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Thermomineral water promotes axonal sprouting but does not reduce glial scar formation in a mouse model of spinal cord injury

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Abstract

Thermomineral water from the Atomic Spa Gornja Trepča has been used for a century in the treatment of neurologic disease. The thermomineral water contains microelements, including lithium and magnesium, which show neural regeneration-promoting effects after central nervous system injury. In this study, we investigated the effects of oral intake of thermomineral water from the Atomic Spa Gornja Trepča on nerve regeneration in a 3-month-old mouse model of spinal cord injury. The mice receiving oral intake of thermomineral water showed better locomotor recovery than those without administration of thermomineral water at 8 and 12 weeks after lower thoracic spinal cord compression. At 12 weeks after injury, sprouting of catecholaminergic axons was better in mice that drank thermomineral water than in those without administration of thermomineral water, but there was no difference in glial reaction to injury between mice with and without administration of thermomineral water. These findings suggest that thermomineral water can promote the nerve regeneration but cannot reduce glial scar formation in a mouse model of spinal cord injury.

Key Words: *astrocyte scar; catecholaminergic innervations; lithium; magnesium; thermomineral water; locomotor recovery; microglia; neuroprotection; magnesium; spinal cord injury; nerve regeneration*

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Introduction

For a long time period, severe spinal cord injury with paralysis has been considered an irreversible condition, because neurons in the central nervous system (CNS), unlike peripheral nerves, do not regrow their once injured axons (Steuer and Guertin, 2009). Additionally, glial scar which forms after spinal cord injury by the reactive astrocytes to prevent further inflammation and neurodegeneration (Bush et al., 1999; Faulkner et al., 2004) is an active barrier which hinders axonal regeneration by containing numerous growth-inhibiting molecules (Silver and Miller, 2004). The range of available treatments for spinal cord injury is very limited, and the provision of medical care for individuals with injury burdens society with high expenses. Present research is focused on advanced interventions that provide hope for regeneration and functional restoration, as well as chronic treatments which could promote partial functional rehabilitation. Therefore, more research efforts focusing on combinatorial approach are needed to improve the clinical outcome after injury (Xu et al., 2011; Lee et al., 2012).

Because thermomineral water from the Atomic Spa Gornja Trepča has been used for a century in patients with autoimmune disorders such as multiple sclerosis and rheumatoid arthritis, as well as after acute CNS trauma, we hypothesized that it might have regeneration-promoting effects after spinal cord injury. The thermomineral water of the Atomic Spa is volcanic water, mixed with infiltration water on its way through the ground. The hot curative waters of Gornja Trepča had been known to local population since the ancient times, while it enjoyed the reputation of a monastery spa during the Middle Ages. These waters contain Mg and Ca as main cations, and hidrocarbonate and Cl as main anions. The water also contains microelements, including Li, Cs, Se, known for their neuroprotective properties (Bräuer and Savaskan, 2004; Su et al., 2007; Chiu and Chuang, 2011; Godoi et al., 2013). The temperature of the water is 27–31°C and pH is 7.4–7.5, so the water is considered to be neutral with alkaline tendency. It has low radioactivity with the emission of 29.6 Bq/L. The water is low mineralized because of the low concentration of anions like HCO_3^- (385 mg/mL) and cations like Mg^{2+} (61 mg/mL), and is free from H_2S and

Fe. The dominating microelement in the water is Li with the concentration of 0.0032 mg/mL. Recent studies have reported that Li is beneficial in animal models of brain injury (Yu et al., 2012), stroke (Chiu and Chuang, 2010, 2011), Alzheimer's disease (Chuang, 2004), amyotrophic lateral sclerosis (Dill et al., 2008), spinal cord injury (Su et al., 2007), and that it stimulates neurogenesis both *in vitro* and *in vivo* (Hashimoto et al., 2003). Lithium also protects neurons from glutamate-induced excitotoxicity and so prevents apoptosis (Chalecka-Franaszek et al., 1999; Young, 2009). Additionally, magnesium, a dominant macroelement in the water, has already been used in clinical trials in patients with spinal cord injury as an adjuvant treatment (Kwon et al., 2010).

The aim of this study was to investigate the effects of the oral intake of the thermomineral water from the Atomic Spa Gornja Trepča on the injured spinal cord of the C57BL/6J wild-type mice. We studied how oral intake of this mineral water influences the outcome of spinal cord injury using motor behavior testing and morphometric approach.

