EFFECTS OF MAGNESIUM SOFT MATTER VESICLES CARRIER ON THE BEHAVIORAL MANIFESTATIONS IN MICE[#]

DANIELA BÎNDAR, LILIANA TARȚĂU**, ANA GÂRLEA*, LOREDANA NIȚĂ***, V. MELNIG**

*Faculty of Physics, COMB Laboratory, "Al.I. Cuza" University, 11A, Carol I Blvd., Iaşi, Romania
**Faculty of Medicine, "Gr.T. Popa" University of Medicine and Pharmacy, 16, Universității St., Iaşi, Romania
***LAMINAST Laboratory, "Petru Poni" Institute of Macromolecular Chemistry, 41A, Grigore Ghica Vodă St., Iaşi, Romania

Abstract. This paper is focused on the experimental researches of the preparation and effects of magnesium chloride soft matter vesicles carrier, in a behavioral model in mice. This model evaluates exploratory activity and motor coordination in mice. The soft matter vesicles were made by magnesium chloride immobilization inside lipid vesicles and their stabilization with chitosan. Their dimensions are ranging between 78 and 300 nm and present a moderate stability. Magnesium chloride 1 mmol/kg body weight entrapped in soft vesicles was orally administered to one group of 7 mice, another two groups being treated with distilled water (the control group) and with magnesium chloride 1 mmol/kg body weight. It was observed that the magnesium chloride vesicles determined a prolonged decrease of both horizontal and vertical mice movements compared with the same dose of non entrapped magnesium chloride, more than 12 hours after substance administration. Our results show that in the mouse model of behavioral manifestations used, magnesium soft matter vesicles carrier decreased both global motor behavior and the number of escape attempts, which could correspond somehow to sedation in humans.

Key words: magnesium chloride, soft vesicles, behavioral model.

INTRODUCTION

Vesicles are hollow spheres enclosed by a bilayer of amphiphiles and are commonly used to encapsulate labile hydrophilic molecules within their interior. They are of great interest for applications ranging from drug delivery and controlled release to separations and sensing. However, the limited stability of vesicles to external conditions such as pH, temperature or ionic strength has hampered their applicability. Our work was focused on the integration of vesicles with a biopolymer as a route to create vesicles with increased stability.

[#]This study was presented as a poster in the *National Conference of Biophysics*, Cluj-Napoca, October, 2009.

Received: September 2009;
in final form December 2009.

ROMANIAN J. BIOPHYS., Vol. 20, No. 1, P. 23–35, BUCHAREST, 2010

Magnesium is one of the most widespread elements in nature. It is the fourth major cation in the human body (after Ca^{2+}, Na^{+} and K^{+}) and the second most abundant within the cells (after K^{+}). Approximately 50% of body total magnesium is found in bone. The other half is found predominantly inside cells of body tissues and organs. More than 300 enzymatic reactions depend on the presence of magnesium, especially concerning the generation and use of adenosine triphosphate.

Magnesium ion is equally important to the central nervous system (spinal cord) and to the brain itself. Electrophysiology studies show that the motor nerves – those that carry messages by electrical impulse from the brain to the muscles – are dependent on magnesium for the ability to conduct these minute electrical messages properly [7, 8].

Magnesium chloride is a chemical compound that has various uses. One common use is that of a supplement. Since magnesium is important for the human body, especially for the muscles and nerves, magnesium chloride may be prescribed for those with mild or severe hypomagnesemia, or low magnesium levels.

Magnesium chloride can be administered *orally* (tablets, enteric-coated tablets, extended-release tablets, e.g. Slow-Mag – 1 tablet (535 mg magnesium chloride, 64 mg elemental magnesium), powder for oral solution, suspension); *parenterally* (intravenous – e.g. magnesium chloride 10% (0.1 g/mL) – injectable ampoule of 10 mL) or *externally* (transdermal – gel-based magnesium chloride product).

Symptoms of a magnesium chloride overdose include nausea, low blood pressure, a slow heartbeat, drowsiness, coma and death. Due to the side effects induced in organism it is necessary to use encapsulated systems.

