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ORIGINAL ARTICLE

An experimental model of the "dual diagnosis": Effect of cytotoxic brain edema plus peripheral neuropathy on the spontaneous locomotor activity of rats

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Abstract

OBJECTIVE: The aim of the study was to find how a simultaneous impairment of the CNS (cellular brain edema induced by water intoxication) and PNS (blockade of the right forelimb brachial plexus by local anesthewtic – Marcaine) affects spontaneous locomotor activity of adult rats.

METHODS: Rats were divided into groups of animals without water intoxication (without WI) – A,B,C, and those that were water intoxicated (induction of brain edema – after WI) – D,E,F. Both groups were further divided into intact ones (A,D), animals with PNS lesion (Marcaine) (B,E) and sham-operated animals (C,F). Locomotor activity (LA) of the rats was tested by the open field test.

RESULTS: LA of rats with both CNS and PNS impairment (WI + Marcaine) was significantly suppressed compared to the activity of control rats. Comparison of LA of rats with a single lesion – PNS impairment only (Marcaine only), CNS lesion only (WI) to those animals with both lesions (WI + Marcaine) revealed even larger decrease of LA of rats with combined lesions, which represents a model of the dual diagnosis. Also the pattern of behaviour of rats in both sham operated groups was different, which apparently depended on water intoxication.

CONCLUSION: The presented results show that the LA of rats with combined lesions is significantly lower compared to the activity of rats with a single lesion in the CNS or PNS. Results also indicate that the already induced endoneurial edema prevents subsequent accumulation of water applied to the intimate vicinity of the peripheral nervous structures.

Abbreviations:

CNS	- central nervous system
PNS	- peripheral nervous system
WI	- water intoxication
SEM	- standard error of mean
No	- number
i.p.	- intraperitoneally
DW	- distil water
deg C	- degrees Celsius
AQP	- aquaporin
ADH	- antidiuretic hormone

INTRODUCTION

The concept of dual diagnosis appears in the literature since seventies of the previous century in relation to the simultaneous occurrence of two serious clinical conditions in a single individual – mental disorder and addiction to alcohol or drugs. Ridgely *et al.* (1990) published a historical overview of diagnostic and therapeutic procedures of such comorbidity, pointed to the poor results of previous uniform treatment procedures of patients with either diagnosis and stressed the need for a specific treatment of each disease separately in the form of separate treatment protocols. They proved that with only patients treated according the new method an improvement of the mental health can be achieved and coexisting addictions coped. Since the mid-eighties the term dual diagnosis gets somewhat different content. It was used in another field of medicine, referring to the difficulties posed by treatment of two different comorbidities occurring simultaneously in one individual –injury of the spinal cord and the brain. Arzaga *et al.* (2003) and Macciocchi *et al.* (2008) reviewed their experience with such dual diagnosis and they noted that the simultaneous injury to the spinal cord and brain occurs frequently and this comorbidity may become a serious problem for acquiring adequate therapeutic and especially rehabilitation process.

Formerly, treatment procedures were targeted to only one type of injury, regardless of the fact that after overcoming the acute period, rehabilitation must consider the interaction of two different problems (Arzaga *et al.* 2003; Macciocchi *et al.* 2008). It is apparent that the brain injury slows down the functional improvement of spinal lesions. Inoue *et al.* (2013) developed an experimental model of a dual injury in rats and published findings, showing that damage of the contralateral (left) cerebral cortex, together with the spinal cord at the level of the right root C5 leads to considerably more severe damage to the right forelimb compared to isolated spinal cord injury. Daly *et al.* (2007) have shown that the current neurorehabilitation emphasis the well-defined, focused and reliably measurable rehabilitation procedures with specific indicators of the functional status. In their work, they mentioned that for the clinical procedures the results of studies of physical activity of rats in experimental models were essential (Biernaskie *et al.* 2001; Kolb *et al.* 1991; Jones *et al.* 1999).

