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Mannitol treatment is not effective in therapy of rabies virus infection in mice

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ABSTRACT

Rabies is a deadly viral disease with an extremely high fatality rate in humans. Previously, it was suggested that an enhancement of the blood–brain barrier (BBB) permeability, which allows immune cells and/or antibodies to enter the central nervous system (CNS) tissue, is critical to clear the infection. In this study, we utilised mannitol to increase BBB permeability in mice infected with highly pathogenic silver-haired bat rabies virus (SHBRV). We found that intraperitoneal injection of mannitol causes a slight, transient increase of BBB permeability in the treated mice. SHBRV-infected mice were treated with intraperitoneally administered mannitol daily from day 3 or day 4 post-infection, but no effect of this treatment on the time of disease onset, clinical signs or survival was observed. This data indicates that the increase of BBB permeability by mannitol is not efficient in promoting CNS virus clearance in SHBRV-infected mice.

1. Introduction

Rabies virus (RABV), a member of the *Lyssavirus* genus, family *Rhabdoviridae*, is highly neurotropic and the disease caused by the virus is restricted to the central nervous system (CNS). In humans, RABV causes more than 70,000 deaths every year [4]. The most common mode of RABV transmission is through passing virus-containing saliva from an infected to uninfected host, mainly during a bite by a rabid animal. At the wound site, the virus enters neurons in the periphery and travels to the CNS. Once the virus reaches the CNS, the infection is lethal in virtually all cases [4]. However, a small number of survivals despite RABV CNS infection have been reported [17]. It was found that the survival was linked to infection with bat-originated RABV with presumably lower pathogenicity for humans in comparison to dog-originated RABV, and enhanced blood–brain barrier (BBB) permeability. The increased BBB permeability probably enabled immune cells and/or antibodies to enter the CNS and clear the infection [16,17,8]. Using rodent models, it was shown that infection with highly pathogenic silver-haired bat-associated rabies virus (SHBRV) leads to a lethal outcome, but without enhancement of BBB permeability [12]. In comparison, mouse infection with attenuated RABV strain CVS-F3 led to enhanced BBB permeability, migration of immune cells into the CNS and efficient virus clearance from the brain [12]. Moreover, induction of more extensive BBB permeability and CNS inflammation resulted in greater virus clearance and survival in SHBRV-infected mice [13]. Another study demonstrated that enhancement of BBB permeability enabled entry of exogenously administered RABV-neutralizing antibodies into the CNS and this prevented development of lethal infection in both immunocompetent and immunocompromised mice [7]. Based on these results, it was suggested that a pharmaceutically-induced

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increase of BBB permeability could represent a promising strategy for treatment of RABV infection [16].

The most commonly used method of permeabilizing the BBB is mannitol mediated osmotic disruption [3]. Mannitol is a hyperosmolar agent widely used for the control of intracranial pressure [1,5]. It induces osmolarity-driven fluid movement from the cerebral tissue into the intravascular space, causing shrinkage of the endothelial cells forming the BBB which leads to a transient increase of the BBB permeability [5]. Louboutin et al. [11] reported that intraperitoneal injection of 3 ml of sterile 25% mannitol in 0.9% saline per 100 g body weight caused a temporal BBB relaxation and increased delivery of intravenously administered transgenes into the CNS in mice. In this context, we hypothesized that an increase in BBB permeability mediated by mannitol will be beneficial for treatment of RABV-infection in mice.

2. Material and methods

2.1. Animals

Female C57/Bl6 mice were purchased from Harlan (vendor AnLab, Prague) at 6 weeks of age and used at 7–8 weeks of age. Mice were randomly assigned to the experimental groups. All procedures were carried out in strict accordance with the Czech national law and guidelines on the use of experimental animals and protection of animals against cruelty (the Animal Welfare Act Number 246/1992 Coll), and were approved by the Institutional Expert Committee and the Ministry of Agriculture of the Czech Republic, permit No. MZe 1627. The mice were kept in plastic cages at constant temperature and humidity in the ventilated cabinet in the BSL-3 animal facility. Sterilized granular diet and water were available *ad libitum*.