Literature data shows that alterations in intracellular free magnesium concentration may be an injury factor in acute and chronic central nervous system (CNS) injury, as well as the potential for magnesium administration to be neuroprotective under these conditions [14]. Among its biochemical effects in the brain, Mg^{2+} functions as a noncompetitive N-methyl-D-aspartate (NMDA) antagonist by binding to a specific Mg^{2+} recognition site and blocking the NMDA-associated ion channel. Mg^{2+} has been shown to be also necessary for the binding of monoamine neurotransmitters at dopamine (D1, D2), norepinephrine (2-NE, -NE) and serotonine (5-HT1a) receptors [16].

It is well known that magnesium ions (Mg^{2+}) have a well-established depressant effect on the central nervous system and on neuromuscular transmission. On the other hand, magnesium deficiency, even mild, increases susceptibility to various types of neurologic and psychological stressors in rodents, healthy human subjects and diverse groups of patients [9]. Rodent studies suggest that magnesium has a complex relationship with aggressive behaviors [12].

Some studies reveal that mice receiving a low magnesium diet for several weeks display increased immobility time in the forced swim test, indicating enhanced depression-like behavior. In addition, the partial magnesium-depletion increased anxiety-related behavior in the light/dark and open field test, while locomotor activity or motor coordination was not influenced [13]. Much more, other authors demonstrate that magnesium deficiency reduces offensive aggressive behavior, but increases defensive aggressive behavior. On the other hand, magnesium supplements can increase learning and enhance the behavioral response to stimulants [3, 15].

The magnesium induces in the mouse an increase in the motive agitation at a low dose (100 mg/kg) and, on the contrary, a very clear reduction of this agitation at high dose (200 mg/kg) [2].

Experimental researches proved that doses of 20 and 30 mg Mg/kg reduced immobility time in the forced swim test exerting antidepressant-like activity. In the elevated plus-maze test, magnesium at the same doses produced an anxiolytic-like effect. The doses of magnesium active in both tests did not affect locomotor activity [10, 17].

Given the close conceptual associations between stress, anxiety and depression which are intrinsic to most animal models and testing paradigms, a reasonable hypothesis emerges whereby magnesium may be exerting its antidepressant effect through anxiolytic mechanisms [4].

Several clinical studies demonstrated a low serum magnesium level in depressed patients. The results indicate that magnesium induces the antidepressant- and anxiolytic-like effects without tolerance to these activities, which suggests a potential antidepressant and anxiolytic activity of magnesium in these disorders in humans. It was observed an association between severity of anxiety or depression and low plasma magnesium [1].

The goals of our study are the obtaining and the stabilization of lipid vesicles coated in chitosan which entrapped magnesium chloride inside aiming their use for *in vivo* testing by the effects of magnesium chloride soft matter vesicles carrier, in a behavioral model in mice.

MATERIALS AND METHODS

MATERIALS

The lipid used, Egg Yolk L-α-phosphatidylcholine (L-α-lecithin), approximately 99% (TLC) pure was obtained from Sigma Chemical Co, Germany, and magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$) from Chimopar SA, Romania.

The chitosan was provided by Vanson Chemicals Redmond WA, USA. The N-deacetylation degree was 79.7%, the average molecular weight was

M_w = 310,000 g/mol and the polydispersity index was 3.26. The 0.5 (*wt*/*wt*) chitosan homogeneous solutions were prepared in a 1% (*v*/*v)* acetic acid solution.

LIPID VESICLES PREPARATION

$MgCl_2 \cdot 6H_2O_{(cr)}$ was obtained from the supersaturated solution of $MgCl_2 \cdot 6H_2O$ by recrystallization at 160 °C for 24 hours. Lipid vesicles were prepared by dissolving 50 mg lipid in chloroform and solvent removing by evaporation, which led to a dry lipid film. The film was then hydrated using a solution made of distilled water and magnesium chloride (12 mg/mL $MgCl_{2\ (cr)}$). Assuming that all the lipid quantity remains in the system, the concentration from solution was of 0.5 mg/mL L-α-lecithin. To obtain unilamellar vesicles, the lipid solution was sonicated for 20 minutes. As we have done in [5], for a good stabilization of suspended vesicles, these were combined with 0.5% chitosan acidic solution in a ratio of 3/2 lipid vesicles/chitosan (*v*/*v*) solution. The solution of lipid vesicles, magnesium chloride and chitosan was dialyzed for 10 hours in order to reach a neutral pH.