Materials and Methods

Animals

Twenty-four female 3-month-old C57BL/6J mice, weighing 21.4 ± 1.2 g, were obtained from the breeding colony at the animal facility of the Institute for Biological Research "Siniša Stanković", Belgrade. All experiments were conducted in accordance with the European Community laws on protection of experimental animals, and the procedures used were approved by the responsible committee of the Serbian Ministry of Agriculture. All animal treatments and data acquisition were performed by observers blinded to treatment groups.

Surgical procedures

All surgical procedures were performed with special attention to the sterile technique inside of the laminar flow cabinet. For surgery, the mice were anesthetized by intraperitoneal injections of ketamine (100 mg/kg body weight; Ketanest®, Parke-Davis/Pfizer, Karlsruhe, Germany) and xylazine (5 mg/kg body weight; Rompun®, Bayer, Leverkusen, Germany). Laminectomy was performed at the $T_{7–9}$ level, corresponding approximately to spinal cord segments $T_{10–12}$, with mouse laminectomy forceps (Fine Science Tools, Heidelberg, Germany), dorsal and lateral vertebral laminae were carefully removed. A mouse spinal cord compression device was used to elicit compression injury at the level of spinal cord segments $T_{10–11}$ (Curtis et al., 1993). The device is composed of a pair of watchmaker forceps mounted in a metal block attached to a stereotaxic frame. Compression force (degree of closure of the forceps) and duration are controlled by an electromagnetic device. The spinal cord was maximally compressed (100%) according to the operational definition of Curtis et al. (1993) for 1 second by a time-controlled current flow (12 V – maximum voltage) through a custom-made electromagnetic device. The skin was then surgically closed using 6-0 nylon stitches (Ethicon, Norderstedt, Germany). After surgery, mice were kept in a heated room (35°C) for several hours to prevent hypothermia and thereafter singly housed in a temperature-controlled (22°C) room with water and standard food provided *ad libitum*. During the postoperative time period, the bladders of the animals were manually voided twice daily. Immediately after surgery, the mice were randomly divided into two groups: an experimental group mice in which were given thermomineral water (Atomic Spa, Gornja Trepča, Serbia) and a control group mice in which were allowed to drink standard water *ad libitum*. The volume of water drank by each mouse was approximately 5 mL daily, in both experimental and control groups, therefore showing that mice had no preference for, or repulsion from thermomineral water. During the immediate post-operative phase and in the first week after injury, four mice from each group died due to severity of injury and post-surgical complications, leaving eight mice per group for the analysis.

Analysis of motor function

The recovery of ground locomotion was evaluated using the Basso Mouse Scale (BMS) score (Basso et al., 2006), which is a modification of Basso, Beattie, Bresnahan (BBB) locomotor rating scale, originally developed for rats (Basso et al., 1995). This method includes evaluation of mice with spinal cord injury observed in the open field for 4 minutes pre-operatively and at least weekly for 84 days post-operatively. The scale estimates movements of the ankle joint, ability of plantar stepping and walking coordination, by giving score values 0–9 (Basso et al., 2006). Maximal value of the score 9 is given for normal walking with frequent or consistent plantar stepping, normal trunk stability and tail always up, whereas a minimal score of 0 is given for the complete absence of ankle movement (Basso et al., 2006). This approach allows identifying all visually discernable attributes of locomotor recovery for both mildly and severely injured mice. Assessment of all parameters was performed before and at 3 days, and 1, 2, 4, 8 and 12 weeks after the injury. Values for the left and right extremities were averaged.

Tissue fixation and sectioning

At 12 weeks after spinal cord injury, mice were anesthetized with 16% solution of sodium pentobarbital (Narcoren, Merial, Hallbergmoos, Germany, 5 µL/g body weight). The animals were transcardially perfused with fixative consisting of 4% formaldehyde and 0.1% $CaCl_2$ in 0.1 mol/L cacodylate buffer, pH 7.3, for 15 minutes at room temperature. Following perfusion, the spinal cords were left *in situ* for 2 hours at room temperature, after which they were dissected out and post-fixed overnight (18–22 hours) at 4°C in the same solution used for perfusion. Tissue was then immersed into 15% sucrose solution in 0.1 mol/L cacodylate buffer, pH 7.3, for 2 days at 4°C, embedded in Tissue Tek (Sakura Finetek, Zoeterwoude, NL, USA), and frozen by 2-minute immersion into 2-methyl-butane (isopentane) precooled to –80°C. Serial transverse or longitudinal sections were cut using a cryostat (Leica CM3050, Leica Instruments, Nußloch, Germany). Sections, 25-µm-thick, were collected on SuperFrost Plus glass slides (Roth, Karlsruhe, Germany). Sampling of