2.2. Virus infection

Mice were infected with the Silver-haired bat rabies virus (SHBRV-18) provided by the Jefferson University and grown at Erasmus MC on N2a cells (P2 virus). The titer of the virus was determined on BHK-21-C13 cells and calculated to be $10^{6.8}$ TCID50/ml. Mice were infected with 50 µl SHBRV-18 ($10^{6.8}$ TCID50/ml) by intramuscular route in the left hind leg. Mice were monitored two times daily for the development of clinical signs according to Healy et al. [6] (clinical score 0 = no signs; clinical score 1 = twitching in inoculated limb/ruffled fur/hunched back/-paralysis in inoculated leg; clinical score 3 = hind quarter paralysis/severe spasms; clinical score 4 = progressive paralysis; clinical score 5 = prostration, permanent recumbency/death). The cut-off for a humane endpoint for this study was established as clinical score 3. All mice exhibiting disease consistent with clinical score 3 were terminated humanely (cervical dislocation) immediately upon detection.

2.3. BBB permeability assay

The BBB permeability was assayed using sodium fluorescein as described previously [14]. Sodium fluorescein (Sigma-Aldrich), a low molecular mass molecule (376 Da), was used to detect fluid shifts between the circulation and CNS, which occur when BBB permeability becomes enhanced [12]. Mice were intraperitoneally injected with 10 mg of sodium fluorescein in 0.1 ml sterile saline. Forty-five minutes later, animals were anesthetised with Keta mine/xylazine/acepromazine (ketamine 60–100 mg/kg, xylazine 10–15 mg/kg, acepromazine 2–5 mg/kg), and cardiac blood was collected, followed by transcardial perfusion with phosphate-buffered saline (PBS) to remove blood from intravascular compartment. After the perfusion, mice were humanely euthanized and brains were removed, individually weighed, and stored at -80 °C until processing. Homogenization of brain tissues was performed in 1 ml of sterile PBS using TissueLyser II (Qiagen). The homogenate was clarified by centrifugation at 14,000*g* for 10 min, at 4 °C. Protein was precipitated with trichloroacetic acid (TCA) to remove potential background fluorescence, as described previously [14]. The amount of fluorescein in each sample was determined using standards ranging from 125 to 3000 µg on an Infinite M200 fluorometer (Tecan) using an excitation wavelength of 480 nm, and fluorescence was read at 538 nm. The uptake ratio was expressed as the ratio of the amount of sodium fluorescein measured in the brain to the amount measured in serum.

2.4. Mannitol treatment

Twenty-five % solution of mannitol (Sigma-Aldrich) in 0.9% saline was prepared. To identify a dose of mannitol that increases BBB permeability most effectively, groups of mice ($n = 3$ per group) were injected intraperitoneally with mannitol at a range of doses (from 200 to 600 µl) 15 min after intraperitoneal injection of sodium fluorescein. Controls ($n = 3$) were injected intraperitoneally with the same volume of 0.9% saline. Thirty minutes after the mannitol treatment mice were processed as described above and the BBB permeability was assayed.

Repeated administration of mannitol was also tested. Groups of mice ($n = 3$ per group) were treated with (i) a single dose of 500 µl of mannitol, (ii) with 500 µl of mannitol every second day for one week, and (iii) with 500 µl of mannitol on a daily basis for one week. Control mice ($n = 3$) were treated with 500 µl of 0.9% saline on a daily basis for one week. At the last day, all mice were processed as described above and the BBB permeability was assayed.

Effect of mannitol on the development of RABV infection in mice was tested in following experimental groups ($n = 13$ per group): (i) SHBRV-infected mice and untreated, (ii) mice uninfected but treated with mannitol once, two times or three times a day, (iii) SHBRV-infected mice and treated with mannitol once, two times or three times a day. Mannitol groups were treated with 500 µl of 25% mannitol from day 3 or day 4 post-infection, respectively, on a daily basis for 10 days. Control mice were treated with 500 µl of 0.9% saline at the same times as mice treated with mannitol.