The aim of this study was to determine whether and how the spontaneous locomotor activity of adult rats can be affected by the CNS impairment (brain edema induced by water intoxication) and PNS (brachial plexus blockade of right forelimb by local anesthetic – Marcaine). Because spontaneous locomotor activity of the rats depends on the normal forelimb function (Schallert *et al.* 2003), horizontal locomotor activity in the open field was tested. Open field test was introduced by Hall in 1934 and it is currently used in a wide spectrum of experimental studies (Hall, 1934; Aragao *et al.* 2011; Russell *et al.* 2011; Jandová *et al.* 2014; Šlamberová *et al.* 2013; Meng *et al.* 2015).

MATERIAL AND METHODS

All experiments were approved by the Ethical Committee of the First Faculty of Medicine (Charles University) and were in agreement with the Guidelines of the Animal Protection Law of the Czech Republic and Guidelines for the treatment of laboratory animals EU Guidelines 86/609/EEC. Male Wistar rats of our own bread were divided into groups with induced brain edema (water intoxicated – after WI) – D,E,F and those with no edema (without WI) – A,B,C. Both groups were further divided into intact ones (A,D) animals with PNS lesion (Marcaine administration) (B,E) and sham-operated animals (Aqua pro injectione instead of Marcaine) (C,F) (number of animals, their body weight and the group labelling is given in Tab.1).

Surgery

Lesion of the peripheral nervous system was achieved by injection of local anesthetic Marcaine in groups B and E into the vicinity of brachial plexus. Spontaneously breathing rats under isoflurane anaesthesia (Florante®, AbbVie Ltd.) in the supine position with abducted and fixed limbs were injected in the skin fold of the right axilla. During three minutes they received a third of the total dose into the inner aspect of the girdle and the other two thirds were applied above and below the girdle. Marcaine (bupivacaine hydrochloride – Marcain©, AstraZeneca plc) is a local anesthetic of the amide type with average latency time and rapid onset of action. It brings prolonged reversible blockade of the vegetative, sensitive and motor nerves and cardiac conduction. Marcaine blocks the passage of ions through voltage-gated sodium channels, thus blocking the formation and propagation of action potentials. The same procedure with aqua pro injectione instead of Marcaine was used in rats of the group C and F (sham operation).

The dose of anaesthetics used for brachial plexus blockade was determined according to the recommended dosage for adult humans (www.medicines.org.uk/emc/medicine/23926). For the brachial plexus blockade, 0.2 ml of 0.5% Marcaine solution was used. After Marcaine injection, inhalation anesthesia was completed and animals were kept to awake spontaneously on their side. Once the rat rose on all four limbs and began to move spontaneously, the open field test was performed. The time interval between the end of inhalation anesthesia and beginning of the open field test was 25 to 30 minutes depending on the wakening from anesthesia.

Water intoxication

Water intoxication was achieved by fractionized hyperhydration combined with administration of an antidiuretic drug desmopressin. Distil water (DW) in

the total amount of 20% of the animal's body weight was injected intraperitoneally (i.p.) in three consecutive doses within 24 hours. Each water injection was accompanied with i.p. injection of 1/3 of the total dose of desmopressin (0,032 μg/kg). Desmopressin (1-desamino-8-D-arginine vasopressin, OCTOSTIM®, Ferring) is an analogue of the human hormone arginine vasopressin (the antidiuretic hormone, or ADH) (www.rxmed.com/b.main/b2.pharmaceutical/OCTOSTIM.html). Method of water intoxication is derived from the literary data (Olson *et al.* 1994, Vajda *et al.* 2000, Manley *et al.* 2000, Yamaguchi *et al.* 1997, Silver at al. 1999).

Open field test

To test the locomotor activity of rats, we used the system Laboras (Metris, B.V., Netherland) for continuous registration and analysis of the locomotor activity. Laboras system consists of triangular shaped sensing platform (carbon fiber plate 700 mm x 700 mm x 1000 mm x 30 mm), positioned on two orthogonally placed sensor-transducers and third fixed point attached to bottom plate. Makrolon cage (type III, 840 cm^2) is placed on this platform. Any mechanical vibrations caused by the movement of the animal are converted into electrical signals, which are then evaluated using software Laboras. Animals were tested in a darkened room at a constant room temperature 22 to 23 deg C, always in the same time, between 9:00 and 12:00. Horizontal locomotor activity – average time spent in locomotion (s), average distance travelled (m) and average speed of locomotion (m/s) during one hour at time intervals of ten minutes were recorded and analysed.