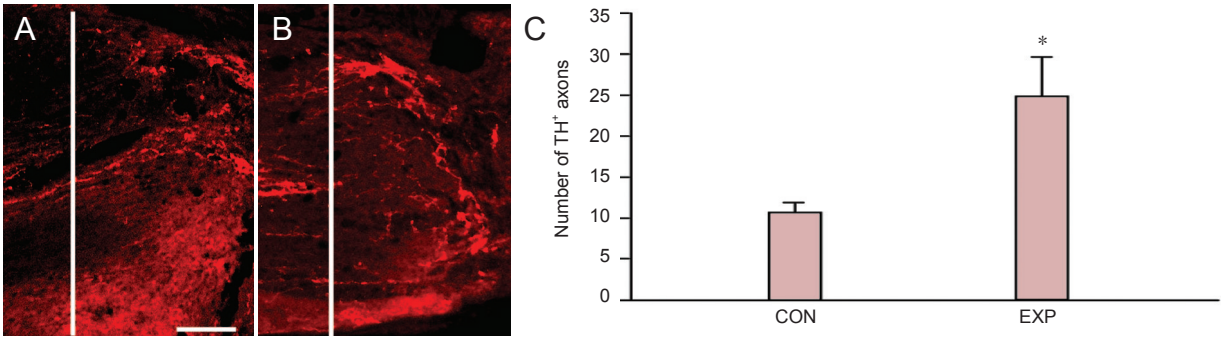


Figure 2 Sprouting of tyrosine hydroxylase (TH)-expressing axons caudal to the lesion site 12 weeks after injury.
(A, B) Representative photomicrographs of immunofluorescent staning for TH (red) in the spinal cord of mice from the control (A) and experimental (B) groups. Scale bar: 100 μm. (C) Number of TH^+ axons in the spinal cord crossing the line drawn 250 μm caudal to the lesion site (white lines in A, B) in mice from the experimental (EXP) and control (CON) groups (n = 8 mice/group). Values are shown as the mean ± SEM. Asterisk indicates significant difference between the groups (P = 0.026; two-tailed t-test).

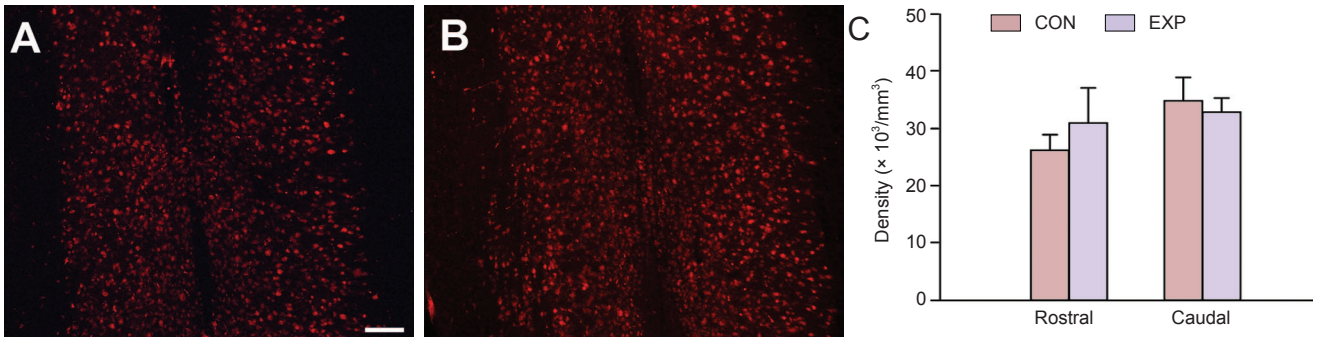


Figure 3 $NeuN^+$ neurons rostral and caudal to the lesion site at 12 weeks after spinal cord injury.
(A, B) Representative photomicrographs of immunofluorescent staning for NeuN (red) in the lumbar spinal cord of mice from the control (A) and experimental (B) groups. Scale bar: 100 μm. (C) Density of $NeuN^+$ cells in the spinal cord rostral and caudal from the injury site in mice from the experimental (EXP) and control (CON) groups (n = 8 mice/group). Values are shown as the mean ± SEM. There were no significant differences between the experimental and control groups (P = 0.50 and 0.62 for rostral and caudal, respectively; two-tailed t-test). NeuN: Neuronal nuclear antigen.