2.5. Real-time quantitative RT-PCR

Levels of different gene-specific mRNAs were measured by quantitative real-time PCR (qRT-PCR). Total RNA was isolated from the brain tissue pellets using the RNeasy Mini Kit (Qiagen), according to the recommendations of the manufacturer. cDNA was synthesized using a High Capacity RNA-to-cDNA Kit (Applied Biosystems), according to the manufacturer's protocol. The synthesized cDNAs were used as templates for real-time PCR. QRT-PCR was performed using pre-developed TaqManH Assay Reagents (assay IDs: Mm00443258_m1, TNF-a; Mm00446190_m1, IL-6; Mm01302428_m1, CCL5/RANTES; Mm00442754_m1, CD4; Mm00438116_m1, CD8b1; Mm00515420_m1, CD19; Mm00445235_m1, IP10/CXCL10; Nm_007393.1, mouse beta actin; Applied Biosystems) and TaqManH Gene Expression Master Mix (Applied Biosystems) on a Rotor Gene-3000 (Corbett Research). Mouse beta actin was used as a housekeeping gene. Amplification conditions were: 2 min at 50 °C; 10 min at 95 °C; 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Quantification of gene expression was performed using the comparative CT method and reported as the fold difference relative to the housekeeping gene. To calculate the fold change in gene expression, the CT of the housekeeping gene was subtracted

from the CT of the target gene to yield the ΔCT. Change in expression of the normalized target gene was expressed as $2^{-\Delta\Delta CT}$ where ΔΔCT = ΔCT samples – ΔCT controls as previously described [10].

Changes in gene expression of the cytokines/chemokines in the brain after mannitol treatment were examined. Groups of mice (n = 5 per group) (i) uninfected and untreated controls, (ii) mice infected with RABV and untreated, (iii) mice uninfected but treated with mannitol, (iv) mice infected with RABV and treated with mannitol were sacrificed at the onset of first RABV clinical signs (day 5 post infection). Mannitol groups were treated with 500 μl of 25% mannitol from day 3 post infection on daily basis until first clinical signs appeared. Control mice were treated with 500 μl of 0.9% saline from day 3 post infection on daily basis until first clinical signs appeared. Brains were removed, individually weighed, and stored at −80 °C until processing. Levels of different gene-specific mRNAs were measured by qRT-PCR as described above.

2.6. Statistics

The data were analyzed using Student's t-test (GraphPad Software, Inc., USA, version 5.04). Effect of number of mannitol administration was analyzed using ANOVA Dunnett's multiple comparison test. Survival curves were compared using Kaplan-Meier method and differences in survival rates were assessed with long-rank (Mantel-Cox) test. Differences with $p < .05$ were considered significant.

