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Memantine Plus Vitamin D Prevents Axonal Degeneration Caused by Lysed Blood

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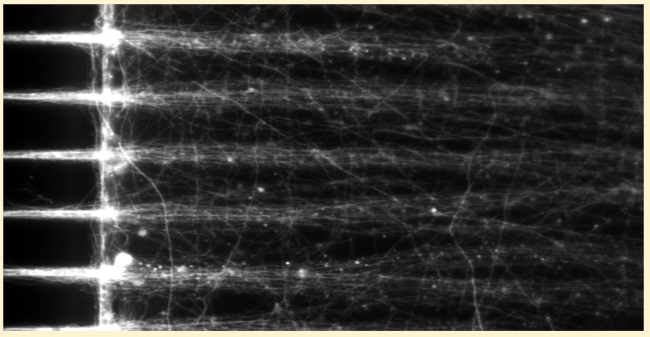
ABSTRACT: Intracranial hemorrhage, whether due to traumatic brain injury or ruptured cerebral aneurysm, is characterized by major neurological damage and a high mortality rate. Apart from cerebral vasospasm and mass effect, brain injury results from the release of unclotted blood that contacts neurons causing calcic stress. The combination of memantine with vitamin D, a neurosteroid hormone, may prevent blood neurotoxicity. Our purpose was to examine the potential protective effects of memantine + vitamin D against lysed or clotted blood in cortical neuronal cultures. We provide the first evidence that cortical axons in contact with lysed blood degenerate less after exposure to lysed blood in microfluidic neuronal cultures enriched with both memantine and vitamin D compared to control medium and cultures enriched with only memantine or only vitamin D. The reported synergistic neuroprotective effect of memantine + vitamin D, the combination originating an effect stronger than the sum, strongly encourages using both drugs following intracranial hemorrhage.



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Intracranial hemorrhage, whether due to traumatic brain injury or ruptured cerebral aneurysm, is characterized by a very high mortality rate estimated around 25−40% at day 28 and major neurological damage typically caused by cerebral vasospasm and mass effect.[1] Importantly, in addition to these ischemic and mechanical insults, brain injury also results from the release of unclotted blood that contacts neurons causing calcic stress.[2] The membrane depolarization resulting from the blockage of the Na^+/K^+-ATPase pump causes a massive release of glutamate into the synaptic cleft, which exceeds the capacity of astrocytic reuptake[3] and causes a massive influx of Ca^{2+} into neurons. This excitotoxic process leads to neuronal necrosis or delayed apoptosis.[4] Memantine, an uncompetitive antagonist of *N*-methyl-D-aspartate receptors (NMDARs), is known to restrict Ca^{2+} neuronal influx.[5] We have recently proposed that vitamin D, a neurosteroid hormone involved in neuronal physiology,[6,7] could enhance the neuroprotective action of memantine.[8] We further showed that cortical axons exposed to amyloid-β peptide and glutamate (i.e., two pathological mechanisms of Alzheimer's disease) degenerated less in cultures enriched with memantine plus vitamin D compared to cultures with only memantine or only vitamin D.[9] However, it remains unclear whether the combination of memantine and vitamin D is also neuroprotective against other neurotoxins such as blood.[2] The purpose of this study was to examine the potential protective effects of memantine plus vitamin D against lysed or clotted blood in cortical neuronal cultures.

We assessed the impact of lysed blood and substances released by blood clots on the axons of mouse cortical neurons cultured in microfluidic chips grown in control medium or medium pretreated with memantine, vitamin D_3, or memantine plus vitamin D_3 (Figure 1A, B). These microfluidic chips compartmentalize cell bodies and axons in fluidically isolated cell culture chambers, enabling the study of stress-induced axonal dying back degeneration and the determination of a fragmentation index (FI).[10,11] First, after a differentiation phase

