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Activated mesenchymal stem cell administration inhibits chronic alcohol drinking and suppresses relapse-like drinking in high-alcohol drinker rats

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ABSTRACT

Neuroinflammation has been reported to follow chronic ethanol intake and may perpetuate alcohol consumption. Present studies determined the effect of human mesenchymal stem cells (hMSCs), known for their anti-inflammatory action, on chronic ethanol intake and relapse-like ethanol intake in a post-deprivation condition. Rats were allowed 12–17 weeks of chronic voluntary ethanol (10% and 20% v/v) intake, after which a single dose of activated hMSCs (5×10^5) was injected into a brain lateral ventricle. Control animals were administered vehicle. After assessing the effect of hMSCs on chronic ethanol intake for 1 week, animals were deprived of ethanol for 2 weeks and thereafter an ethanol re-access of 60 min was allowed to determine relapse-like intake. A single administration of activated hMSCs inhibited chronic alcohol consumption by 70% ($P < 0.001$), an effect seen within the first 24 hours of hMSCs administration, and reduced relapse-like drinking by 80% ($P < 0.001$). In the relapse-like condition, control animals attain blood ethanol ('binge-like') levels >80 mg/dl. The single hMSC administration reduced relapse-like blood ethanol levels to 20 mg/dl. Chronic ethanol intake increased by 250% ($P < 0.001$) the levels of reactive oxygen species in hippocampus, which were markedly reduced by hMSC administration. Astrocyte glial acidic fibrillary protein immunoreactivity, a hallmark of neuroinflammation, was increased by 60–80% ($P < 0.001$) by chronic ethanol intake, an effect that was fully abolished by the administration of hMSCs. This study supports the neuroinflammation-chronic ethanol intake hypothesis and suggest that mesenchymal stem cell administration may be considered in the treatment of alcohol use disorders.

Keywords alcohol preferring rats, alcoholism, binge drinking, GFAP, glutathione, LPS, oxidative stress, TNF-alpha.

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A number of animal and human studies have shown that chronic ethanol consumption leads to an increase in inflammatory cytokines, both in the periphery (Leclercq *et al.* 2012) and in the brain (Crews *et al.* 2015: Crews & Vetreno 2016; Montesinos *et al.* 2016; Schneider *et al.* 2017). Upon alcohol intake, acetaldehyde generated by gut bacteria increases the permeability of the intestinal mucosa, allowing the diffusion of large molecules into the portal blood, including bacterial lipopolysaccharide (Ferrier *et al.* 2006). Lipopolysaccharide entering the liver triggers the release of tumor necrosis factor-alpha (TNF-α) into the circulation, which via brain TNF-α receptors transfers the inflammatory condition to the brain (Qin *et al.* 2007; Crews & Vetreno 2016). The TNF-α receptor knockout prevents lipopolysaccharide and TNF-α-induced neuroinflammation (Qin *et al.* 2007). A direct relationship between neuroinflammation and alcohol consumption is supported by animal studies demonstrating that the systemic administration of a single dose of lipopolysaccharide increases voluntary ethanol intake for a prolonged period (Blednov *et al.* 2011). While in the periphery, the inflammation induced by lipopolysaccharide administration is short-lived, disappearing in 9 hours; in the brain, the inflammation

lasts over 10 months (Qin *et al.* 2007), suggesting the existence of self-perpetuating neuroinflammatory mechanisms.

In addition to neuroinflammation due to systemic influx of lipopolysaccharide, the generation of reactive oxygen species (ROS) induced by brain ethanol *per se* upon chronic ethanol intake may also contribute to neuroinflammation. Marked increases in brain ROS have been reported following chronic ethanol intake in rodents (Montaliu *et al.* 1994; Costa *et al.* 2015). Some reports indicate that ROS lead to inactivation of IkB, the NFkB inhibitor, allowing the entrance of the NFkB p65 moiety into the nucleus, which activates the synthesis of pro-inflammatory cytokines, including TNF-α (Kastl *et al.* 2014). A vicious cycle may develop because TNF-α further uncouples mitochondria, inducing superoxide ion generation (Kastl *et al.* 2014) and the production of several ROS. Two additional mechanisms likely contribute to the generation of brain ROS. Ethanol is known to release dopamine in nucleus accumbens (Imperato & Di Chiara 1986), which is further oxidized by monoamine oxidase in a reaction that generates hydrogen peroxide (Cunha-Oliveira *et al.* 2013). An increased generation of ROS in the brain following chronic ethanol intake, also results from an increase in brain cytochrome P450 2E1 (CYP2E1), which generates superoxide radicals and further hydroxyl radicals (Montaliu *et al.* 1994; Howard *et al.* 2003).

Alcohol use disorders are chronic relapsing conditions (Weiss *et al.* 2001). A valuable animal model of alcohol relapse-like behavior is the 'alcohol deprivation effect' (ADE), the condition in which a marked increase in ethanol intake is seen upon ethanol re-access in animals that have consumed alcohol chronically and are deprived of ethanol for a prolonged period. (Hölter & Spanagel 1999; Tampier *et al.* 2013; Vengeliene *et al.* 2014). Ethanol intake in the relapse-like condition can exceed 2 g ethanol/kg in 60 minutes in high-ethanol drinker lines/strains (equivalent to 10 standard drinks/70 kg in a 1 hour sitting, akin to human 'binge drinking'). Because a long maintenance period of ethanol intake is needed to elicit ADE (Hölter *et al.* 2000), the mechanism is not clear, although drugs currently used in the treatment of alcohol use disorders (e.g. naltrexone, nalmefene and acamprosate-Ca,) reduce ADE-stimulated ethanol intake (Meinhardt & Sommer 2015).