The drug was dissolved in water, prepared immediately before use and was administered orally (using an esogastric tube) in the same volume of solution (0.3 mL).

MEASUREMENTS

The size distribution and Zeta potential measurements were determined using a Malvern Zetasizer Nano ZS, ZEN-3500 model.

Thermal stability was studied with the Mettler Toledo TGA-SDTA 851e apparatus. Thermogravimetric data were recorded in the temperature range of 20 °C to 600 °C, in air atmosphere, with a 10 °C/min heating rate.

The *in vivo* experiment was carried out on white male mice (20–25 g) distributed into 3 groups of 7 animals each, treated orally as follows: Group I: distilled water 0.3 mL (Control); Group II (Mg): magnesium chloride 1 mmol/ kilogram body weight (kbw); Group III (Mg vesicles): magnesium chloride 1 mmol/kbw entrapped in soft vesicles.

Standard laboratory food and tap water were freely available, except during the time of the experiments. Before the experiment mice were placed on a raised wire mesh, under a clear plastic box and allowed 2 hours to acclimatize to the testing room.

The mice psycho-motor abilities were tested in the LE-8811 Actimeter device (Panlab), in order to investigate both the global motor behavior and the number of escape attempts. This device that allows the study of spontaneous locomotors activity is a complete Infra Red microelectronic controlled and stands alone equipment (cages dimensions – 450 (W) × 450 (D) × 200 (H) mm), used to

measure free surface movements and rearing behavior on mouse or rat. The system is basically composed of a two dimensional square frame, a frame support and a control unit. Each frame counts with 16 × 16 infrared beams for optimal subject detection.

The system is completely modular: each frame may be used for evaluation of general activity (one or several animals), locomotor, rearings and stereotype movements. The infrared photocell system can be set with up to 15 levels of sensitivity in order to adapt the frames to the typology of the animal (rats, mice). The frames can be controlled by independent control units or directly through SeDaCom computer software, which allows easy exportation of data (through RS 232 serial port) in a format compatible with Excel™.

Mice were placed on the cage device and each movement produced a signal caused by variation in inductance and capacity of the apparatus resonance circuit. These signals were automatically converted to numbers. Horizontal or vertical activity is defined as the total number of beam interruptions over a certain period. In this experiment the horizontal and vertical mice movements were automatically counted in a 2 minutes period, every 2 hours. Each animal of the studied groups is subjected to successive tests (for the same two minutes interval), the animal being placed on the work surface and repeatedly replaced in its respective cage.

Values are recorded electronically and displayed according to prior programming an electronic system for data processing.

The data was presented as +/– standard deviation and significance was tested by SPSS Statistics for Windows version 13.0 and ANOVA (Analysis of Variance) method, followed by Neumann Keuls test as *post hoc*. P-values less than 0.05 are considered statistically significant as compared with those of the control group.

Experimental protocol was implemented, according to recommendations of the committee of research and ethics of "Gr.T. Popa" University of Medicine and Pharmacy. For ethical reasons, all the animals were sacrificed at the end of the experiment.

RESULTS AND DISCUSSION

MAGNESIUM CHLORIDE HEXAHYDRATE THERMAL ANALYSIS

Magnesium chloride hexahydrate is very soluble in water and dissociates into Mg^{2+} and Cl^{-}. To prevent complete dissociation it was recrystallized to 160 °C, this temperature being chosen according to its thermal degradation behavior, which is presented below.

It is well known that $MgCl_2 \cdot 6H_2O$ begin to lose water with the temperature increase beyond 116.7 °C [6]. As can be seen in Figure 1, $MgCl_2 \cdot 6H_2O$ showed a spectacular mass loss at 190 °C. This is due to the majority of its hydration water

loss at this temperature and the hydrolysis onset which give HCl following the reaction:

$$MgCl_2 \cdot 6H_2O \rightarrow Mg(OH)Cl + HCl_{(g)} + 5H_2O\,,$$

$$Mg(OH)Cl \rightarrow MgO + HCl_{(g)}\,.$$

However, at 480°C a minor mass loss is displayed in the TG curve.