Statistical evaluation

The results of all measurements were statistically evaluated using the tests of the GraphPad Prism program (parametric ANOVA and nonparametric Kruskal-Wallis test, the statistical significance was set at 5%).

Tab. 1. Number of animals, their body weight and the group labelling.

Animals without water intoxication	A	B	C
	Control group	Marcaine group	Sham group
No	11	8	8
(Mean ± SEM) g	415.5±9.413	417.0±11.62	408.8±10.35
Animals after water intoxication	D	E	F
	Control group	Marcaine group	Sham group
No	8	8	8
(Mean ± SEM) g	409.8±7.762	409.4±7.250	397±7.184

RESULTS

Results in graphs give the average horizontal locomotor activity in all studied categories: average time spent in locomotion (s), average distance travelled (m) and average speed of locomotion (m/s) during one hour.

Overall results in all groups are given first: A, B, C rats without WI and D, E and F rats after WI (see also Table 1). Locomotor activity of all experimental animals was smaller than that of the control group (A). (Figure 1)

Locomotor activity of rats without water intoxication (groups B – Marcaine and C – Sham) was significantly lower in all studied categories when compared to the activity of the rats in the control group A. No difference was found between groups B (Marcaine) and C (Sham) (Figure 2).

When locomotor activity of rats after water intoxication (groups D, E, F) was compered, the data revealed that the locomotor activity of rats in group E (WI + Marcaine), which represents the dual diagnosis, was significantly lower compared to the activity of rats in

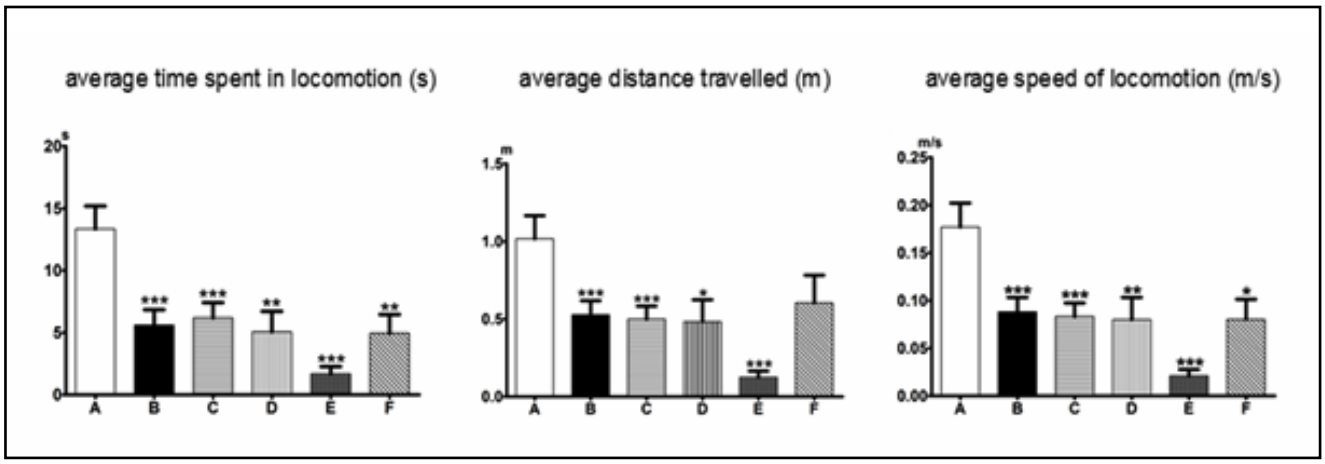

Fig. 1. All groups. Legend: horizontal axis: groups of animals A-F (see Tab. 1), vertical axis: units of the locomotor activity in the studied categories: s = second, m = metre, m/s = metre/second, *p<0.05, **p<0.01, ***p<0.001; results are given in averages ±SEM

groups D (WI only) and F (WI Sham). Between the groups D and F no significant difference in the locomotor activity was found (Figure 3).