Mesenchymal stem cells (MSCs) have been found to be highly immunosuppressive (Prockop & Oh 2012) by secreting anti-inflammatory cytokines (Zachar *et al.* 2016). MSCs also trap ROS (Valle-Prieto & Conget 2010). Because MSCs are not innately immunosuppressive, they acquire this function in response to the action of the pro-inflammatory cytokines TNF-α and IFN-γ. Upon activation, MSCs secrete a broad range of anti-inflammatory molecules including IL10, IL5 and TSG-6 that regulate both innate and adaptive immunity and repolarize cells from pro-inflammatory to anti-inflammatory phenotypes (Rasmusson *et al.* 2005). Additionally, MSCs secrete the enzyme indoleamine 2,3-dioxigenase (IDO), which inhibits the activation and proliferation of T cells through tryptophan depletion (Li *et al.* 2012) and the production of kynurenine, a tryptophan metabolite, that suppresses T-cell proliferation (Frumento *et al.* 2002). In the present studies, human MSCs (hMSCs) were activated by in vitro incubation with TNF-α and IFN-γ to increase the generation of anti-inflammatory cytokines prior to their intracerebroventricular administration.

Overall, the aim of this study was to determine whether the administration of activated hMSCs (1) inhibits chronic ethanol intake, (2) reduces ethanol intake in the ADE relapse-like model, (3) inhibits brain ethanol-induced oxidative stress and (4) reverses ethanol-induced morphological changes in hippocampal astrocytes, which characterize neuroinflammation.

MATERIALS AND METHODS

Animals

Adult female Wistar-derived rats selectively bred as alcohol consumers (University of Chile Bibulous; UChB) (Quintanilla *et al.* 2006), weighing 180 to 280 g at the time of experiments, were used in the study. The 2-month-old rats were housed in individual cages (45 × 23 × 20 cm) at a temperature-controlled and humidity-controlled room under 12-hour light/dark cycle (lights off at 7 PM) for 1 week to acclimate animals to the testing conditions. During this time, food and water were freely available. To minimize environmental and handling stress, the UChB animals are bred and maintained with strict access control on separate animal quarters from all other animals on the main Faculty of Medicine animal rooms. Alcohol intake was determined every 24 hours from volume difference of 100 ml inverted graduated cylinders. The tip of spout was 3 mm internal diameter, such that no spillage losses occur. When rats were allowed the 60-minute re-access after the deprivation period, 10 ml inverted (sealed) pipettes were used to gauge ethanol intake. Ethanol intake was expressed as gram of pure ethanol consumed per kilogram body weight. All experimental procedures were conducted during the light phase. Animal experimental procedures were approved by the Ethics Committee for Studies with Laboratory Animal at the Faculty of Medicine (Protocol CBA#0507, FMUCH) and by the Council for Science and Technology Research of Chile.

MSC isolation, ex-vivo expansion and characterization

Human adipose tissue-derived MSCs (hMSCs) were isolated from fresh subcutaneous adipose tissue samples (abdominal region) obtained from liposuction aspirates of patients undergoing cosmetic liposuction at Clínica Alemana, Santiago, Chile, as previously described by Oses *et al.* (2017). A written informed consent was obtained. The protocols were approved by the Ethics Committee of Facultad de Medicina Clínica Alemana-Universidad del Desarrollo. After two subcultures, cells were characterized according to their adipogenic and osteogenic differentiation potential, by the presence of putative hMSC markers (CD29, CD13, CD105, CD73 and CD90) and the absence of markers characteristics of other cell lineages (CD235a, CD31 and CD 45) as previously described by (Oses *et al.* (2017).

Activation of human MSC

Human MSCs (passage 3) at 70% confluency were activated for 40 hours in alpha-MEM supplemented with 10% FBS containing 10 ng/ml TNF-α and 15 ng/ml IFN-γ (R&D Systems, Minneapolis, MN). After activation, cells were washed two times with phosphate-buffered saline (PBS) and trypsinized. The number of living cells was estimated with trypan blue solution dye test.

Quantification of mRNA levels of anti-inflammatory factors in activated hMSCs

After activation of hMSCs with pro-inflammatory cytokines, total RNA was purified using Trizol (Invitrogen, Grand Island, NY) following the manufacturer instructions. RNA isolated from hMSCs without activation (naïve) was used as control. Two micrograms of total RNA were used to perform reverse transcription with MMLV reverse transcriptase (Invitrogen) and oligo dT primers. Real time polymerase chain reactions were performed in a 10 µl final volume containing: 50 ng cDNA, polymerase chain reaction LightCycler-DNA Master SYBERGreen reaction mix (Roche, Indianapolis, IN), 3 mM $MgCl_2$ and 0.5 µM of the primers to amplify the anti-inflammatory factors IL-5, IL-10, IDO and TSG-6 using a Light-Cycler 1.5 thermocycler (Roche). To ensure that amplicons were generated from mRNA and not from genomic DNA, controls without reverse transcriptase during the reverse transcription reaction were included. Analysis of melting curve was used to ensure that only one product was amplified during the reaction. Agarose gel electrophoresis was used to characterize amplicon sizes. Relative quantifications were performed by the ΔΔCT method. The mRNA level of each target gene was normalized against the mRNA levels of the housekeeping genes elongation factor 1 alpha 1 (EEF1A1) and ribosomal protein L13A (RPL13A) of the same sample.