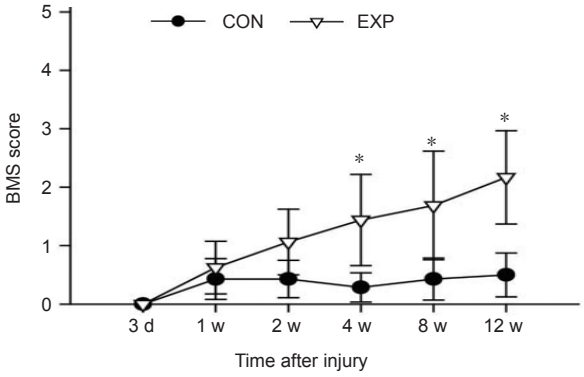


Figure 1 Time course of functional recovery after compressive spinal cord injury in mice from the experimental (EXP) and control (CON) groups.
The values are shown as the mean ± SEM (n = 8 mice/group) of the open field locomotion score (Basso Mouse Scale (BMS)) at 3 days (d) and at 1, 2, 4, 8 and 12 weeks (w) after injury. Asterisks indicate significant differences between the groups (P < 0.05; two-way analysis of variance followed by Tukey's *post-hoc* test).

sections was always done in a standard sequence so that six sections 250 μm apart were present on each slide.

Immunohistochemistry

Procedures for immunohistochemistry have been described previously (Jakovcevski et al., 2007). Water-bath antigen de-masking was performed in 0.01 mol/L sodium citrate solution, pH 9.0, for 30 minutes at 80°C for all antigens. Non-specific binding was blocked using 5% normal goat serum dissolved in PBS, pH 7.3 and supplemented with 0.2% Triton X-100, 0.02% sodium azide for 1 hour at room temperature. Incubation with the primary antibody, diluted in PBS containing 0.5% lambda-carrageenan (Sigma/Aldrich) and 0.02% sodium azide, was carried out for 3 days at 4°C. The following primary antibodies were used: for detection of cathecholaminergic axons, rabbit anti-tyrosine hydroxylase (Chemicon, Temecula, CA, USA; 1:800), for detection of neurons, mouse monoclonal anti-neuron-specific nuclear antigen (NeuN; Chemicon; 1:1,000), for detection of astrocytes and glial scar, mouse anti-glial fibrillary acidic protein (GFAP; Sigma-Aldrich, St Louis, MO, USA; 1:1,000), and rabbit anti-ionized calcium binding adaptor-1, for detection of microglia/macrophages (Iba-1; Sigma/Aldrich; 1:1,500). After washing in PBS (3 × 15 minutes at room temperature), goat anti-mouse or anti-rabbit Cy-3 conjugated secondary antibody (Dianova, Hamburg, Germany) diluted 1:200 in PBS-carrageenan solution was applied for 2 hours at room temperature. After a subsequent wash in PBS, cell nuclei were stained for 10 minutes at room temperature with

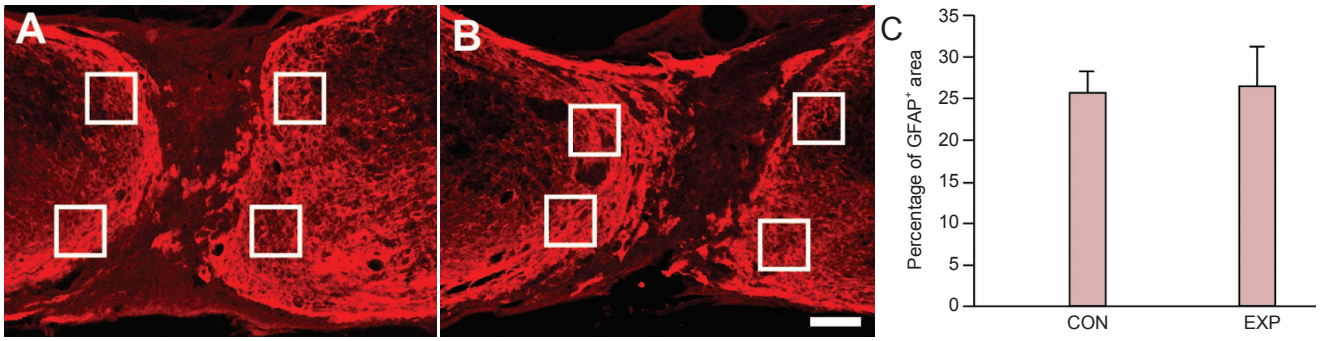
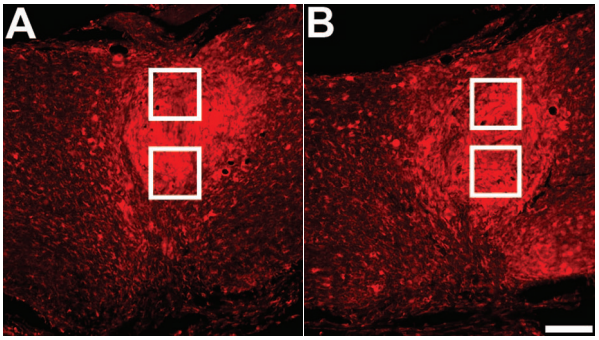