3. Results and discussion

In this study, we examined the effect of increased BBB permeability induced by mannitol on the development of lethal rabies infection in mice. To determine the optimal conditions for BBB disruption by intraperitoneal administration of mannitol, the leakage of the sodium fluorescein from circulation into the CNS tissues was measured in brains of the treated mice. Mannitol was injected intraperitoneally at a range of doses (from 200 to 600 μl). Mice injected with 300, 500, and 600 μl of mannitol solution exhibited slight, but statistically significant increase of sodium fluorescein concentration in brain tissue (Fig. 1A). No clinical signs of potential toxic effect of mannitol treatment (e.g. ruffled fur, apathy, prostration, permanent recumbency, problems with movement, the presence of fluid in the peritoneum, reluctance to eat) were seen in the treated mice. Intraperitoneal administration of 500 μl of 25% mannitol in 0.9% saline was found to represent an effective dose for the enhancement of BBB permeability and has no visible effects in mice. This confirmed a previous report by Louboutin et al. [11] demonstrating that intraperitoneal injection of mannitol causes a transient BBB relaxation. The effect of repeated administration of mannitol on the intensity of BBB permeability was also investigated in groups of mice that were treated with a single dose of 500 μl of mannitol, with 500 μl of mannitol every second day for one week, and with 500 μl of mannitol daily for one week. The results revealed that repeated treatment with mannitol has no effect on the intensity of BBB permeability, and the fluorescence signal in the brains in all treated groups was significantly higher in comparison to controls (Fig. 1B). No clinical signs of potential toxic effect of mannitol treatment were seen in the mice To investigate if transiently increased BBB permeability has any therapeutic effect, mice were infected with SHBRV-18 by the intramuscular route and treated with 500 μl of mannitol by intraperitoneal route. Groups of mice were treated with mannitol once a day, two-times and three-times per day from 3rd and 4th day post infection for 10 days. One dose of mannitol per day had no effect on disease onset, clinical score and survival of RABV-infected mice (Fig. 2). Administration of mannitol two- or three-times per day was found to be toxic and caused severe side effects if the treatment with two- or three doses per day was conducted for 2–3 days. Toxic effects of the repeated treatment included apathy, prostration, permanent recumbency, problems with movement, the presence of fluid in

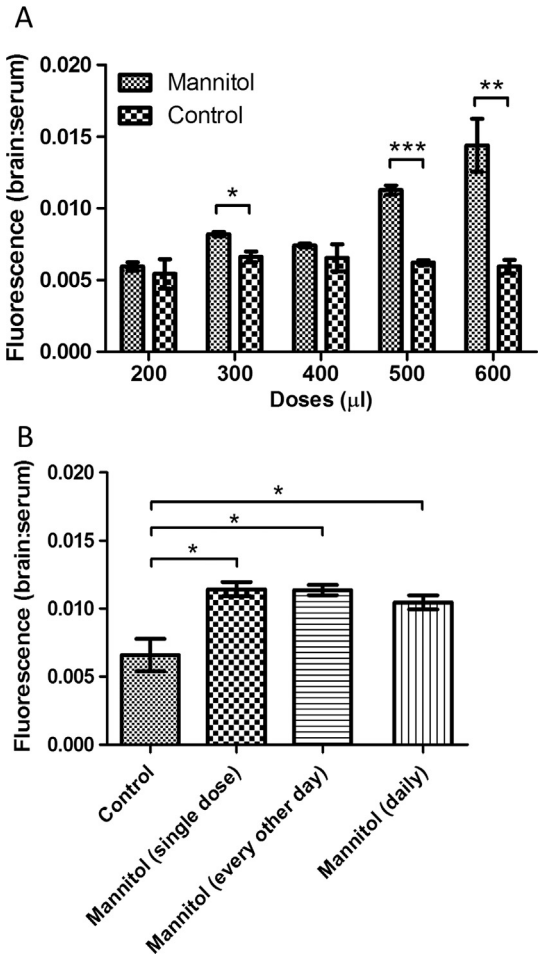

Fig. 1. BBB permeability changes in the brains of mice treated intraperitoneally with mannitol. (A) Forty-five minutes after intraperitoneal injection of sodium fluorescein and thirty minutes after intraperitoneal injection of different doses of mannitol, the sera and brains were processed as described in the Material and Methods. BBB permeability is expressed as brain:serum ratio (%). (B) Groups of mice (n = 3 per group) were treated with a single dose of 500 μl of mannitol, with 500 μl of mannitol every second day for one week, or with 500 μl of mannitol daily for one week. Control mice (n = 3) were treated with 500 μl of vehicle daily for one week. At the last day, forty-five minutes after intraperitoneal injection of sodium fluorescein, the sera and brains were processed as described in the Material and Methods. BBB permeability is expressed as brain:serum ratio (%). $^{*}p < .05$; $^{**}p < .01$, $^{***}p < .001$.

