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The Neuroprotective Ability of Polyethylene Glycol is Affected by Temperature in Ex Vivo Spinal Cord Injury Model

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Abstract Immediate membrane sealing after spinal cord injury (SCI) can prevent further degradation and result in ultimate functional recovery. It has been reported that polyethylene glycol (PEG) can repair membrane damage caused by mechanical insults to the spinal cord. Furthermore, membrane fluidity and its sealing process vary at different temperatures. Here, we have assessed the possible synergistic effects of PEG and temperature on the repair of neural membranes in an SCI model. The effects of PEGs (400, 1,000 and 2,000 Da) were studied at different temperatures (25, 37 and 40 °C) by means of compound action potential (CAP) recovery and a lactate dehydrogenase (LDH) assay. Isolated spinal cords were mounted in a double sucrose gap chamber, where the amplitude and area of CAPs were recorded after implementing injury, in the presence and absence of PEG. Moreover, the LDH assay was used to assess the effects of PEG on membrane resealing. Data showed that the least CAP recovery occurred at 25 °C, followed by 37 and 40 °C, in all treated groups. Moreover, maximum CAP amplitude recovery, 65.46 ± 5.04 %, was monitored in the presence of PEG400 at 40 °C, followed by 41.49 ± 2.41 % in PEG1000 and 37.36 ± 1.62 % in PEG2000. Furthermore, raising the temperature from 37 to 40 °C significantly increased CAP recovery in the PEG2000 group. Similar recovery patterns were obtained by CAP area measurements and LDH assay. The results suggest that application of low-molecular weight PEG (PEG400) in mild hyperthermia conditions (40 °C) provides the optimum condition for membrane sealing in SCI model.

Keywords Double sucrose gap technique · Compound action potential (CAP) · Membrane sealing · Hyperthermia

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Introduction

Following acute spinal cord injury (SCI), two broad pathophysiological events happen: primary structural injury and a delayed secondary injury (Profyris et al. 2004; Young 1993). Direct mechanical trauma that causes primary injury results in local destruction of neural tissue. However, the axonal tracts that are left intact by the primary injury receive a progressive wave of secondary injury that is more profound and leads to the loss of nerve impulse conduction and ultimate destruction (Beattie et al. 2002). Proposed mechanisms reported for the delayed damage include ischemia, abnormal exchange of intracellular ions (e.g., Na^+ and Ca^{2+}), production of reactive oxygen species (Luo et al. 2002b), lipid peroxidation and excitotoxicity (Baptiste and Fehlings 2006). Thus, neuroprotective and regenerative strategies should be implemented immediately after primary insult to limit further tissue loss.

It has been shown that the destruction of neuronal membrane caused by mechanical injury plays a critical role in the mentioned destructive processes (Luo et al. 2002a) and causes failure of the axolemma to function as a smart barrier in the prevention of unregulated exchange of ions (Honmou and Young 1995). Thus, the immediate sealing of membrane lesions might rescue neurons from progressive (Profyris et al. 2004) degeneration. Different approaches aimed at resealing the disrupted membranes have been

effective at preventing tissue loss (Shi and Borgens 2000; Duerstock and Borgens 2002), restoring functional recovery and increasing the survival rate of injured neurons following central nervous system or peripheral nervous system traumatic injury (Borgens et al. 2002; Britt et al. 2010).

Biomaterials such as poloxamer188, poloxamine1107 and polyethylene glycol (PEG) are polymers and surfactants that possess sealing capability; and they have been widely used in the treatment of SCI (Hannig et al. 1999, 2000; Luo et al. 2002a). The neuroprotective effects of PEG, a biodegradable hydrophilic polymer, and its capability to seal membrane ruptures have been approved in SCI models (Luo et al. 2004; Luo and Shi 2007). Furthermore, improved neurological function has been reported in both in vivo and in vitro SCI models following PEG application (Borgens et al. 2002; Laverty et al. 2004; Nomura et al. 2006; Spaeth et al. 2012). Previous reports have shown that the effects of PEG on damaged membranes and cell fusion are exerted in a size- and concentration-dependent manner (Vijayalakshmi et al. 1999; Nakajima and Ikada 1995; Davidson and Gerald 1976). However, there are studies that do not support the significant effect of concentration and molecular weight of the applied PEGs on the sealing and fusion of membranes (Shi and Borgens 1999, 2000, 2001).