Intracerebroventricular stereotaxic administration of activated hMSCs

Female UChB rats that had consumed ethanol chronically were pre-anesthetized with chloral hydrate (280 mg/Kg, i.p.) and mounted on a David Kopf stereotaxic frame with the skull oriented according to the Paxinos & Watson (1998) rat brain atlas for rats of approximately 250 g. Anesthesia was kept with a mixture of air and isoflurane along the full procedure. The skull was exposed and a hole drilled for implanting the tip of a 10-µl Hamilton syringe into the left lateral ventricle (A −0.8; L −1.6; V −3.4), the ventral coordinate calculated from the surface of the brain. Ten microliters of a solution containing 5×10^5 activated hMSCs in 10 µl saline containing 10% of rat serum was injected; the syringe removed and the wound sutured, adding an antiseptic solution. Control animals were injected with 10 µl of saline containing 10% rat serum—the standard control in this field. Thereafter, animals were returned to their cages according to the indicated protocols. Ethanol intake was recorded daily for both groups. Results are expressed as gram of total ethanol consumed per kilogram body weight on a daily basis (24-hour intakes) and as milliliter of water consumed per kilogram body weight daily.

Drugs and solutions

The 10% and 20% ethanol drinking solutions were obtained by dilution of ethanol (95%, Merck Darmstadt, Germany) with distilled water (10% v/v or 20% v/v ethanol solution in 100 ml). Glutathione reductase (G3664), NADPH (N1630) and DTNB *(*5,5′-dithiobis-2-nitrobenzoic acid*)* (D8130) were purchased from Sigma-Aldrich.

PROCEDURES

Blood ethanol determinations

After the rats had a 60 minute of re-access to ethanol solutions (10% and 20%) and water (after 14 days of ethanol deprivation), 100 µl of blood samples were collected from the tip of the tail (Murphy *et al.* 1986), while under moderate sedation with acepromazine (1 mg/kg i.p.). The blood samples collected without anticoagulants were immediately added to 0.9 ml of distilled water at 4°C in a glass vial sealed with Mininert valves (Supelco, Bellefonte, PA). Samples were kept on ice for 15 minutes before ethanol determination, for which the diluted blood samples were incubated 15 minutes at

60°C, and 1.0 ml of gas was removed and analyzed by head space as chromatography (Perkin Elmer SRI 8610) as described earlier by Quintanilla *et al.* (2007).

GSSG/GSH ratio assay

Rats were anesthetized with chloral hydrate (280 mg/kg, i.p.) and perfused intracardially with 100 ml of 0.1 M PBS (pH 7.4). Hippocampus were extracted and mixed with three volumes of ice-cold potassium buffer containing 5 mM EDTA, pH 7.4, and flash-frozen and stored at −80°C until homogenization. Reduced glutathione (GSH) and glutathione disulfide (GSSG) contents were determined as described previously by Rahman *et al.* (2006) and Perez-Lobos *et al.* (2017). Briefly, in the assay for total GSH and GSSG, GSSG in the sample was first converted into GSH with glutathione reductase and NADPH. The total free thiol group of GSH was reacted with the sulfhydryl reagent DTNB (5,5′-dithiobis-2-nitrobenzoic acid) yielding a product that absorbs at 412 nm. GSSG *per se* in the homogenate was measured by adding 2-vinyl pyridine, a thiol scavenger that traps GSH, preventing GSH from binding to DNTB. The excess of 2-vinyl pyridine was neutralized with triethanolamine. Thereafter, GSSG was converted into GSH by glutathione reductase and NADPH, DNTB was added and absorbance measured at 412 nm.

Tissue preparation and astrocyte immunohistochemistry

Preparation of brain tissue for immunocytochemistry was performed according to Morales *et al.* (2008) and Perez-Lobos *et al.* (2017). Briefly, rats were anesthetized with (280 mg/kg i.p.) chloral hydrate and perfused intracardially with 100 ml of 0.1 M PBS (pH 7.4), followed by 200 ml formalin solution (4% paraformaldehyde; in 0.1-M PBS, pH 7.4). The brain was removed from the skull, post-fixed for 24 hours at 4°C in the same fixative, and immersed in 30% sucrose in 0.1 M of PBS at 4°C for 2–3 days. Thereafter, coronal sections of the hippocampus (20 μm thick) were obtained using a cryostat (Thermo Scientific Microm HM 525 Cryostat). The sections were mounted on silanized slides and stored at −20°C pending further experiment. Coronal sections were washed with 0.1 M PBS and treated with blocking solution (6.5% normal goat serum 2% BSA and 0.3% Triton X-100 in PBS) and then incubated with primary antibody against glial fibrillary acidic protein (GFAP, astrocyte marker, Sigma-Aldrich, 1:500 dilution in blocking solution) overnight at 4°C. Samples were rinsed with PBS, incubated with goat anti-mouse secondary antibody [Alexafluor 488, 1:500 dilution in 1% normal goat serum (Thermo Fisher Scientific, Whaltam MA) in PBS 0.1 M], and nuclei were counter-stained with 4,6 diamino-2-phenylindol (DAPI; Invitrogen 0.02 M; 0.0125 mg/mL) for 2 hours. After rinsed, the samples were mounted with Fluoromount and examined by confocal microscopy (Olympus-fv10i).

Microscopy and image analysis of GFAP positive cells

Microphotographs (five–six) were taken from *lacunosum moleculare stratum* of hippocampus in the field of an Olympus FV10i microscope (Center Valley, PA, USA), using 60× objective lens (NA1.30). Images were captured using FV10-ASW-2b software (Olympus). The area inspected for each stack was 0.04 mm^2 and the thickness (Z axis) was measured for each case. FIJI image analysis software (http://fiji.sc/Fiji) was used to project the Z-stack for all acquired images per tissue section. Total length and thickness of primary processes of astrocytes were assessed for six GFAP positive cells per Z-stack according to Tavares *et al.* (2017).