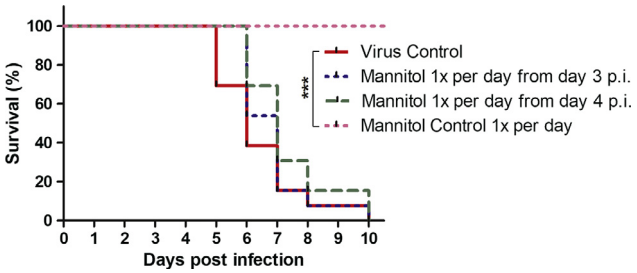

Fig. 2. Survival of RABV-infected mice treated with mannitol. C57Bl/6 mice (n = 13 per group) were infected with SHBRV-18 by intramuscular route in the left hind leg. The mice were treated with 500 μl of 25% mannitol in 0.9% saline or with 0.9% saline. The therapy was initiated on day 3 or 4 p.i. and was applied once a day. All mice exhibiting disease consistent with clinical score 3 were terminated. $^{***}p < .001$.

the peritoneum, reluctance to eat or sudden death of the treated mice. After the observation of these side effects, the experiment was immediately terminated (data not shown). These data indicate that a temporary opening of the BBB once a day is well tolerated, but if the BBB opening occurs more than once a day and this is repeated for a couple of days, it might have harmful consequences.

Brains from mice infected with SHBRV-18 were collected on day of the first clinical signs (day 5 p.i.), and homogenates were subsequently assayed for the expression of cytokine/chemokine and immune cell surface markers mRNA expression by real-time PCR. As shown in Fig. 3, mannitol treatment had no effect on proinflammatory cytokine/chemokine mRNA expression in the brain, and no significant changes in the mRNA expression of immune cells surface markers in the brains were observed at the time of the appearance of the first signs of neuroinfection. RABV infection induced no or little upregulation of cytokine/chemokine mRNA expression at the time of first neurological signs of the infection, which correlates with previous studies showing that SHBRV infection stimulates only limited innate immune response in the brain in comparison to a response triggered by laboratory strains [15,18,19]. In general, virulence of RABV infection relies on escape of host immune response including reduction of an innate immune response [9]. Mannitol treatment significantly reduced the mRNA levels of TNF-α ($p < .05$) and CCL-5 ($p < .01$) in brains of RABV-infected mice. From all immune cells surface markers mRNA, only CD19, a marker of B cells, was slightly upregulated in SHBRV-infected mice at the time of the first neurological signs, but there were no significant differences if compared to mRNA levels in brains of SHBRV-infected mice treated with mannitol or mannitol-treated uninfected controls ($p > .05$).

The results indicate that BBB breakdown induced by a daily intraperitoneal injection of mannitol is not strong enough to be effective in treating RABV-infection in mice. This may be explained by a short duration of BBB relaxation after mannitol administration, local variations of BBB disruption, and/or insufficient level of BBB permeation for the entry of immune cells and antibodies into the CNS [2]. Indeed, the studies by Phares et al. [12], and Roy and Hooper [13] indicate much stronger BBB opening after the infection with low-virulent CVS-F3 RABV strain leading to virus clearance from the CNS, than what we see after mannitol treatment in our study. One may speculate that administration of mannitol intravenously could induce more extensive BBB relaxation, and this will be a subject of our next study. Also, testing of other molecules than mannitol is needed to identify possible methods to effectively and safely induce BBB relaxation and facilitate RABV clearance from the brain.

Another possibility for the ineffectiveness of mannitol is that the BBB permeability may be not a sole factor affecting viral clearance in the CNS. Given that SHBRV infection does not efficiently induce expression of inflammatory cytokines/chemokines in the CNS [18], it is highly possible that, in addition to the increased BBB permeability, the cytokine/chemokine production is important for recruitment of effector cells into the CNS and thereby for viral clearance. The role of cytokines/chemokines in the BBB opening and virus clearance from the CNS will be addressed in our future studies.