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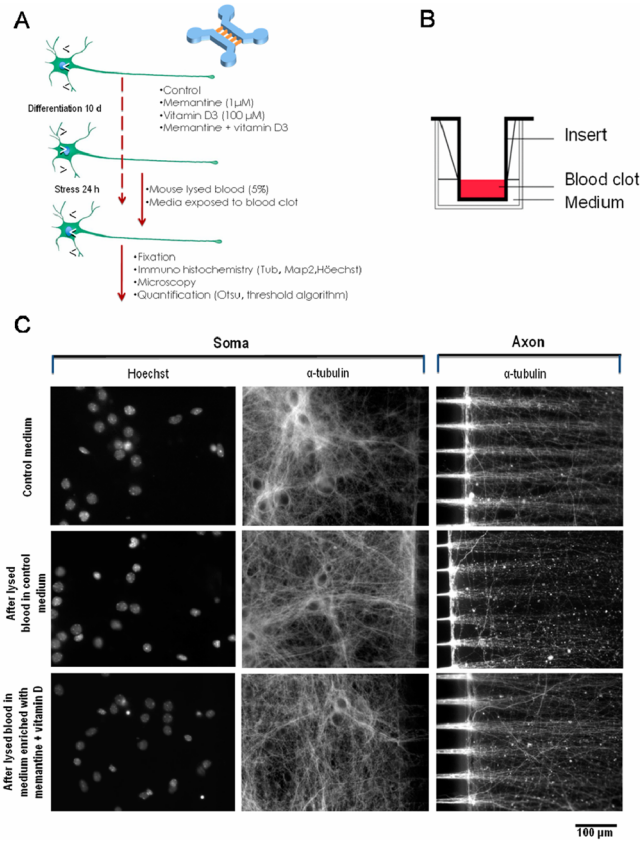

Figure 1. Methods. (A) Experimental timeline. (B) Hanging cell culture insert membrane (pore size 0.4 μm) to produce neuronal cell culture media incubated with a blood clot. A volume of 50 μL of fresh blood was added to the inset in contact with 250 μL of neuronal cell culture media in the 24-well plate. At 24 h after contact with blood clot, the cell culture media was added to the somatic compartment of the cortical cultures. (C) Representative examples of axonal degeneration in the presence or absence of somatic stress by direct application of lysed blood (5%) for 24 h (cell nuclei are stained with DAPI and axons with β3-tubulin).

for 10 days, we found that the mean FI was identical in the four media (P = 0.72) (Figure 2A). In particular, there was no difference between the medium enriched with memantine plus vitamin D (FI = 3.1 ± 0.7%; mean ± standard deviation) and the control medium (FI = 3.3 ± 0.9%, P = 0.50).

Second, we added the stressor (i.e., either mouse lysed blood, or media in contact for 24 h with blood clot) to the somatic compartment of the cortical cultures. Axonal fragmentation was quantified after 24 h (Figure 1C). The change in fragmentation index (Δ_{FI}) following stressor addition was defined as Δ_{FI} = [(FI after stressor − FI before stressor)/((FI after stressor + FI before stressor)/2)] × 100.[9] We found that, in the control medium, blood clot did not increase the FI (Figure 2A, B); however, lysed blood induced an axonal dying back pattern (Figure 1C, and pink color in Figure 2B), beginning at 12 h at the axonal tip with retrograde progression until the whole axon was fragmented, as revealed by real-time phase contrast observation (data not shown). At 24 h after lysed blood addition, the final FI was 13.4 ± 1.0% in the control medium (before-and-after P = 0.04). Conversely, in the medium enriched with memantine plus vitamin D, the FI did not change compared to the differentiation phase (final FI, 4.2 ± 1.0%; before-and-after P = 0.23) (Figures 1C and 2C). Comparing treatments at 24 h, the memantine + vitamin D treatment maintained a significantly lower FI compared to the other three groups (P = 0.04) (Figure 2A). The Δ_{FI} was 24 ± 45% with memantine + vitamin D, significantly lower than that for control (127 ± 12%, P = 0.04), memantine-enriched (81 ± 30%, P = 0.04), and vitamin D-enriched media (81 ± 26%, P = 0.04) (Figure 2D).