Studies on the effect of a single intracerebroventricular injection of activated hMSCs on (a) chronic ethanol intake and (b) brain GSSG/GSH ratio

A group of 18 animals was divided into three subgroups (n = 6 each). (1) The water group was allowed only water and chow and euthanized on day 139, (2) the ethanol + hMSCs group was allowed 10% and 20% ethanol and water from three bottles for 139 days but animals were injected intracerebroventricular (ICV) 5×10^5 activated hMSCs on day 118 and euthanized on day 139 (i.e. 3 weeks after hMSC injection). (3) The ethanol + vehicle group (control) was allowed 10% and 20% ethanol and water from three bottles and euthanized after determining the prior 24-hour intake on day 139 but was injected ICV 10 μl of vehicle on day 118. Ethanol and water consumption were recorded daily in the ethanol + hMSCs and ethanol + vehicle groups to determine the effect of hMSCs on ethanol and water intake (Fig. 1). The GSSG/GSH ratio was determined in the hippocampus of the three groups of animals euthanized at day 139.

Studies on the effect of a single injection (ICV) of activated hMSCs on (a) chronic ethanol intake, (b) ADE-ethanol intake and (c) hippocampal astrocyte immunoreactivity

Eighteen rats were divided into three subgroups (n = 6 per group). (1) The water group was allowed only water and chow and euthanized on day 121; (2) The ethanol + hMSCs group was administered 10% and 20% ethanol for 107 days while on day 100, animals were injected ICV 5×10^5 activated hMSC. On day 107, ethanol was removed for 2 weeks (deprivation starts) and on day 121, animals were allowed ethanol re-access

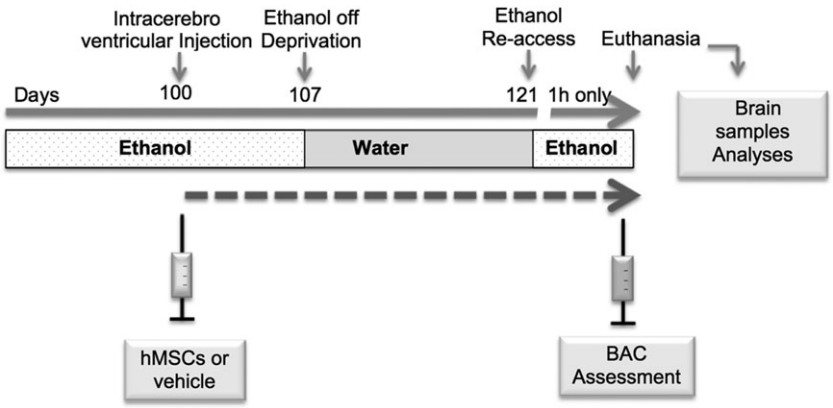

Figure 1 Time-line of study shown in Fig. 4. hMSCs, human mesenchymal stem cells

(10% and 20%) for 60 minutes (i.e. 3 weeks after MSCs injection). Immediately after the 60 minutes of ethanol re-access, animals were euthanized for analyses of blood ethanol levels and hippocampal astrocyte GFAP immunofluorescence. (3) The ethanol + vehicle group (control) followed the same time schedule as group 2, but animals were injected with an equivalent volume of vehicle instead of hMSCs. The time line of this experiment is described in Fig. 1.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism (SanDiego, CA). Data are expressed as means ± SEM. Two-way (treatment × day) analysis of variance (ANOVA) followed by Bonferroni's *post hoc* test or ANOVA for repeated measures when required was conducted to compare the ethanol intake or water intake between vehicle and hMSCs groups. One-way ANOVA followed by Tukey's *post hoc* test was used to analyze total length and thickness of primary processes of GFAP positive astrocytes and for GSSG/GSH ratios data. No animal selection was made either prior of after the experiments. When appropriate, a Student's *t*-test was conducted to determine if two sets of data were significantly different from each other. A level of $P < 0.05$ was considered for statistical significance.

RESULTS

Activation of human mesenchymal stem cells

Figure 2 shows the effect of TNF-α and IFN-γ activation on the expression of hMSCs anti-inflammatory factors IL-5; IL-10; IDO and TSG-6. It is shown that hMSC activation increased by over one order of magnitude (*t*-test; $P < 0.001$) the expression of the anti-inflammatory factors. These activated hMSCs showed maximal effects on ethanol intake immediately after their ICV administration (*vide infra)*.

Rapid and long-lasting effects of a single intracerebroventricular administration of activated hMSCs. Inhibition of chronic ethanol intake and reduction of ethanol-increased brain GSSG/GSH ratio.

Figure 3 shows that rats that had consumed 10% and 20% ethanol for 16–17 weeks consumed 10 to 11 g ethanol/kg/day before receiving hMSCs. The ICV administration of activated hMSCs (5×10^5 cells) inhibited chronic ethanol intake by 70–75% within 24 hours of hMSC administration (Fig. 3a). F-ANOVA for the full 3 weeks following the single administration of activated hMSCs showed that ethanol intake was significantly inhibited ($F_{\text{treatment (1, 210)}} = 1525$, $P < 0.0001$). Bonferroni tests showed that the single dose administration of activated hMSCs significantly inhibited ($P < 0.01$) chronic ethanol intake at each of the times studied within the 3 weeks. An inhibitory effect on ethanol intake of 50% was still observed at the end of the 3 weeks after hMSCs administration, which was statistically significant versus the inhibition observed on the first day following hMSCs administration (the regression of ethanol intake within the 3 weeks after hMSC administration was significant: $r^2 = 0.796$; $P < 0.0001$). Figure 3b shows that animals increased their water intake within the 3 weeks after activated hMSC administration [two-way ANOVA; $F_{\text{treatment (1,210)}} = 290$, $P < 0.0001$]. The increase in water intake likely results from the reduction in the intake of water contained in the ethanol solutions, thus allowing animals to maintain their water homeostasis. Figure 3c shows the GSSG/GSH ratio in hippocampus of the (1) water, (2) ethanol + vehicle and (3) ethanol + activated hMSCs groups. One-way ANOVA revealed significant differences between the groups [$F_{(2, 15)} = 13, < 0.001$]. *Post hoc* comparisons indicated that the GSSG/GSH ratio was significantly increased (250%) in the hippocampus of animals that had consumed ethanol for 20 weeks and received vehicle versus the group consuming water only ($P < 0.001$) (Fig. 3c). The effect of ethanol intake on the GSSG/GSH