Effects of different types of lesions are compared at Figure 4. Lesion of the peripheral nerve induced by the local anesthetic (Group B) was compared with the CNS lesion (group D – brain edema induced by water intoxication) and animals with both types of lesion (group E). Animals with combined lesion (group E) were less active compared to the rats in groups B and D (Figure 4).

Presented figures indicate that the locomotor activity of rats with induced lesions in the groups B through F (WI, Marcaine) was significantly suppressed compared with locomotor activity of control rats in group A. Locomotor activity of animals that were not intoxicated with water (experimental groups B – Marcaine and C – Sham) was significantly smaller compared to the activity of the rats in the control group A; however, there was no difference between groups B and C. All animals with water intoxication (WI) were very different from those who were not. Locomotor activity of rats in group E with two induced lesions (WI + Marcaine) was significantly inhibited compared to the activities of rats in groups D (WI only) and F (WI Sham), but physical activity in groups D and F were the same. This finding shows difference in the behaviour between sham-operated animals in the dependence of the hydration status. Comparison of locomotor activities of rats with one lesion only (group B – Marcaine only and D – WI only) versus the group E with both lesions (WI + Marcaine) demonstrates a statistically significant decrease of locomotor activity of rats with both lesions, which may represent the dual diagnosis.

DISCUSSION

To explain changes in horizontal activities, described in present study, effect of single lesions should be mentioned first. Among the groups without water intoxication (groups A and B) a significant decrease in locomotor activity in group B (Marcaine group) was found compared with healthy rats (group A) in all categories examined (see Figure 2). This finding corresponds to the effect of the local anesthetic on the conductivity of the nerve fibers. As we stated in the methodology,

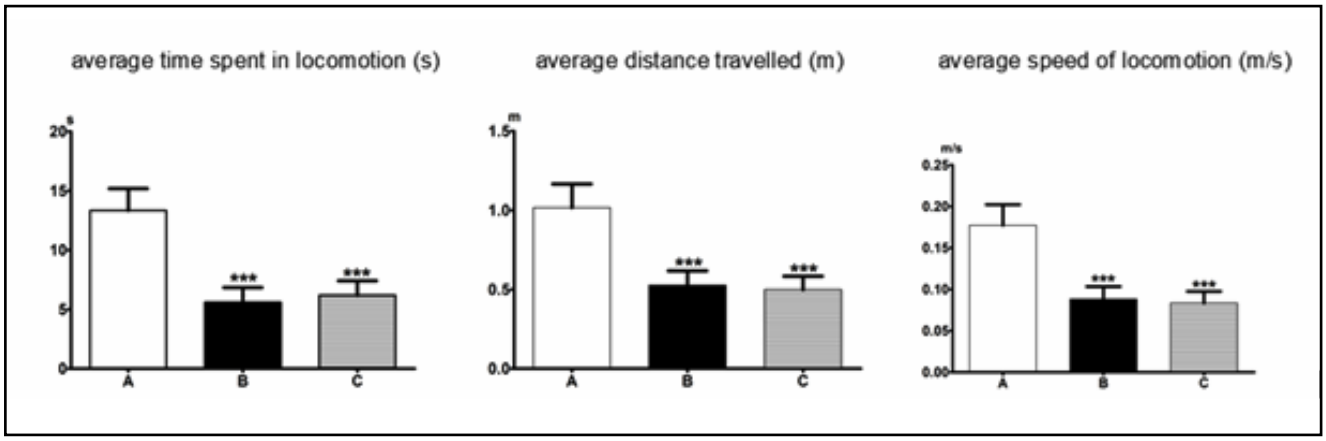

Fig. 2. Rats without WI. Legend: horizontal axis: groups of animals A-C (see Tab. 1), vertical axis: units of the locomotor activity in the studied categories: s = second, m = metre, m/s = metre/second, *p<0.05, **p<0.01, ***p<0.001; results are given in averages ±SEM