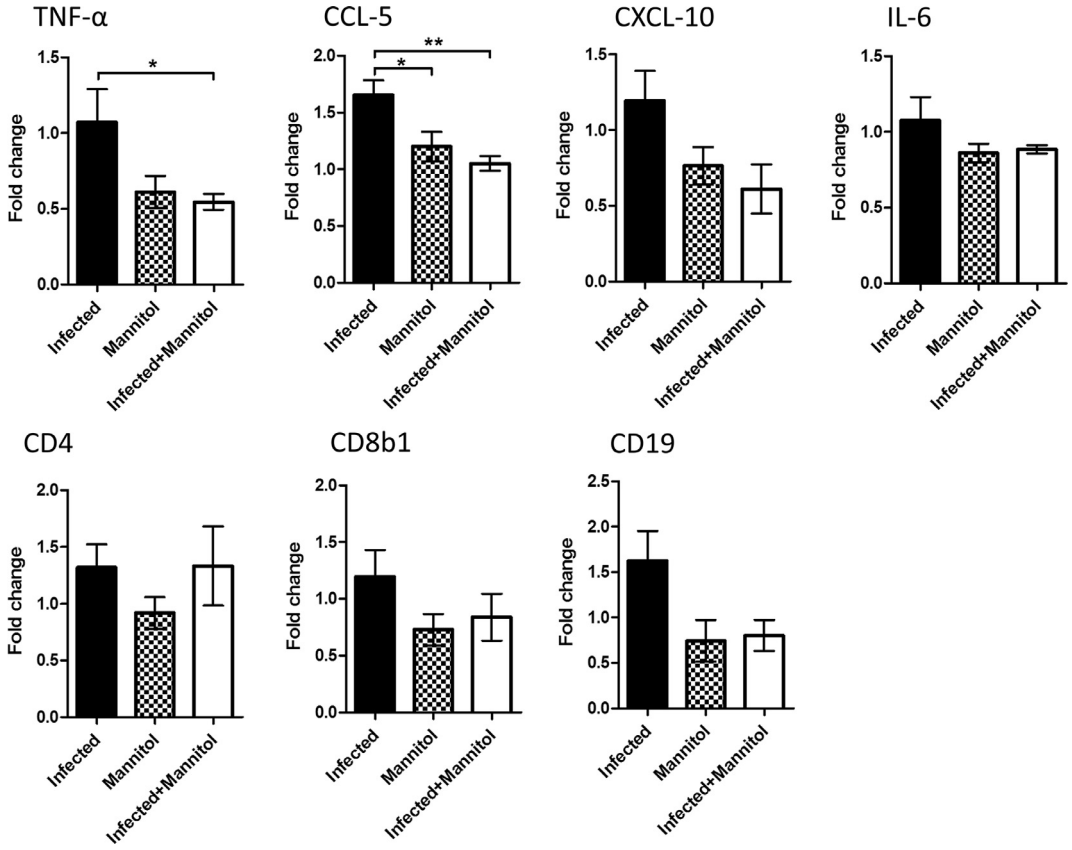

Fig. 3. Changes in mRNA expression of proinflammatory cytokine, chemokine and cell surface markers of immune cells in brains of RABV infected mice treated with mannitol. Mice ($n = 5$per group) were infected with SHBRV-18 by intramuscular route in the left hind leg. The mice were treated intraperitoneally with 500 μl of 25% mannitol in 0.9% saline. Infected control mice were treated intraperitoneally with 500 μl of 0.9% saline. Uninfected control mice ($n = 5$) were treated intraperitoneally with 500 μl of 25% mannitol in 0.9% saline. The therapy was initiated on day 3 p.i. and was applied daily until day 5 p.i. (first appearance of clinical signs). mRNA levels of the cytokines/chemokines TNF-α, CCL-5, CXCL-10, IL-6 and cell surface markers of $CD4^+$, $CD8^+$ T-cells, and B-cells in brains were assayed by real-time PCR as described in Material and Methods. Data are expressed as the mean + SEM of the fold increase of expression of the mRNA levels in brains of uninfected and untreated control mice ($n = 5$) after normalization to the housekeeping gene (mouse beta actin). $^*p < .05$; $^{**}p < .01$.

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