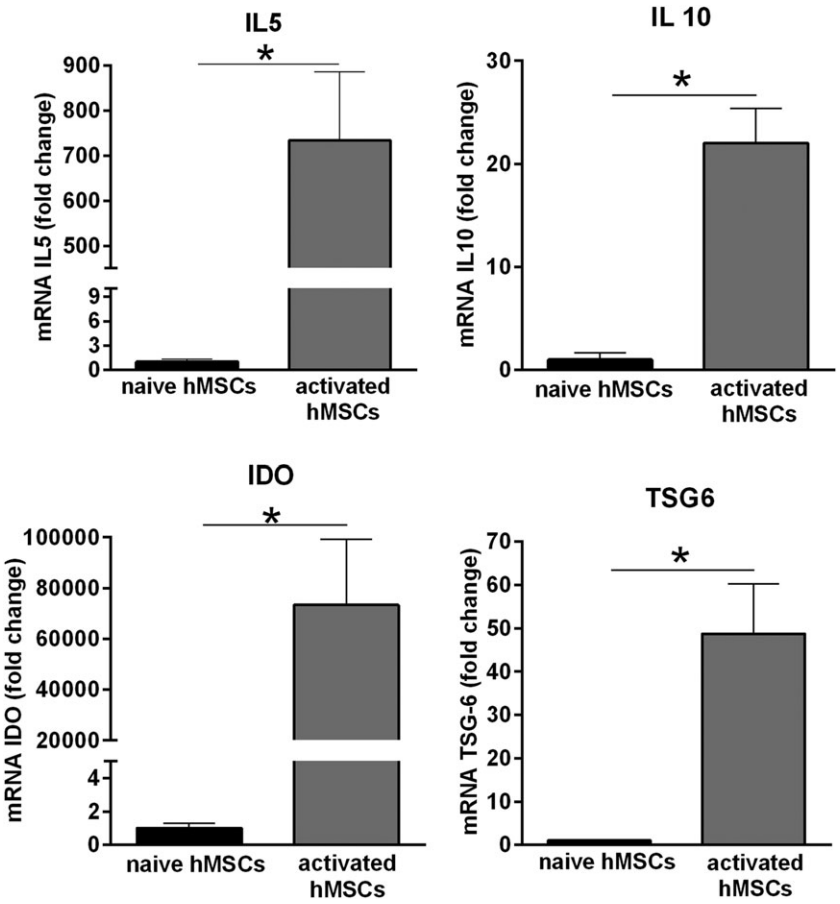

Figure 2 Activation of human mesenchymal stem cells (hMSCs) increases the gene expression of anti-inflammatory factors. Total RNA was obtained from hMSCs exposed to 10 ng/ml TNF-α and 15 ng/ml IFN-γ (activated MSCs) or vehicle (naive hMSCs) for 40 hours and subjected to quantitative reverse transcriptase-PCR analysis for quantification of the mRNA levels of the anti-inflammatory factors IL-5, IL-10, IDO and TSG-6. Data for each target gene was normalized against the mRNA levels of the housekeeping genes EEF1A1 and RPL13A of the same sample and presented as fold change of expression in activated MSCs versus naive MSCs. Data are shown as means ± SEM. $N = 4$ per experimental group. Asterisk indicates statistically significant differences between the activated hMSCs and naïve hMSCs: *$P < 0.05$ (Student's *t*-test)

ratio was significantly reduced in the animals injected with hMSCs (ethanol + hMSCs group) 3 weeks earlier ($P < 0.01$).

Inhibitory effect of a single intracerebroventricular injection of activated hMSCs on (a) chronic ethanol intake, (b) ethanol intake in the ADE relapse-like model and (c) hippocampal astrocyte immunoreactivity.

Figure 4a—left shows the effect of a single dose (5×10^5) of activated hMSCs on chronic ethanol consumption. ANOVA for repeated measures of data in Fig. 4a—left showed that the administration of the single dose of activated hMSCs to rats that had consumed ethanol chronically for 100 days reduced chronic ethanol intake by 72% versus the control group [$F_{\text{treatment}(1,\ 104)} = 185.7$, $P < 0.0001$). *Post hoc* analysis revealed that the effect of a single dose of activated hMSCs reduced ethanol intake during all the 7 days that lasted the initial part of the study compared to the control group ($P < 0.001$, Bonferroni test). As also seen in Fig. 3, the maximum inhibitory effect of activated hMSCs was seen within 24 hours and remaining constant throughout the 7 days of chronic ethanol consumption. In these studies, animals were subsequently deprived of ethanol for 2 weeks after which ethanol (10% and 20%) re-access was allowed for 60 minutes (as in timeline shown in Fig. 1). Figure 4a—right shows the effect of the single dose of activated hMSCs on relapse-like ethanol intake ADE. Figure 4a—right shows that during the 60-minute ethanol re-access on day 121 (3 weeks after hMSC administration), control animals showed an ADE relapse-like ethanol intake of 1.60 ± 0.04 g ethanol/Kg/60 minutes, whereas animals that were injected with activated hMSCs reduced the ethanol intake to 0.32 ± 0.15 g ethanol/kg/60 minutes, thus 80% lower than the intake of the ethanol + vehicle group (*t*-test = 8.24; $P < 0.001$, $n = 6$/group). Figure 4b shows the effect of a single dose of activated hMSCs on blood ethanol levels (mg/dl) resulting from the ethanol consumption during the 60 minutes of ethanol re-access (values in gram ethanol per kilogram intake shown in Fig. 4a—right). Results indicate that during alcohol reinstatement, the control group displayed blood ethanol levels >80 mg/dl (95.8 ± 10). By the contrast, the group injected with activated hMSCs showed blood ethanol levels of 19.4 ± 8.6 mg/dl, (*t*-test = 8.245; $P < 0.001$), thus 80% lower than the control group. This effect was seen 21 days after the single dose administration of activated hMSCs. At the end of the 60-minute ethanol re-access, animals (including the water control animals) were euthanized to determine hippocampal astrocyte