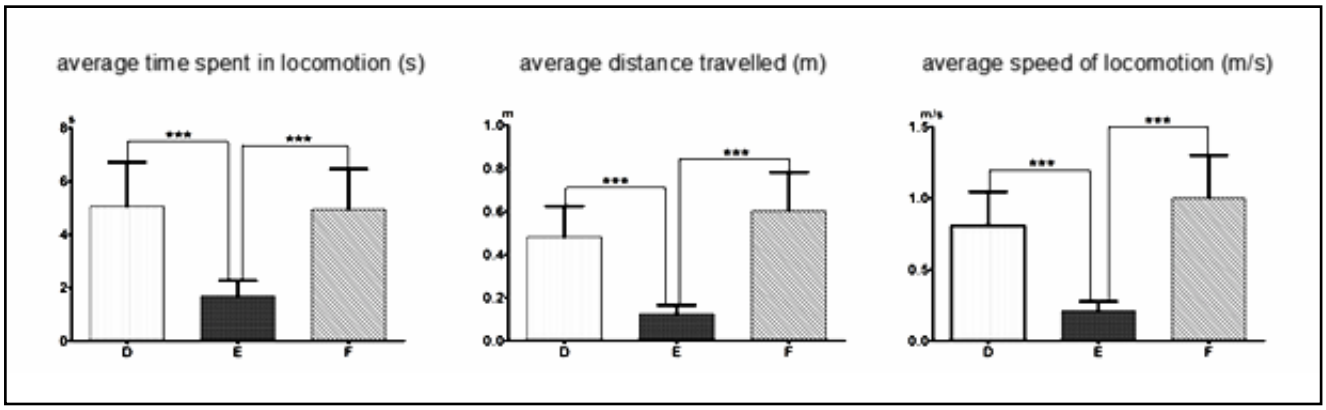

Fig. 3. Rats after WI. Legend: horizontal axis: groups of animals D-F (see Tab. 1), vertical axis: units of the locomotor activity in the studied categories: s = second, m = metre, m/s = metre/second, *p<0.05, **p<0.01, ***p<0.001; results are given in averages ±SEM

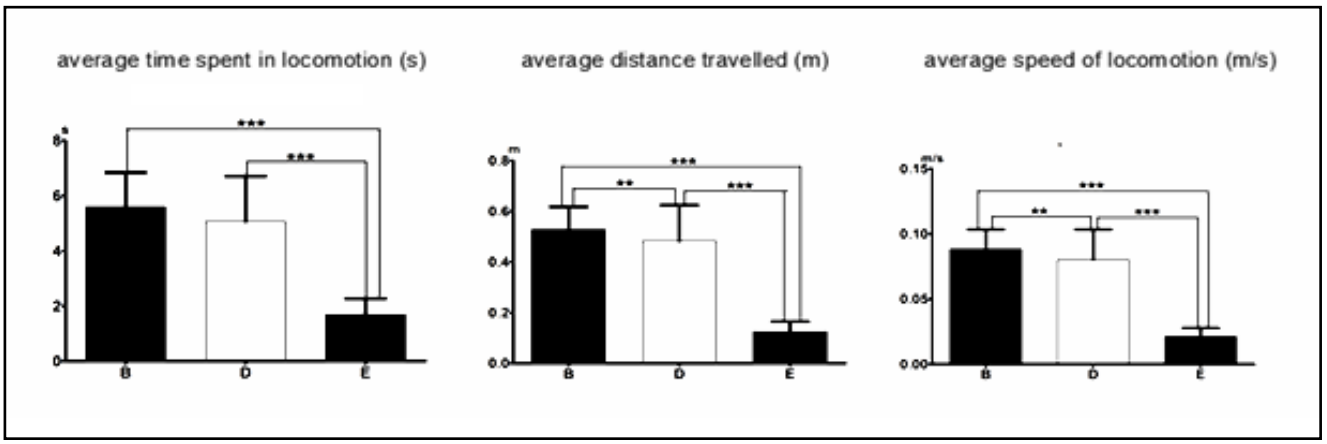

Fig. 4. Groups B (Marcaine only), D (WI only), E (Marcaine + WI). Legend: horizontal axis: groups of animals B, D, E. (see Tab. 1), vertical axis: units of the locomotor activity in the studied categories: s = second, m = metre, m/s = metre/second, **p*<0.05, ***p*<0.01, ****p*<0.001; results are given in averages ±SEM