Our results show that the combination of memantine with vitamin D is not toxic to cortical neurons (Figure 2A), and protects axons against the damage caused by lysed blood (Figure 2). No significant protective effect against lysed blood was observed when each compound was used separately, suggesting potentiation and synergistic benefits of the combination. These findings are in concordance with prior studies that demonstrated neuroprotection provided by these two compounds in combination against other neurotoxins such as amyloid-β peptide and glutamate.[9,12]

Recent findings indicate that an intracerebral hematoma is toxic to neighboring cells.[2] The entry of circulating Ca^{2+} activates several intracellular enzymes (i.e., phospholipases, proteases, endonucleases) and neuronal nitric oxide synthase (nNOS).[3] This deleterious cascade promotes the formation of superoxide ions resulting in the genesis of free radicals involved in lipid and protein oxidation and DNA degradation. Animal data support the neuroprotective potential of NMDAR antagonists as antiexcitotoxic agents in traumatic brain injury,[13] and good clinical evidence indicates that NMDAR antagonists are well-tolerated and may improve outcome in brain-injured patients.[14] In addition, growing evidence suggests that vitamin D, a neurosteroid hormone with receptors on cortical neurons,[6] impacts brain function[15−17] and regulates intraneuronal calcium homeostasis,[18,19] oxidative stress[20,21] and inflammatory changes,[22] genetic expression of neurotrophins,[23] and vaso-constriction.[24] As illustrated here, these neuroprotective properties may be complementary and synergistic with those of memantine, with the combination providing greater protection than the sum, thereby preventing the axonal degeneration induced by lysed blood.

The present finding, together with the good tolerance profile of memantine and vitamin D, strongly encourages testing these drugs in vivo during the acute phase of intracranial hemorrhage.

METHODS

Materials and Methods. We assessed the impact of lysed blood and substances released by blood clots on the axons of mouse cortical neurons cultured in microfluidic chips grown in control medium, or pretreated with 1 μM memantine, or 100 nM vitamin D_3, or memantine plus vitamin D_3 (Figure 1).

Microfluidic Chip Production. Using photolithography, we patterned two layers of negative photoresistant SU-8 on a silicon wafer as previously described in more details.[10,11] The resulting master of the microfluidic device has a complementary positive relief pattern of the cell culture compartment and microchannels. This master was used to produce several culture chambers using polydimethylsiloxan (PDMS, Sylgard 184, Ellsworth Adhesives, Pontoise, France; Figure 1). PDMS was mixed with curing agent (9:1 ratio) and desiccated under vacuum. The resulting preparation was poured onto a resin replicate SU-8 and reticulated at 70 °C for 2 h. The elastomer polymer print was detached and reservoirs were created using a biopsy puncher. The resulting piece was cleaned with isopropanol and air-dried. The polymer print and a glass coverslip were treated for 1 min in an air plasma generator (40% of power, 300 mTor; Diener, Ebhausen, Germany) and bonded together. Chips were placed under UV for 15 min for sterilization, before being coated with a solution of poly-D-