Figure 4 Glial fibrillary acidic protein (GFAP) expression around the lesion site at 12 weeks after spinal cord injury.
(A, B) Representative photomicrographs of the GFAP-immunostained (red) injured spinal cords of mice from the control (A) and experimental (B) groups. White squares depict areas in which the measurements were performed. Scale bar: 200 μm. (C) Percentage of GFAP-immunoreactive area normalized to the total image area (shown as the mean ± SEM). There was no significant difference between the experimental and control groups (P = 0.77, two-tailed t-test; n = 8 mice/group). CON: Control group; EXP: experimental group



(A, B) Representative photomicrographs of the Iba-1-immunostained (red) lesion sites of spinal cords of mice from the control (A) and experimental (B) groups. White squares depict areas in which the measurements were performed. (C) Percentage of Iba-1-immunoreactive area normalized to the total image area (shown as the mean ± SEM). There was no significant difference between the experimental (EXP) and control (CON)groups (P = 0.30, two-tailed t-test; n = 8 mice/group). Scale bar: 200 μm. Iba-1: Ionized calcium-binding adapter molecule 1.

Figure 5 Iba-1$^+$ microglia within the lesion site at 12 weeks after spinal cord injury.

bis-benzimide solution (Hoechst 33258 dye; Sigma/Aldrich; 5 μg/mL in PBS). Finally, the sections were washed again, mounted in anti-fading medium (Fluoromount G; Southern Biotechnology Associates, Biozol, Eching, Germany) and stored in the dark at 4°C.

Quantifications of axons at the lesion site

Parasagittal spinal cord sections stained for tyrosine hydroxylase (TH) were used to analyze the numbers of catecholaminergic axons in the thoracic spinal cord caudal to the lesion site at 12 weeks after spinal cord injury. All TH$^+$ axons crossing an arbitrarily selected border 250 μm caudally to the lesion site were counted in every 5th parasagittal serial section from the spinal cord of each mouse on an Axiophot microscope (Zeiss, Oberkochen, Germany) equipped with a motorized stage and Neurolucida software-controlled computer system (MicroBrightField, Magdeburg, Germany). This method for estimation of axonal sprouting beyond the lesion site was previously shown to correlate well with locomotion recovery score (Apostolova et al., 2006; Jakovcevski et al., 2007), and gives a reasonable estimate of overall axonal sprouting in the lumbar spinal cord (Pan et al., 2014).

Stereological analysis of neuronal density

Cell counts were performed on an Axioscope microscope (Carl Zeiss Meditec, Inc., Dublin, CA, USA) equipped with a motorized stage and Neurolucida software-controlled computer system (MicroBrightField) using the optical disector method as described (Jakovcevski et al., 2009; Mehanna et al., 2010; Wu et al., 2012). We counted NeuN-immunoreactive cells 1.5 mm rostral and 1.5 mm caudal to the edges of the lesion site, in the spinal cord segment comprising a rectangle of 0.5 mm in length. Therefore, we did not measure neuronal death in immediate vicinity to lesion site caused by injury, but rather the amount of remote neurodegeneration at 3 months after injury. Longitudinal spinal cord sections from the 0.5-mm-long segment starting at the beginning of the lumbar enlargement, as well as the segment of the same length of the thoracic spinal cord were used for counting. The sections were observed under low-power magnification (10× objective) with a 365/420 nm excitation/emission filter set (blue fluorescence). The nuclear staining was used to delineate spinal cord area. The numerical density of NeuN-immunoreactive neurons was estimated by counting nuclei of immunolabeled cells within systematically randomly spaced optical disectors. The parameters for this analysis were guard space depth 2 μm, base and height of the dissector 900 μm^2 and 10 μm, respectively, distance between the optical dissectors 30 μm, using the objective Plan-Neofluar × 40/0.75. Left and right spinal cord areas were evaluated in six sections 250 μm apart each. All results shown are averaged bilateral values. The counts were

performed by one observer in a blinded fashion.