Hypothermia decreases basic enzymatic activity, reduces the need for energy in the cell and, thus, saves the cell's ATP supplies. As a result, hypothermia has shown therapeutic effects and caused improved recovery in various types of neural injury, due to its obstructing role against the spread of damage caused by primary injury (Biagas and Gaeta 1998; Hansebout et al. 1984). However, animal studies focused on SCIs have not shown a consistent neuroprotective effect of hypothermia, either locally or systemically (Kwon et al. 2008). Membrane sealing is inhibited at low temperatures in invertebrate giant axons (Yawo and Kuno 1985) and adult mammalian spinal cord fibers (Shi and Pryor 2000). Nehrt et al. (2010) reported that if hypothermic treatment is necessary and desired, application of PEG (2,000 Da) at lower temperature (25 °C) is more effective than hypothermia in the absence of PEG.

To our knowledge, the combined potential effects of PEG, in different sizes and hypo-/hyperthermic conditions, on membrane sealing in damaged spinal cords have not been reported so far. The present study was designed to assess the effects of PEGs (400, 1,000 and 2,000 Da) at three different temperatures (25, 37 and 40 °C) on the repair of disrupted membrane. The effects were evaluated based on the extent of recovery of compound action potential (CAP) amplitude and area in an ex vivo model of SCI. In addition, a lactate dehydrogenase (LDH) assay was used to evaluate the extent of membrane resealing after PEG application.

Materials and Methods

In these experiments, we made every effort to minimize the suffering and the number of animals used. The experimental protocols were reviewed and approved by the Faculty of Veterinary Medicine, University of Tehran, and the Society for Prevention of Cruelty to Animals.

Spinal Cord Preparation

A total of 47 adult male Wistar rats (weight 250–300 g) were deeply anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine (12 mg/kg) (Alfasan, Woerden, the Netherlands). To remove blood and lower the cord temperature, transcardial perfusion was performed using 200 ml oxygenated cold Krebs solution (NaCl 124 mM, KCl 2 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.3 mM, $CaCl_2$ 2 mM, dextrose 20 mM, sodium ascorbate 10 mM, $NaHCO_3$ 26 mM; Sigma, St. Louis, MO). Following rapid excision of the spine, a complete laminectomy was performed to expose the spinal cord. The spinal cord was then removed from the vertebrae, stabilized by incubation in oxygenated Krebs solution and bubbled with an O_2/CO_2 gas mixture for 1 h at room temperature. The whole procedure was based on the preparation method of white matter strips in guinea pigs (Shi and Borgens 2000; Nehrt et al. 2010).

Ex Vivo Recording Chamber

The double sucrose gap chamber constructed of Plexiglass™ consists of three large compartments (each with a volume of 2 ml) separated by two narrow reservoirs (each with a volume of 1 ml), as described previously (Peasley and Shi 2003) (Fig. 1). The middle compartment, representing extracellular medium, was filled with oxygenated Krebs solution; and the other two compartments, at either end, which were in contact with the cytosol of the spinal cord fibers, were filled with isotonic potassium chloride (120 mM). The separating compartments were filled with nonconductive isotonic sucrose, in order to separate the intracellular medium of the spinal cord fibers from the extracellular side, electrically. Complete isolation of the compartments was achieved with silicone grease. Peristaltic pumps continuously circulated the oxygenated Krebs solution and isotonic sucrose in their respective compartments. Stimulation of the spinal cord at the proximal end produced CAP signals, obtained from the summation of action potentials from many different axons that

were recorded by silver/silver chloride electrodes at the distal end. The middle reservoir was grounded.

Intact spinal cord fibers were stimulated by square, unipolar current pulses, 0.1 ms in duration and 0.8 V in amplitude; and they produced typical CAP responses, with a peak amplitude of 87.66 ± 7.4 μV (Fig. 2).