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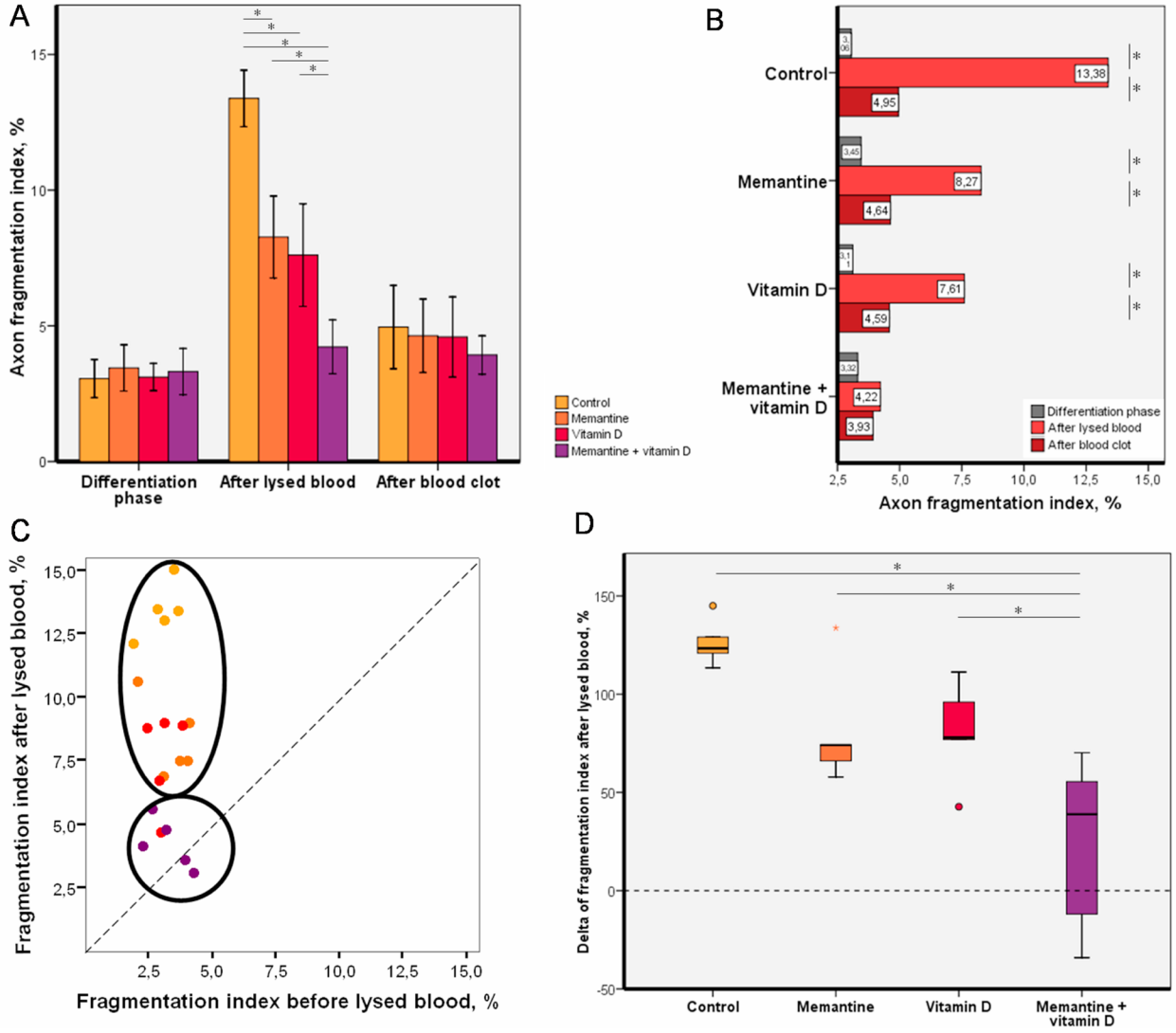

Figure 2. Axonal fragmentation index (FI). (A) Bar plots for comparison of means and standard deviations of FI following addition of blood lysate or blood clot for 24 h. (B) Bar plots for comparison of means of FI by medium cultures. (C) Changes in FI following application of lysed blood. Each data point represents the FI for an experiment before (*X*-axis value) and after (*Y*-axis value) application of lysed blood. Points lying around the line of identity and on its right (lower encircled area: mainly experiments with memantine plus vitamin D) indicate that FI was similar after stressor. Points to the left of the line (upper encircled area: mainly the three other experiments) indicate that FI was higher after stressor. (D) Box plot for Δ_{FI} after application of lysed blood. *$P < 0.05$.

lysine (10 μg/mL, Sigma-Aldrich, Saint-Quentin, France) overnight and washed with phosphate-buffered saline (PBS).