These results allow us to recrystallize $MgCl_2 \cdot 6H_2O$ at 160 °C considering that the magnesium chloride hexahydrate does not suffer a major degradation but only a slight decrease of hydrated water molecules stoichiometry.

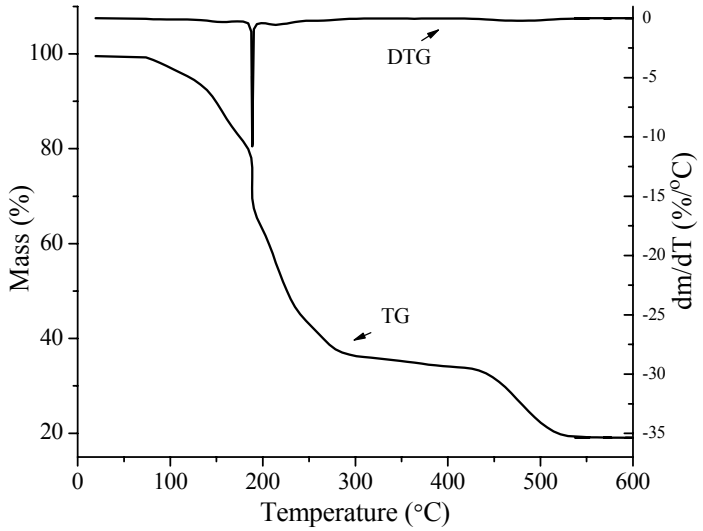

Fig. 1. TG and DTG curves for magnesium chloride hexahydrate.

VESICLES CHARACTERIZATION

The pH values for the systems used as control-sample and those with vesicles are given in Table 1.

Table 1

Solutions pH values

Solution	**pH**
Magnesium chloride	8.92
Lipid vesicles	8.31
Lipid + magnesium chloride vesicles	8.57
Lipid + magnesium chloride + chitosan vesicles (before dialysis)	3.63
Lipid + magnesium chloride + chitosan vesicles (after dialysis)	6.98

In Figure 2 are shown the size distributions of lipid vesicles prepared in different conditions related to the initial magnesium chloride hexahydrate crystallites size. The area under the curves cannot be correlated with the vesicle concentrations because the measurement apparatus procentually scales the maximum value of concentration. The average sizes of the systems are presented in Table 2. It can be observed that the $MgCl_2 \cdot 6H_2O$ nanocrystallites are present in the aqueous solution, these having an average size of 55.93 nm, which proves that there has not been a total dissociation of it. It can be seen that the magnesium chloride vesicles are smaller than the simple lipid vesicles and $MgCl_2 \cdot 6H_2O$ crystallites. This is due, on the one hand, to the $MgCl_2 \cdot 6H_2O$ crystallites size reduction by sonication and, on the other hand, to the ionic strength influence of the magnesium solution on the magnesium chloride vesicles stability. The magnesium chloride vesicles with chitosan have the biggest size. Thus, the lipid vesicles have an average size of 81.16 nm, the magnesium chloride vesicles of 36.89 nm and the magnesium chloride vesicles with chitosan of 129.56 nm.

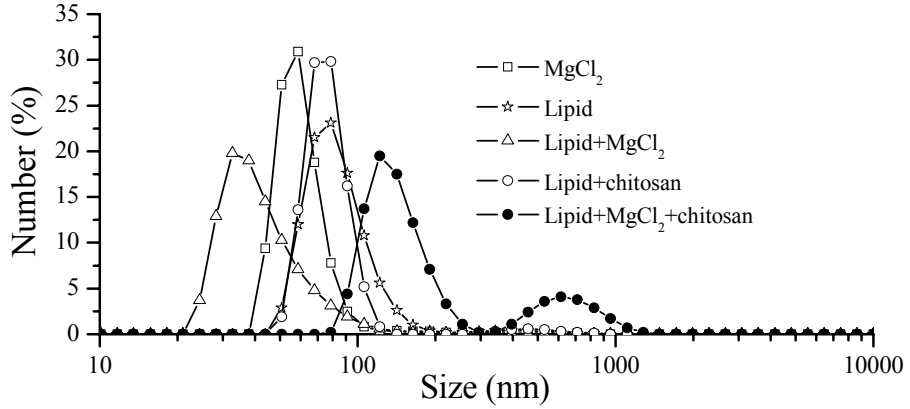

Fig. 2. Size distribution by number for the lipid vesicles solutions.