Estimation of the glial scar volume and the expression of GFAP and Iba-1 in the glial scar

Spaced serial 25-μm-thick longitudinal spinal cord sections 250 μm apart were immunostained for GFAP and used for estimations of the scar volume using the Cavalieri's principle. This method for estimation of the volume of irregularly shaped objects as a sum of regularly sampled areas multiplied by the distance between them was adopted by Howard and Reed (1998) and has been previously used to assess brain and spinal cord volumes (*e.g.*, Apostolova et al., 2006; Djogo et al., 2013). Areas of the scar required for volume estimation were measured directly under the microscope using the Neurolucida software (MicroBrightField). To investigate expression of GFAP and Iba-1 rostral and caudal to the lesion site, we estimated the area of the immunopositive structures normalized to the total image area using ImageJ software. This estimation was performed on images obtained on an LSM 510 confocal microscope (Carl Zeiss) using a 40× oil-immersion objective and a digital resolution of 1,024 × 1,024 pixels. The confocal images were converted to grey scale to consistently adjust the threshold based on the histogram shape of the optimal color intensity. Images of the GFAP or Iba-1 immunostained area rostral and caudal to the lesion site taken from each of six longitudinal sections were analyzed per animal, and mean values from individual animals were used to calculate group mean values.

Photographic documentation

Photographic documentation was made on an Axiophot 2 microscope equipped with a digital camera AxioCam HRC and Axio Vision Software (Zeiss) at the resolution of 1,024 × 1,024 pixel (RGB) and with a Leica confocal laser scanning microscope (Leica). The images were processed using Adobe Photoshop CS5.1 software (Adobe System Inc., San Jose, CA, USA) and manipulations were limited to crop and brightness/contrast functions.

Statistical analysis

Statistical analysis was performed using Sigma Plot 12.5 software (SPSS, IBM, Armonk, NY, USA). Data are shown as the mean ± SEM, and were tested by the two-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test or two-tailed *t*-test, as appropriate. For all comparisons, the degree of freedom was determined by the number of animals. The accepted level of significance was 5%.

Results

Mice that drank thermomineral water show improved locomotor recovery after spinal cord injury

Spinal cord compression caused severe disabilities in both groups of mice as assessed by BMS scores which were close to 0 at 3 days after injury (**Figure 1**). Between 1 and 12 weeks, the walking abilities improved in both groups, but the improvement was significantly better in the group of mice that drank thermomineral water than in control mice, as revealed by analyses of BMS scores at 4, 8 and 12 weeks after injury ($P < 0.05$; **Figure 1**). This represents the difference between the slight ankle movements seen in the majority of mice in the control group, while in the experimental group, the majority of animals displayed extensive ankle movement. Recovery of locomotor function was analyzed by two-way analysis of variance with treatment and time as factors. Data show that the main effect of the treatment was statistically significant ($P = 0.002$), *i.e.*, mice that drank thermomineral water recovered significantly better when compared with control animals. Time after injury had also significant influence on recovery (analysis of variance; $P = 0.047$), indicating that in both groups, locomotor function improved over time, whereas analysis showed no significant interaction effect between the treatment and time factors ($P = 0.30$). Subsequent Tukey's *post-hoc* tests showed statistical significance for differences between treatments at 4 weeks ($P = 0.036$), 8 weeks ($P = 0.018$) and 12 weeks ($P = 0.011$). Overall, our results indicate a better locomotor recovery after spinal cord injury in mice that drank thermomineral water compared with controls.

Enhanced axonal regrowth/sprouting of catecholaminergic axons at the site of injury in the spinal cord of mice treated with thermomineral water

To assess monoaminergic reinnervation of the spinal cord caudal to the injury site, previously shown to correlate well with motor recovery (Jakovcevski et al., 2007, 2013; Devanathan et al., 2010), we counted numbers of catecholaminergic (dopaminergic and noradrenergic, TH^+) axons crossing an arbitrarily selected border 250 μm caudal to the lesion site in spaced serial parasagittal sections at 12 weeks after the injury (**Figure 2A, B**). Numbers of TH^+ fibers at 12 weeks were more than double in mice that drank thermomineral water compared with control mice ($P < 0.05$; **Figure 2C**).