Primary Cortical Neuronal Cultures. All animals in this study were ethically maintained and used in compliance with the Policy on Ethics approved by the Society for Neuroscience. Cerebral cortices were microdissected from Swiss mouse embryos at E14 (Elevage Janvier, Le Genest-Saint-Isle, France). All steps of the dissection were completed in PBS (without $CaCl_2$ and $MgCl_2$) + 0.1% glucose (Merck, France). Dissected structures were digested with trypsin 0.05% ethylenediaminetetraacetic acid (EDTA) solution (Gibco, Life Technologies SAS, Saint-Aubin, France) for 10 min at room temperature and mechanically dissociated with a Pasteur pipet. After several PBS rinses, cells were resuspended in DMEM growth medium (Dulbecco's modified Eagle's medium, Gibco, Life Technologies SAS, Saint-Aubin, France) at a final density of 40 or 50 million cells/mL. Cell suspensions were then seeded in the somatic compartment by introducing 2 μL of the cell suspension in the upper reservoir: the cells flow into the chamber and adhere to the substrate within 1−2 min. Cell culture medium was then added in equal amounts (40 μL) in the four reservoirs. Cortical neurons were grown in DMEM glutamax (Invitrogen, Life Technologies SAS, Saint-Aubin, France) + streptomycin/penicillin (Invitrogen, Life Technologies SAS, Saint-Aubin, France) + 10% fetal bovine serum (FBS, PAA laboratories, Velizy-Villacoublay, France) + N2 (Invitrogen, Life Technologies SAS, Saint-Aubin, France) + B27 supplement without antioxidant (Invitrogen, Life Technologies SAS, Saint-Aubin, France). Microfluidic chips were placed in Petri dishes containing water in order to prevent evaporation of the culture medium and then placed in a 5% CO_2 and 37 °C incubator. Culture medium was renewed every 4 days. Ten days after neuronal differentiation, the astrocytes represented 3−4% of the cells.

Neuronal Cell Culture Treatments. The microfluidic chips compartmentalize cell bodies and axons in fluidically isolated cell culture chambers (Figure 1A), enabling the study of stress-induced axonal dying back degeneration.[10,11] Prior to the different pharmacological or disruptive treatments, cortical neuronal cells grown for 10 days in the microfluidic device were examined by phase contrast microscopy to select acceptable cultures based on three criteria. Cultures presenting a somatic cell death above 10%, or cultures with less than 80% of microchannels containing axons, or axons with blebs were discarded. Before pharmacological treatment, the cell culture medium was completely replaced. To ensure fluidic isolation, a

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hydrostatic pressure difference was generated by overpressurizing the nontreated chamber as previously described by Taylor et al.[25] Therefore, pharmalogical compounds applied in the somatic compartment were unable to diffuse into the distal compartment or vice versa. At day one after seeding of the neuroblasts, 1 μM memantine and 100 nM 1,25-dihydroxyvitamin D_3 (Santa Cruz Biotech, Clinisciences, Nanterre, France) were added (Figure 1A).

Neuronal cell culture blood stress was achieved by direct contact of the neuronal cell bodies, in the somatic compartment of the microfluidic device, with neuronal cell culture media either containing 5% lysed blood or media that had been in contact for 24 h with a blood clot. To obtain accurate results for axonal degeneration, the time window of axon fragmentation should be in a linear progression phase.[10] Pilot experiments revealed that the optimal time-window was 18−36 h for the stressors used in this experiment. For this reason, we chose 24 h as an optimal time point.

Lysed blood was obtained by one freezing/thawing cycle of mouse blood. Pilot experiments revealed that one freezing/thawing cycle was sufficient to induce a relatively constant toxicity on the neuronal cell cultures. To create a blood clot, 12 mm diameter hanging cell culture inserts (Millicell, Millipore, Billerica, MA; cat. #PIHT12R48, membrane pore size 0.4 μm) were placed into 24-wells containing neuronal cell culture media. Mouse blood (50 μL/well) was then rapidly placed on the membrane surface of each insert. The culture plates were then returned to the incubator. After 24 h, 100 μL of cell culture media that was exposed to the blood clot was transferred to the somatic compartment of the microfluific cultures. Cultures were fixed after 24 h prior to immunochemistry analysis.