Table 2

Average values of the dimension and Zeta potential for the investigated systems

System	**Average dimension (nm)**
Magnesium chloride	55.93
Lipid vesicles	81.16
Lipid + magnesium chloride vesicles	36.89
Lipid + magnesium chloride + chitosan vesicles	129.56

In Figure 3 is shown the Zeta potential distribution of the same systems presented above.

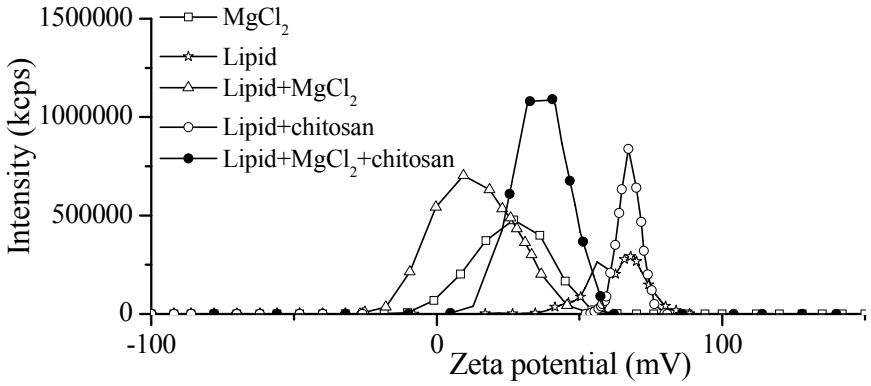

Fig. 3. Zeta potential distribution for the lipid vesicles solutions.

From the Zeta potential distribution (Fig. 3), we conclude that all the systems of vesicles made are predominantly positively charged.

The colloidal solutions stability is defined according to the average value of Zeta potential [11] (Table 3). We must specify that the stability conditions defined in Table 3 were established for suspension of a homogeneous positively spherical particles model, in which the affinity of the particle surface with water creates a structured surface water layer, and this structure changes considerably the properties of the particle – water – polymer from those in the bulk. This electrostatic field, in combination with the thermal motion of the ions, creates a countercharge, and thus screens the electric surface charge. The net electric charge in this screening diffuse layer is equal in magnitude to the net surface charge, but it has the opposite polarity. In the case of soft matter vesicles the surface charged is determined by the pH dependent protonation/deprotonation processes. The classification of positively charged systems stability is done in the same consideration and corresponds to the same domains as those from Table 3 with the opposite sign.

Table 3

Colloidal solutions stability – Zeta potential relation

Stability	**Average Zeta potential (mV)**
Extreme to very good stability	–100 to –60 mV
Reasonable stability	–60 to –40 mV
Moderate stability	–40 to –30 mV
Threshold of light dispersion	–30 to –15 mV
Threshold of agglomeration	–15 to –10 mV
Strong agglomeration & precipitation	–5 to +5 mV

Comparing the average values of Zeta potential from Table 2 of vesicles assemblies obtained at previously mentioned parameters with stability condition expressed in Table 3 it can be observed that the magnesium solution is at threshold of light dispersion (ZP = +25.3 mV) and lipid – magnesium vesicles are at threshold of agglomeration (ZP = +13.2 mV). In contrast, the lipid – magnesium vesicles confined with chitosan have a moderate stability (ZP = +36.1 mV). We can conclude that the systems correspond to the criteria of suspension solutions and the chitosan determines all the vesicles to become positively charged and more confined, which leads to repulsion forces between them.

IN VIVO TESTING

Using the behavioral experimental model, we obtained three types of information regarding the animal spontaneous motor activity: total, horizontal and respectively vertical movements.