Number of $NeuN^+$ neurons in the spinal cord, rostral and caudal to the site of injury

To investigate whether the intake of thermomineral water could protect neurons from dying after injury, we performed stereological counts of neurons in the thoracic and lumbar spinal cord at 12 weeks after injury. Neurons were counted in both the ventral and dorsal horn of the spinal cord gray matter (**Figure 3A, B**). There was no significant difference in the density of neurons (number of cells per volume) between the two treatment groups, either rostral or caudal to the injury site (**Figure 3C**). Thus, thermomineral water intake did not influence the number of surviving neurons after spinal cord injury.

Glial scar volume and GFAP expression at the site of injury

To investigate whether thermomineral water intake leads to alterations in the formation of glial scar, we estimated $GFAP^+$ astrocyte scar volume at 12 weeks after injury. This is deemed important, because functional recovery after spinal cord injury strongly depends on the lesion severity, usually assessed by size of the astroglial scar (Jakovcevski et al., 2007;

Wu et al., 2012). The volume of the GFAP$^+$ astroglial scar was similar between the experimental (0.92 ± 0.17 mm^3) and control mice (1.1 ± 0.23 mm^3). We also sampled areas rostral and caudal to the lesion center and measured the size of the area immunostained for GFAP (**Figure 4A, B**). Congruent with measurements of the scar volume, there was no significant difference between both groups (**Figure 4C**). Thus, GFAP$^+$ astroglial scar was not influenced by the thermomineral water intake.

Iba-1$^+$ microglia/macrophages at the site of injury

It is well recognized that immune response after injury influences the regenerative response of the CNS (Ankeny and Popovich, 2009; Wu et al., 2012). Microglia/macrophages were immunofluorescently labeled with Iba-1 antibody, which labels both activated and quiescent cells (Streit et al., 1999), and the size of immunostained area was measured at the lesion site (**Figure 5A, B**). There was a tendency towards lower Iba-1 expression in animals that drank thermomineral water, but it did not reach statistical significance (**Figure 5C**).

Discussion

Injury to the spinal cord causes massive tissue destruction and total neuronal loss at the site of injury, coupled with progressive neurodegeneration of the structures caudal to the site of injury (Rossignol et al., 2007; Blesch and Tuszynski, 2009; Filli and Schwab, 2012). Because no therapies are available to cure spinal cord injury, any approach that would benefit locomotor function and improve rehabilitation after injury presents a considerable advance. The objective of this study was to examine if the balance of minerals present in the Atomic Spa water improves locomotor recovery in an experimental animal model, thus eliminating possible placebo effects that could be present in patients. Additionally, we aimed to show that not only single elements in high therapeutic doses, but also mixtures of such elements in much lower concentrations are capable of producing beneficial effects, avoiding possible adverse side effects.

The improvement of locomotor recovery after injury was modest in mice of both experimental and control groups, indicating high severity of injury. However, a large difference in BMS scores between the mice that drank thermomineral water and control mice at 2 and 3 months after injury indicates that the effects of thermomineral water can improve locomotion even upon severe spinal cord injury. The results of histological analysis of the injured spinal cords at 3 months upon injury indicate that thermomineral water improved axonal sprouting, but did not alter neuronal survival or glial response to injury. Notably, monoaminergic axons were sprouting markedly more in mice treated with thermomineral water than in controls. These axons, originating in brainstem nuclei, were previously shown to be important for recovery of motor function after spinal cord injury (Jakovcevski et al., 2007, 2013; Lieberoth et al., 2009; Chen et al., 2010; Devanathan et al., 2010; Pan et al., 2014). This increase in fiber numbers could be due to regrowth of axons across the lesion scar or sprouting of spared axons caudal to the lesion site, indicating a more vigorous regenerative response in the injured spinal cord of mice treated with thermomineral water. The number of NeuN$^+$ neurons, however, was not affected by the thermomineral water intake, either rostral or caudal from the site of injury. The loss of neurons, and in particular motoneurons, due to deafferentiation is well documented in several animal models of spinal cord injury (Young, 1966; Eidelberg et al., 1989; Dong Teng et al., 1999), but it is less clear whether the protection from neuronal loss would impact motor recovery (Dong Teng et al., 1999; Mehanna et al., 2010). Previous studies that have reported neuroprotective effects always coupled increase in neuronal numbers with increased axonal sprouting, with the latter being more likely involved in improved restoration of motor function (Cui et al., 2011; Xu et al., 2011).