Immunocytochemistry. Immunostaining was performed directly on the chips, by applying reagents to the various compartments. Neurons were fixed with 4% paraformaldehyde in PBS (Sigma-Aldrich, Saint-Quentin, France) for 20 min at room temperature. Cells were then washed twice with PBS for 5 min and permeabilized for 45 min with 0.2% triton X-100 and 1% BSA in PBS. Solutions of primary antibodies, diluted in PBS, were incubated at 4 °C overnight. Cultures were rinsed 2 times for 5 min with PBS and further incubated with the corresponding secondary antibodies for 2 h at room temperature. Cells were observed after rinsing and filling of the reservoirs with PBS. Image acquisition was performed using an Axiobserver Z1 (Zeiss, Marly Le Roi, France) microscope fitted with a cooled charge coupled device camera (CCD; CoolsnapHQ2, Roper Scientific, Evry, France). The microscope was controlled using Micro-Manager software. Neurons were immunostained with monoclonal anti-β-tubulin-III (1:300; Sigma-Aldrich, Saint-Quentin, France) and FITC-conjugated monoclonal anti-α-tubulin (mouse immunoglobulin G, 1:500; Sigma-Aldrich, Saint-Quentin, France). Cells were stained with Hoechst 33342 (4′,6-diamidino-2-phenyl indole dihydrochloride, DAPI; 1:25,000; Sigma-Aldrich, Saint-Quentin, France) to reveal nuclei.

Quantification of Axonal Degeneration. The analysis of axonal degeneration was performed after immunostaining of axonal tubulin. Intact axons present a homogeneous and linear tubulin staining delineating the axon shaft, while blebbed or severed axons exhibit a fragmented tubulin network. Around 5 to 8 cortical axons exit the microchannels to reach the axonal chamber. Cortical axonal degeneration was evaluated by automated image analysis (Figure 1C). Cortical neurons emit very long, thin, and branched bundles, making it difficult for a single axon to be reliably identified. In order to assess cortical axonal fragmentation, we used a macro developed for the NIH ImageJ software (http://rsbweb.nih.gov/ij/) based on the Otsu thresholding algorithm. We quantified a ratio of discrete tubulin staining (reminiscent of fragmented axons) over linear continuous tubulin staining (intact axons). Tubulin staining presenting as spots with a circularity of 0.9 was considered as fragmented. The total area of such regions was calculated and this value was normalized by the total axonal area, which was measured from the threshold image.[10] This ratio, termed the fragmentation index (FI), is an indicator of the average axonal fragmentation level and was used for statistical comparisons. Each point was examined in five independent experiments for lysed blood and five independent experiments for blood clot. Random locations were selected in the culture chambers, and up to five axonal and three somatic 40× images were captured per condition. The change in fragmentation index (Δ_{FI}) following stressor addition was defined as Δ_{FI} = (FI after stressor − FI before stressor)/((FI after stressor + FI before stressor)/2)] × 100.[9]

Statistical Analyses. Two-tailed nonparametric Wilcoxon signed-ranks tests were used throughout this study to compare groups, and to compare the FI before and after stressor addition in the same group. When more than two groups were compared, the two-tailed nonparametric Friedman test was used. P-values < 0.05 were considered significant. All statistics were performed using SPSS (v19.0, IBM Corporation, Chicago, IL).

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Author Contributions

C.A. has full access to all of the data in the study, takes responsibility for the data, the analyses and interpretation, and the conduct of the research, and has the right to publish any and all data, separate and apart from the attitudes of the sponsor. Study concept and design: D.C., C.A., and O.B. Acquisition and analysis of data: B.B. and M.B. Interpretation of data: C.A. and O.B. Drafting of the manuscript: C.A. Critical revision of the manuscript for important intellectual content: O.B., D.C., R.B., and B.B. Obtained funding: O.B. Administrative, technical, or material support: O.B. and B.B. Study supervision: C.A. and O.B. All of the authors reviewed the manuscript prior to submission.

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Notes

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The authors declare no competing financial interest.

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