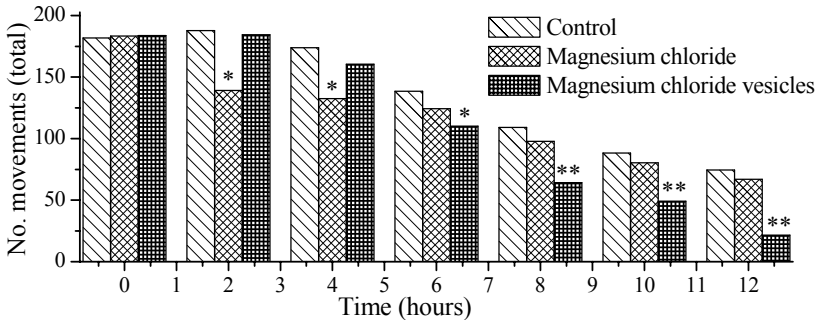

Fig. 4. Activity Cage – total movements. Each point is the mean ± SEM of number of total movements for seven mice. * $p < 0.05$ vs control; ** $p < 0.01$ vs control.

The number of total movements (Fig. 4) represents a feature that reflects the overall intensity of animal behavior in a certain period of time and is also a good way of corroboration and verification of data biological significance.

Magnesium chloride (1 mmol/kbw) determined a rapid and progressive reduction of mice total movements, statistically significant, as compared with the control group, only in the first 2 and 4 hours ($p < 0.05$) after oral administration.

In the same experimental conditions, oral administration of magnesium chloride entrapped in soft vesicles resulted in a decrease of mice total movements after 4 hours, statistically significant after 6 hours ($p < 0.05$) and more pronounced after 8 hours ($p < 0.01$), effect that kept on more than 12 hours ($p < 0.01$).

The number of movements in the horizontal plane provides data on the general behavior exploration of the environment, but also about the self-maintenance of animal personal hygiene (paw-licking, hair combing and nose cleaning by self-directed, possibly recuperative, behavior – autogrooming).

The results of spontaneous motor activity measurement can be extrapolated in a Gaussian curve (Fig. 5), in which the small values presented on the left side represent a strongly repressed expression of the behavior corresponding to psychomotor inhibition and sedation, and respectively, the high values presented on the right side typify the hyperactivity appropriate to psychomotor unrest and anxiety.

The fact that for the mice group that received magnesium chloride solution it cannot be obtained a Gaussian fit demonstrates, once again, that this method of administration is not efficient. The well known effect of magnesium chloride, mainly due to its higher solubility and rapid absorption, consists of a sudden decrease in motor activity in the first 2 hours after administration. After that, it can be considered as having a Gaussian-type evolution which, unfortunately, leads to a behavior with values slightly lower than those obtained in case of the control group who was given water.

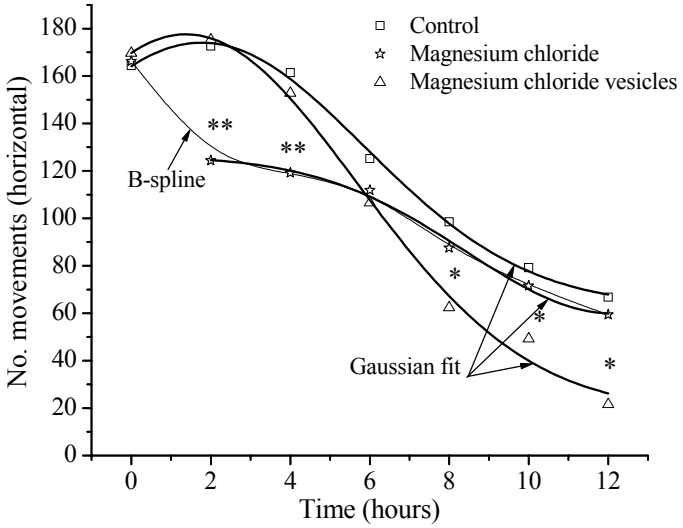

Fig. 5. Activity Cage – horizontal movements and Gaussian fit of spontaneous motor activity measurement. Each point is the mean ± SEM of number of horizontal movements for seven mice. * $p < 0.05$ vs control; ** $p < 0.01$ vs control.