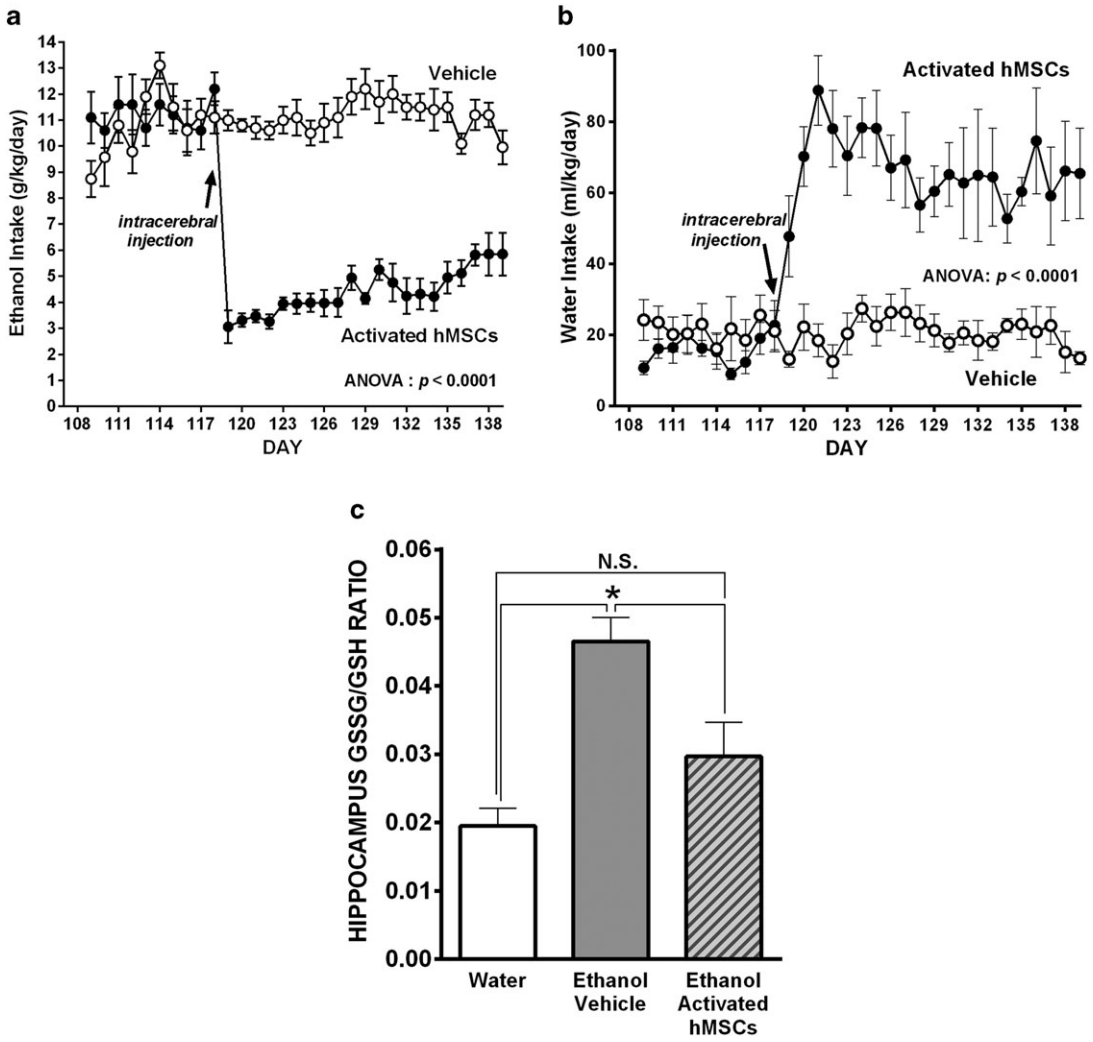

Figure 3 The intracerebroventricular administration of activated hMSCs, inhibited chronic ethanol intake, increased water intake and reduced the ethanol-induced increase in the hippocampus GSSG/GSH ratio. A single dose of activated hMSCs (5×10^5) injected into the left cerebral ventricle of rats consuming ethanol for 17 weeks inhibited chronic ethanol intake for 21 days versus rats injected with vehicle ($P < 0.0001$; two-way ANOVA, Bonferroni *post hoc* significant for 21/21 days) (a) increased the water intake ($P < 0.0001$; two-way ANOVA, Bonferroni *post hoc* significant for 20/21 days) and (b) reduced ethanol-induced increases in hippocampal GSSG/GSH ratio. Data are means ± SEM ($n = 6$ rats per group). Asterisk indicates statistically significant differences between the (ethanol + activated hMSCs group) and the (ethanol + vehicle group): *$P < 0.01$ (One-way ANOVA and Tukey's *t*-test). hMSCs, human mesenchymal stem cells; ANOVA, analysis of variance; GSSG, glutathione disulfide; GSH, glutathione

immunoreactivity. Figure 4c (center) shows that compared to water control, chronic ethanol intake markedly and significantly ($P < 0.001$; Fig. 4d & e) increased the immunoreactivity of GFAP, a characteristic marker of neuroinflammation. The administration of activated hMSCs fully reversed these changes ($P < 0.001$), both on thickness of the primary astrocyte processes (Fig. 4d) and on their length (Fig. 4e).