Marcaine 0.5% causes a prolonged reversible blockade of the vegetative, sensitive and motor nerves that prevents the ionic flows across the nerve fiber membrane and thus blocks generation and propagation of action potentials. This effect was induced in our study by Marcaine injection into the proximity of the brachial plexus and resulted in the weakening of the right forelimb together with the decline of the monitored locomotor activity.

Locomotor activity of rats with water intoxication (group D – after WI) was lower than that in control rats (group A) (see Figure 1). This decrease was caused by the induced cellular edema. Cellular edema in general reduces the initiation and conduction of upper limbs movements during locomotion, without functional or anatomical distress of the pathway that controls the movements (Inoue *et al.* 2013). Origin, timing and consequences of cellular brain edema induced by water intoxication are known. Water intoxication reduces the amount of solutes in the extracellular compartment due to their marked dilution and causes hypoosmolality. This creates an osmotic gradient that activates the movement of water from the extracellular space into the cell. Accumulation of water in the cell triggers a cascade of events that leads to failure of cellular metabolism and development of cytotoxic edema (Klatzo 1967; Go 1997; Kimlberg 1995). Recent view on the pathophysiology of cytotoxic edema was described by Liang *et al.* (2007). Cytotoxic edema is a premorbid cellular process (synonyms a cellular edema, oncotic cell swelling or oncosis) whose primary attribute is an influx and intracellular accumulation of extracellular Na^+ and other cations into neurons and astrocytes. Influx of cations leads to an influx of anions in order to maintain electroneutrality, and combinations of these phenomena controls the influx of water into the cells. Water passes membrane through specific water channels – aquaporins (AQP). An essential role for the movement of water in the CNS and for the formation of cellular edema has AQP4 (Agre *et al.* 2004; Manley *et al.* 2000; Wells 1998; Pasantes-Morales *et al.* 2002; Papadopoulos & Verkman 2007; Hsu *et al.* 2015). Cytotoxic edema itself does not lead to brain swelling, but that depletes the extracellular space for Na^+, Cl^- and water, creating a new gradient for the flow of those molecules and water from the capillaries of the gliovascular complex (BBB). As a result of the loss of selective permeability of the BBB, the new gradient created by cytotoxic edema leads to formation of ionic edema, the process known as brain edema, which is characterized by increased water content and the volume of the brain (Klatzo, 1967; Michinaga & Koyama, 2015).

Reversible form of the cellular edema induced by water intoxication is currently used as routine experimental model (Olson *et al.* 1994; Vajda *et al.* 2000; Manley *et al.* 2000; Yamaguchi *et al.* 1997). Though supposed to be reversible, cytotoxic edema (oncotic, ischemic, traumatic) defined as premorbid cellular process (Liang *et al.* 2007), leads to various functional and anatomical abnormalities (Marešová *et al.* 2014; Kozler & Pokorný 2012; Creed *et al.* 2011; Onaya 2002; Tolunay *et al.* 2015; Placha *et al.* 2016).

For the interpretation of changes in horizontal activities documented in our study, effects of combined and simultaneous lesions should be analysed. Rats in Group E were intoxicated with water and Marcaine was injected into neural structures of brachial plexus. Locomotor activity of those rats was significantly more suppressed in all the criteria compared to the groups B (Marcaine only without WI) and D (WI only) (see Fig 4). Combined lesions lead to a greater deficit than the isolated lesions. In Group B the effect was due to a local anesthetic only and in group D it was effect of the induced cellular edema. In group E conditions of the dual diagnosis were fulfilled. The above finding can thus be summarized: between groups A and D, the group D (WI only) had lower activity which confirms the dampening effect of brain edema on locomotion. In

group B (Marcaine only) the malfunction of the right forelimbs developed due to Marcaine effect only, meanwhile in group E, this effect is potentiated by general depressant effect of brain edema, and the horizontal locomotor activity is worst among all categories examined compared to groups B (Marcaine only) and D (WI only).