For the mice group receiving magnesium chloride vesicles, the great majority of the records on the naive animal is included in the corresponding zone of bell curve and will reveal the average values suggestive for normal behavior.

Oral administration of magnesium chloride (1 mmol/kbw) produced a rapid and progressive reduction of mice horizontal movements, statistically significant as compared with control group, only in the first 2 and 4 hours ($p < 0.01$) of the measurement in Activity Cage test (Fig. 5). Using the magnesium chloride

entrapped in soft vesicles we obtained a decrease of mice horizontal movements after 4 hours, statistically significant after 8 hours ($p < 0.05$), and prolonged for more than 12 hours (0.05).

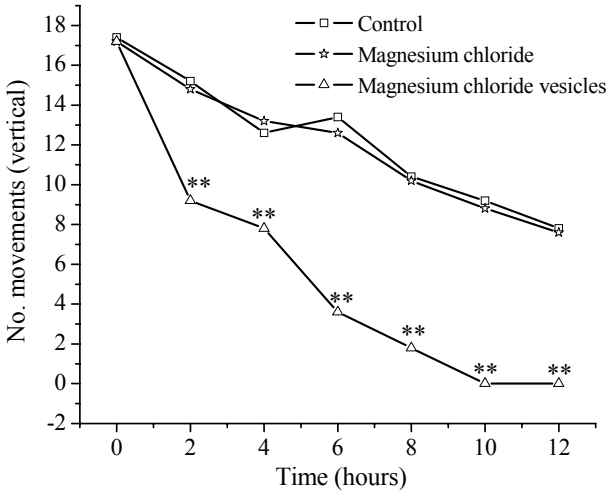

Fig. 6. Activity Cage – vertical movements. Each point is the mean ± SEM of number of vertical movements for seven mice. ** $p < 0.01$ *vs* control.

The number of movements in vertical plane can be also counted by direct observation. This parameter reveals the animal testing, trying to climb on the transparent walls of registration cage, and is suggestive for attempt behavior, a correspondent for the indirect fear and anxiety states.

Magnesium chloride (1 mmol/kbw) administered orally did not significantly influence mice vertical movements, as compared with the control group at any moments of behavior manifestations measurement.

Magnesium chloride entrapped in soft vesicles determined a decrease of mice vertical movements, statistically significant ($p < 0.01$), immediately after substance administration, and prolonged for more than 12 hours (Fig. 6).

The results of our experimental research proved that the use of soft matter vesicles as carrier for magnesium chloride presents the advantage of a sustained release of drug as compared with non entrapped substance, presumably due to the particularities of the active substance release from the vesicles carrier.

CONCLUSIONS

The present paper describes an original method to entrap magnesium chloride in soft vesicles and the effects of this new carrier system on animal psycho-motor abilities. We achieved a method of magnesium chloride incorporation into vesicles which will transport and control their release in the animal body.

The size distribution measurements reveal that the dimension of the vesicles varies from tens of nanometers to hundreds depending on the concentrations and the physical parameters of precursors.

By the addition of associating chitosan, the size and morphology are changed. Also, chitosan determines all the vesicles to become positively charged and more confined, which leads to repulsion forces between them. Therefore, the systems are more stable.

Using a behavioral model to evaluate exploratory activity and motor coordination in mice, we determined that in our experimental conditions, oral administration of 1 mmol/kbw magnesium chloride entrapped in soft vesicles resulted in a prolonged reduction of vertical movements number and those in horizontal plane, both variations being statistically significant, as compared with the control group and with the same dose of magnesium chloride non entrapped.

These results reflect a decrease of escape attempts number, within the context, significant diminution of exploratory and self-maintenance spontaneous behavior. Corroboration of the two changes observed provides a type of behavior that at humans corresponds to the decrease of anxiety.

Our results show that in the mouse model of behavioral manifestations used, magnesium soft matter vesicles carrier diminished both global motor behavior and the number of escape attempts, which could correspond somehow to general behavioral inhibition or to sedation in humans.