DISCUSSION

The main question addressed in this study was whether the intracerebral administration of anti-inflammatory hMSCs inhibits chronic ethanol intake and prevents relapse-like ethanol ('binge-like') drinking in an animal model of high ethanol consumption. hMSCs, considered to be the 'guardians' against inflammation (Prockop & Oh 2012), were shown to markedly inhibit both chronic ethanol intake and relapse-like drinking. Activated hMSCs also reversed the increased immunoreactivity against astrocyte GFAP in the hippocampus of rats that had consumed ethanol chronically. Taken together these findings support the view that ethanol induces neuroinflammation, which contributes to the perpetuation of ethanol intake (Blednov *et al.* 2011; Crews *et al.* 2015). Also in line with an inhibitory effect of anti-inflammatory drugs on ethanol intake, the administration of ibudilast, a drug shown to reduce TNF-α levels, was reported to inhibit

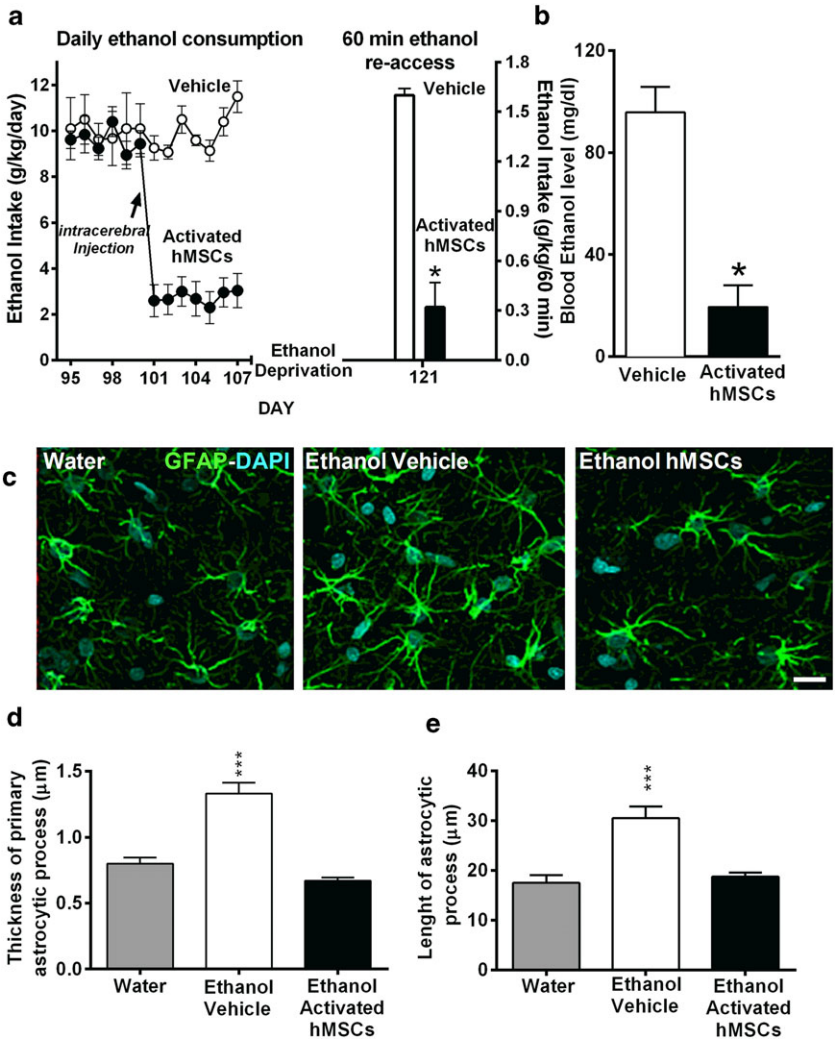

Figure 4 The intracerebroventricular administration of activated hMSCs reduced (1) chronic ethanol intake, (2) relapse-like ethanol intake in the ADE post-deprivation condition and the ethanol blood level achieved in the relapse-like condition and (3) ethanol-induced increased immunoreactivity of glial fibrillary acidic protein (GFAP) in hippocampal astrocytes. A single dose (5×10^5) of activated hMSCs injected into the left cerebral ventricle of rats consuming ethanol for 14 weeks (a—left) reduced their ethanol intake (means ± SEM) compared with the vehicle injected group ($P < 0.0001$; repeated measures ANOVA); (a—right) reduced the relapse-like ADE-ethanol consumption (g ethanol/kg/60 minutes) during the 60 minutes of re-exposure to ethanol versus the vehicle group (*$P < 0.001$; Student's *t*-test); (b) reduced ethanol blood levels induced by the 60-minute relapse-like ethanol consumption after 14 days of deprivation (ADE) and (d and e) reversed the ethanol-induced increase of the thickness and length of astrocytic processes (***$P < 0.001$, one-way ANOVA, Tukey's *t*-test). ADE, alcohol deprivation effect; ANOVA, analysis of variance, hMSCs; human mesenchymal stem cells

by 50% both chronic ethanol intake and ADE-relapse-like ethanol intake in *P* and high alcohol drinking (HAD) alcohol preferring rats (Bell *et al.* 2015).