Reactive astrocytes are prominent feature of the cellular response to spinal cord injury (Faulkner et al., 2004). Scar tissue formed in part by reactive astrocytes has long been implicated a major impediment to axon regeneration (Ramon y Cajal, 1928; Clemente and Windle, 1954; Reier et al., 1983; Liuzzi and Lasek, 1987; Rudge and Silver, 1990). Numerous studies report that inhibition of astrocyte reaction to injury has beneficial effect on recovery (Jakovcevski et al., 2007; Hellal et al., 2011; Cregg et al., 2014). Our current study, however, did not show an effect of thermomineral water on astrocyte scaring. Another component of glial response to spinal cord injury is microglia/macrophages proliferation/invasion of injured tissue (Streit and Kreutzberg, 1988; Streit et al., 1999). Previous studies on the influence of microglia/macrophages on recovery after injury have been inconclusive, revealing both pro-inflammatory and neuroprotective roles of microglia. While some view microglia/macrophages as integral components of the regenerative response, others emphasize their contribution to delayed neuronal apoptosis and demyelination in secondary response during subchronic phase after injury (Ankeny and Popovich, 2010; David and Kroner, 2011; Streit, 2002; Wu et al., 2012). Activated microglia/macrophages secrete extracellular matrix components, such as keratin, dermatan and chondroitin sulfate proteoglycans which have an overall negative impact on axonal regeneration (Grimpe and Silver, 2002; Jones and Tuszynski, 2002; Akyüz et al., 2013). Our finding of non-significantly lower microglia/macrophage infiltration of injury site in mice treated with thermomineral water could be due to lower rate of elimination of damaged fibers compared with controls, thus not being a direct effect of thermomineral water intake on microglia/macrophages.

Lithium has been used for more than 60 years to treat bipolar disorders due to its complex activity in modulation of the neurotransmitter systems at multiple levels of signaling in the brain (Manji and Lenox, 1998; Jope, 1999; Geddes and Miklowitz, 2013). Neuroprotective effects of lithium have been reported in the 1990s (Jope, 1999), but only recently the possible mechanisms of these effects have been described (Chuang, 2004; Chiu and Chuang, 2010). Chronic treatment with lithium reduces microglia and macrophage activation (Su et al., 2007), and also increases neurogenesis, both in

cultured brain neurons and *in vivo*, and prevents inhibition of proliferation induced by glutamate or glucocorticoids (Hashimoto et al., 2003; Chiu and Chuang, 2010). Long-term treatment with lithium effectively protects primary cultures of neurons from glutamate-induced toxicity mediated by N-methyl-D-aspartate (NMDA) receptors. Lithium prevents the activation of Cdk5 which regulates signaling mediated by NMDA receptors and whose sustained activity is believed to be involved in the pathogenesis of many neurodegenerative diseases (Cruz and Tsai, 2004; Chiu and Chuang, 2010). Additionally, lithium induces the expression of the brain-derived neurotrophic factor and its receptor and stimulates neurogenesis in primary cultures of neurons (Hashimoto et al., 2002).

Many other ions which are present in the thermomineral water from the Atomic spa have neuroprotective/neurotrophic properties. The neuroprotective effects of magnesium in fetal CNS development have been well established and the use of magnesium sulfate in the prevention of brain injury in preterm infants is supported by relevant, evidence-based clinical guidelines (Salmeen et al., 2014). However, despite plenty of evidence for neuroprotective benefits of magnesium in animal models of spinal cord injury, its clinical use in human medicine has been restricted by the fact that doses needed for neuroprotection (300–600 mg/kg) far exceed human tolerability for magnesium (Kwon et al., 2009), thus a necessity for developing special formulations, such as combination with polyethylene glycol. This formulation allows much lower doses of magnesium to be clinically effective (Kwon et al., 2009). However, the concentration of magnesium in the Atomic spa water is relatively low (61 mg/mL), so the neuroprotective properties of this water observed in patients cannot be attributed solely to its magnesium content.

Another important consideration regarding the thermomineral water is that in patients with spinal cord injury, it has been extensively used during the chronic phase, *i.e.*, during rehabilitation after injury. Our results in a rodent model demonstrate that, taking individual health status of patients into consideration, it could also contribute to the restoration of innervation in the acute and sub-chronic phases after injury.

Finally, we would like to emphasize that none of the macro- or microelements contained within the thermomineral water tested here has previously been reported to be therapeutically effective in such low doses. Thus, it is our hypothesis that the optimal ratio between the ions contained within this natural spring water, rather than single ions, has beneficial effect. Although further studies are needed to confirm this hypothesis, our study provides evidence that the thermomineral water from the Atomic Spa Gornja Trepča promotes recovery after spinal cord injury in a mouse model.

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