Extrapolation of these results is however difficult, because of the huge differences in behavior, cognitive function and motor coordination skills, between lab animal and human.

Acknowledgements. This study was financially supported by PN II TD 17/2008.

REFERENCES

1. DECOLLOGNE, S., A. TOMAS, C. LECERF, E. ADAMOWICZ, M. SEMAN, NMDA receptor complex blockade by oral administration of magnesium: comparison with MK-801, *Pharmacol. Biochem. Behav.*, 1997, **58**, 261–268.
2. DURLACH, J., M. BARA, *Le magnésium en biologie et en médecine*, Ed. Med. Int. Paris, 2000.
3. DURLACH, J., P. BAC, M. BARA, A. GUIET-BARA, Physiopathology of symptomatic and latent forms of central nervous system hyperexcitability due to magnesium deficiency: a current general scheme, *Magnes. Res.*, 2000, **13**, 293–302.
4. FROMM, L., B. PHARM, D.L. HEATH, R. VINK, A.J. NIMMO, Magnesium attenuates post-traumatic depression/anxiety following diffuse traumatic brain injury in rats, *J. Am. Coll. Nutr.*, 2004, **23**, 529S–533S.
5. GÂRLEA, A., M.I. POPA, V. POHOAȚĂ, V. MELNIG, Ibuprofen/ketoprofen entrapment in chitosan based vesicle carrier, *Rom. J. Biophys*, 2007, **17**, 157–168.
6. HOLLEMAN, A.F., E. WIBERG, *Inorganic Chemistry*, Academic Press, San Diego, 2001.
7. LANGLEY, W.F., D. MANN, Central nervous system magnesium deficiency, *Arch. Int. Med.*, 1991, **151**, 593–596.

8. MOUSAIN-BOSC, M., M. ROCHE, J. RAPIN, J.P. BAL, Magnesium VitB6 intake reduces central nervous system hyperexcitability in children, *J. Am. Coll. Nutr.*, 2004, **23**, 545S–548S.
9. NECHIFOR, M., P.J. PORR, *Magnesium Involvements in Biology and Pharmacotherapy*, Casa Cărții de Știință, Cluj-Napoca, 2003, 160–177.
10. POLESZAK, E., B. SZEWCZYK, E. KDZIERSKA, P. WLAŹ, A. PILC, G. NOWAK, Antidepressant- and anxiolytic-like activity of magnesium in mice, *Pharmacol. Biochem. Behav.*, 2004, **78**, 7–12.
11. RIDDICK, T.M., Control of Colloid Stability through Zeta Potential, Livingston Pub. Co., 1968.
12. SARIS, N.L., E. MERVAALA, H. KARPPANEN, J.A. KHAWAJA, A. LEWENSTAM, Magnesium: an update on physiological, clinical and analytical concepts, *Clin. Chim. Acta*, 2000, **294**, 1–26.
13. SINGEWALD, N., C. SINNER, A. HETZENAUER, S.B. SARTORI, H. MURCK, Magnesium-deficient diet alters depression- and anxiety-related behavior in mice—influence of desipramine and *Hypericum perforatum* extract, *Neuropharmacol.*, 2004, **47**, 1189–1197.
14. TURNER, R.J., R. VINK, Magnesium in the central nervous system, in: *New Perspectives in Magnesium Research – Nutrition and Health*, Y. Nishizawa, H. Morii, J. Durlach Eds., Springer, London Pub., 2007, Part 10, 338–355.
15. WERBACH, M.R., Nutritional influences on aggressive behavior, *J. Orthomol. Med.*, 1995, **7**, 45–51.
16. WONG, E.H.F., J.A. KEMP, Sites for antagonism of the N-methyl-D-aspartate receptor channel complex, *Annu. Rev. Pharmacol. Toxicol*., 1991, **31**, 401–425.
17. ZIEBA, A., R. KATA, D. DUDEK, M. SCHLEGEL-ZAWADZKA, G. NOWAK, Serum trace elements in animal models and human depression: III. Magnesium. Relationship with copper, *Hum. Psychopharmacol. Clin. Exp.*, 2000, **15**, 631–635.