Our findings are fully in agreement with the already published data on the damage of the contralateral (left), cerebral cortex, together with the spinal cord at the level of the right C5 root. The combined lesion leads to considerably more severe damage to the right forelimb compared to isolated spinal cord injury (Inoue *et al.* 2013). The issue of dual diagnosis was studied by other authors and results of experimental studies in rodents confirmed greater attenuation of physical activity for combined lesions of the brain and spinal cord in comparison with isolated lesion (Kokotilo *et al.* 2009; Schallert *et al.* 2003; Strong *et al.* 2009). All these studies concern combined lesions of the two CNS structures – the brain and spinal cord. Our results showed that combined CNS impairment (induced cellular edema) and PNS (Marcaine effect) also leads to a significant attenuation of locomotor activity of rats in comparison with the isolated lesions. No literary data could be found about the dual diagnosis combining CNS and PNS lesion.

Our results showed no differences between treatment groups B (Marcaine) and C (Sham) (see Figure 2). It can be expected that the volume of 0.2 ml of water that formed a fluid depot in the intimate proximity of the nerves in brachial plexus induced by its volumetric effect a compressive neuropathy.

The fact that of compression of nerve fibers brings impairment of their function was demonstrated in rats in experimental models: Compression with a miniclamp for 2 seconds or a compression by a minicuff inflated to 50 mmHg resulted in an increased endoneural pressure and subsequent impairment of axonal conduction (Igarashi *et al.* 2005; Rydevik *et al.* 1980).

Locomotor activity of rats in the Sham group without WI (C) was entirely different from the locomotor activity in Sham group after WI (F). Results in the Group F were similar to those in the group D, which means that the locomotor activity impairment is an effect of induced cellular brain edema, without concomitant effect of neuropathy (see Figure 3). This finding can be explained on the basis of endoneurial environment homeostasis (Grambalova *et al.* 2015). Water intoxication induces intracellular edema, not only in the CNS but also in the PNS (Fishman 2006). Under physiological conditions, the function of axons and myelin sheaths is maintained by the homeostasis of endoneurial environment within the peripheral nerve. Homeostasis has several mechanisms, but the main role has endoneurial microvessels, which have, with the exception of brain capillaries, least permeable endothelium of all the other blood vessels in the body (Mizisin *et al.* 2011; Olsson 1990). Water intoxication undoubtedly affects the endoneurial homeostasis (Fishman 2006) and the development of cytotoxic edema (as described above in connection with induced cellular edema) leads to intracellular – endoneurial – hyperosmolality. In the so changed endoneurial microenvironment, water injected to the intimate proximity of peripheral nerve cannot accumulate and induce nerve compression, because due to the direction of the osmotic gradient water quickly moves into the endoneurial hyperosmolar environment. Absorption of 0.2 ml of water thus cannot affect the nerve function. Volume of Aqua pro injection was the same as the dose of Marcaine. It can explain the different locomotor activity of both sham groups (C, F) and very similar locomotor activity of rats in the sham group F and group D (WI only).

CONCLUSION

The aim of our study was to determine whether and how the spontaneous locomotor activity of adult rats may be affected in conditions of the parallel impairment of CNS (brain edema induced by cellular water intoxication) and PNS (right forelimb brachial plexus blockade by local anesthetic). The most important aspect in the presented results is the evidence that locomotor activity of the rats with combined lesions is significantly worse than the activity of rats with isolated lesions. This finding is consistent with the current literature data on the effects of dual diagnosis on locomotor activity. It should be emphasized that we employed a combination of the CNS and PNS lesions, which has not yet been published. The results of our study also showed that the induced endoneurial edema prevents the accumulation of water applied to the intimate proximity of peripheral nerve.

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