Stimulation, amplification and recordings were made by an ADIMI 1484 Signal Generator-Amplifier-Recorder (ADIMI, Tehran, Iran). Compression injury was induced by compressing the spinal cord in the middle compartment of the chamber with a calibrated titanium clip (FT 726T; Aesculap, Tuttlingen, Germany), applying 1.16 N at the L_{11} level for 15 s, which caused a drastic decline in CAP conduction.

PEG Application

PEGs (400, 1,000 and 2,000 Da) were obtained from Sigma and diluted in Krebs solution (50 % w/w). After injury, the compressed spinal cord, located in the central bath, was incubated with PEG for 3–5 min. Then, the PEG solution was removed and substituted with oxygenated Krebs solution. Continuous recording of CAP signals was carried out before injury, after injury and after the PEG treatment stages.

Measurement of CAP Amplitude and Area

The nerve conduction (CAP) of the isolated spinal cord sealed in the chamber was recorded before compression, as preinjury, and compared with CAP after compression both in the presence and in the absence of PEG at three different temperatures (25, 37 and 40 °C). The effect of temperature on CAP recovery was examined prior to the introduction of PEGs to the chamber. The stimuli were repeated five times, and the averaged value was used. To quantify the CAP results, the area under the curve (CAP area) and the peak amplitude of the CAP were used by different groups (Stys et al. 1991; Pryor and Shi 2006). CAP area was measured by integrating the area under the CAP peak from the beginning of peak 1–2 ms. Amplitude was measured from the peak of one polarity to the next peak of the opposite polarity (Fig. 2). The ratios between CAP amplitude and CAP area before and after compression and after PEG treatment were calculated in different temperature groups and represented as CAP recovery percentage.

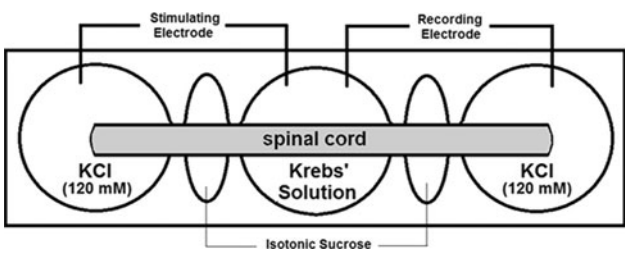

Fig. 1 Schematic CAP recording setup. Oxygenated Krebs solution and isotonic sucrose were perfused continuously by peristaltic pumps via tubing connected to each compartment. Compression of the spinal cord at the L_{11} level, positioned in the middle compartment, was performed with a calibrated clip exerting a closing force of 1.16 N. The silver electrodes used to apply the stimulating signal and to record CAP are also shown. The middle compartment was grounded

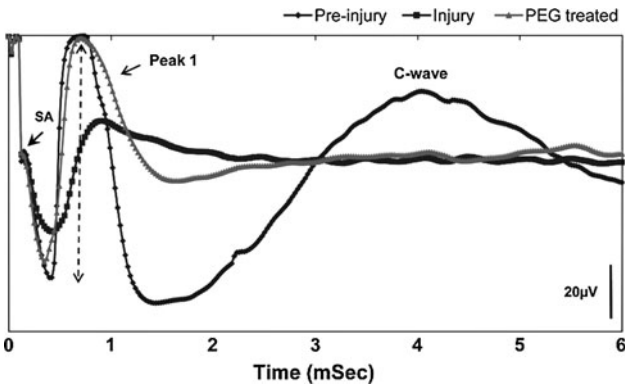

Fig. 2 Representative CAP signals recorded in the isolated spinal cord in the preinjury state, after compression injury and after treatment with PEG1000 at 40 °C. CAP partially recovered after PEG treatment ($n = 5$). Amplitude was measured from the peak of one polarity to the next peak of the opposite polarity, as shown by the *dashed arrow* C-wave reflects the activity of nonmyelinated axons. *SA* stimulation artifact