Hippocampal damage has been reported in alcoholics (Sullivan *et al.* 1995) and in animals ingesting ethanol for prolonged periods (Walker *et al.* 1980; Franke *et al.* 1997). Damage starts with an increased immunoreactivity against GFAP and progresses to cells loss (Franke *et al.* 1997). In the present studies, a marked GFAP immunoreactivity was observed in animals that had consumed 10% and 20% ethanol solutions for 20 weeks, which was fully reversed by the administration of activated hMSCs 3-weeks prior to the hippocampal immunocytochemistry analysis. As indicated above, marked increases in ROS are also observed in the brain of ethanol treated animals (Montaliu *et al.* 1994; Costa *et al.* 2015), an effect that may be both a direct effect of ethanol on the generation of ROS or a secondary effect of increases in TNF-α that are reported to activate mitochondrial reactive oxygen generation (Kastl *et al.* 2014), which could lead to a vicious cycle where neuroinflammation and ROS potentiate and perpetuate each other. Increases in hippocampal TNF-α and other pro-inflammatory cytokines have been reported in rats orally administered 2 g ethanol/kg twice daily for 30 days, while the administration of N-acetyl cysteine, a potent antioxidant, suppressed the inflammatory effects induced by ethanol treatment (Schneider *et al.* 2017).

A likely mechanism by which neuroinflammation perpetuates chronic ethanol intake is the reduction of astrocyte glutamate transporter GLT-1 levels, with concomitant increases in extracellular glutamate (Das *et al.* 2015). The administration of the antibiotic ceftriaxone reverses the ethanol-induced reduction of GLT-1 activity and markedly inhibits chronic ethanol self-administration (Das *et al.* 2015). Studies by David *et al.* (2016) showed that neuroinflammation *per se* induced by the intracranial administration of the parasite *Toxoplasma gondii* leads to a reduction in the activity of the GLT-1 glutamate transporter, increases extracellular glutamate levels, increases astrocyte volume and enhances astrocyte GFAP immunoreactivity. The

inhibitory effect of N-acetyl cysteine on chronic ethanol intake (Quintanilla *et al.* 2016) and on ethanol seeking (Lebourgeois *et al.* 2017) could conceivably be explained by its anti-inflammatory effects (Schneider *et al.* 2017). Further, the GLT-1 transporter has mechanisms (Trotti *et al.* 1998) in which cysteine residues sense the redox state of the cell (e.g. GSSG/GSH), which as shown in the present study, was partially normalized by activated hMSCs administration.

In a recent study on the effect of adipose tissue-derived rat MSCs (rMSCs) on chronic ethanol intake (Israel *et al.* 2017), a single intracerebral administration of non-activated rMSCs resulted in a lower (40–50%) but significant reduction of chronic ethanol intake. Thus, a prior activation of MSCs is not an absolute requirement to reduce chronic ethanol intake. The administration of non-stimulated rMSCs also inhibited ADE relapse-like intake to the same extent (80–85%) as activated hMSC. However, the inhibitory effect of activated hMSCs on chronic ethanol intake in the present study was faster and fully expressed within 24 hours of their administration, suggesting an action of trophic factors secreted by the activated hMSCs. The demonstration of marked inhibitory effects of hMSCs on ethanol intake in animal models and the reduction of neuroinflammation markers is of additional interest for possible translational studies.

Treatment of alcohol use disorders without prior waiting for a protracted alcohol withdrawal appears preferable from a translational point view. This view was applied to both experiments in this study, in which the single dose of activated hMSCs was administered during the chronic ethanol maintenance phase. hMSCs are hypo-immunogenic and therefore not readily rejected by the immune system, thus allowing a long-lasting allogenic transplantation (Shi *et al.* 2010).

It is noteworthy that the 60-minute ADE relapse-like drinking resulted in blood alcohol levels >80 mg ethanol/dl considered to result from 'binge drinking' in humans. The administration of activated hMSCs led the blood alcohol levels of 20 mg/dl, akin to levels found after the intake of one standard drink in humans.

It is noted that a limitation of the present study is the use of only female rats. For rats selectively bred for their high-ethanol intake e.g. the Indianapolis bred rats, females have an equal (HAD-1) or higher (HAD-2) ethanol intake than males (Dhaher *et al.* 2012). For Sardinian high-ethanol intake bred rats, females show higher levels of ethanol intake than males (Loi *et al.* 2014). Males of the UChB rat line show a 20% lower ethanol intake than females (unpublished). It is noted that the weight and ethanol intake of female rats remains relatively constant after adulthood. Male rats on the contrary increase their weight (mostly body fat) but their livers do not grow at the same rate. Thus, ethanol metabolism expressed per unit body weight is reduced as males get heavier. Animals in the present studies were caged separately, such that estrous cycles are not synchronized and no 5-day (estrous) cycles are observed on ethanol intake.

The results of these studies indicate that the administration of activated MSCs appears promising towards the treatment of alcohol use disorders and preventing relapse. Further studies are required to determine the full duration of the anti-alcohol action of activated hMSCs before engaging in any invasive procedures. Studies should also examine the possible intravenous or intranasal delivery of the small vesicles (exosomes) that contain the anti-inflammatory cytokines secreted by activated MSCs (Hanson & Frey 2008; Lee *et al.* 2012), which although with a shorter half-life would allow repeated noninvasive re-administration. Exosomes have been used in several conditions that require an anti-inflammatory and neuroprotective actions (Zhang *et al.* 2015). The choice of treatment will also depend on the stage of alcohol use disorders and the morbidities in each patient. Overall, the studies presented suggest new options for the treatment of alcohol use disorders.

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Authors Contributions

F. E., M. E. Q. and Y. I conceived the work, M. E. Q., F. E., P. M., C. L. –P., M. H. M. and M. E. conducted the studies. Y. I. and M. E. Q. drafted the manuscript. All the authors contributed to analyzing the data and approved the final version of the manuscript.

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