injected) would be an even better treatment approach. Furthermore, as many as 213 different AD animal models are available[45] and, therefore, we cannot rule out that other models may be more suitable and reflect human AD better than the model used here. Future studies should examine all these factors as well as study the potential undiscovered molecular effects of exercise training and high cardiorespiratory fitness that might reduce the risk of or even have therapeutic effects on AD.

5. Conclusion

The present study demonstrates that blood plasma from exercise-trained donors can promote neuronal viability and potently enhance adult hippocampal neurogenesis, but with limited effect on cognitive function. The reduced levels of pro-inflammatory cytokines in plasma from exercise-trained donor rats provide evidence to suggest the effects of exercise training on the brain are at least partly mediated by blood-borne factors.

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Authors' contributions

CSN participated in the cell study, planning and carrying out of the animal study and the amyloid plaque staining, and drafted the manuscript; AMH participated in the animal study, carried out immunohistological staining and data analyses, and drafted the manuscript; RNR participated in the animal study and the immunohistological staining; LB helped with obtaining funding and participated in planning the study; KJ participated in the immunohistological staining and data analysis for neurogenesis; RMdS participated in the animal study; LR performed the cytokine multiplex assay on donor rat plasma; BS participated in the animal study; JBNM participated in planning the study; AKF participated in planning the study; MPW participated in planning the study; NS carried out the cell study, participated in the animal study and data analysis for neurogenesis, and helped draft the manuscript; ART obtained funding, planned and coordinated the cell and animal study, and drafted the manuscript. All authors commented and critically revised previous drafts. All authors have read and approved the final version of the manuscript, and agree with the order of presentation of the authors.

Competing interests

The authors declare that they have no competing interests.

Supplementary materials

Supplementary materials associated with this article can be found in the online version at doi:10.1016/j.jshs.2023.07.003.

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