Assessment of Membrane Permeability and Resealing

Membrane integrity and resealing were evaluated by the LDH, 9–160 kDa, assay. LDH is a cytoplasmic enzyme that is normally present in low concentrations in the extracellular fluid. Leakage of this molecule into the extracellular space is indicative of membrane disruption (Koh and Choi 1987). This assay is based on the reduction of NAD by the action of LDH to form a tetrazolium dye, which is measured spectrophotometrically at 492 nm. Here, 10 μl of the solution bathing the spinal cords was collected before injury, after compression injury and after PEG treatment; and the LDH activities were measured using an LDH assay kit (Sigma). Modified Krebs solution was used here in order to avoid interference of sodium ascorbate during the LDH assay. The amount of released LDH was expressed as a percentage of the control (injured/untreated) group.

Statistical Analysis

The amplitude and area of every CAP were analyzed and expressed as mean ± SD. The results were statistically

evaluated using Student's t tests, and differences were considered statistically significant at $p < 0.05$. ANOVA was used to assess the CAP recovery between groups treated with different PEGs and at different temperatures.

Results

Control CAPs recorded in intact, isolated spinal cords showed the typical pattern and proved the reliability of the technique. Fast peak (peak 1) and C-wave, which reflects the activity of nonmyelinated axons (Velumian et al. 2010), were recorded. The compression injury caused a sudden decline in CAP amplitude and area, which were partially recovered after PEG treatment (Fig. 2). However, the C-wave that disappeared after injury was not recovered after PEG treatment.

The combined effects of PEG and temperature were also studied for three different molecular weights of PEG (400, 1,000 and 2,000 Da) at three temperatures (25, 37 and 40 °C). CAP amplitudes were calculated preinjury, after compression and after PEG treatment condition. Figure 3 shows a comparison between the CAP amplitude recoveries (%) in the different groups.

As shown in Fig. 3, the percentage of CAP amplitude recovery, compared with the control group, declined in all three treatment groups as the size of the applied PEG was increased. Moreover, in general, higher recovery was monitored at higher temperatures in all groups treated with different sizes of PEG. For instance, in the PEG2000-treated group, CAP recovery at 40 °C (37.36 ± 1.6 %) was significantly ($p < 0.05$) higher than in the groups treated at 37 °C (33.28 ± 2 %) and 25 °C (16.32 ± 6.3 %). The results of CAP amplitude measurement showed no

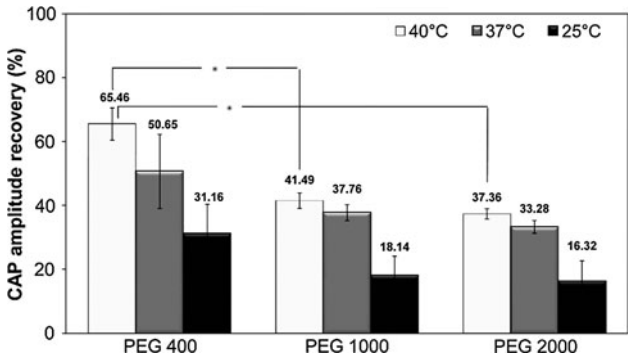

Fig. 3 CAP amplitude recovery (%) in groups treated with PEGs of different molecular weights (400, 1,000 and 2,000 Da, 50 % w/w) at different temperatures (25, 37 and 40 °C). The results of CAP recovery after treatment with PEGs, compared with the control group (injured/untreated), showed the effectiveness of treatment with smaller PEG (400 Da) at 40 °C on CAP recovery (65.46 ± 5 %, $n = 6$) versus PEG1000 (41.49 ± 2.4 %, $n = 5$) and PEG2000 (37.36 ± 1.6 %, $n = 5$). *$p < 0.001$

significant differences between groups treated with different PEG molecular weights at low temperature (25 °C).

In the hypothermic (25 °C) condition, recovery was less than at 37 and 40 °C, i.e., 31.16 ± 9.2 % ($p = 0.44$, $n = 5$), 18.14 ± 5.9 % ($p < 0.05$, $n = 6$) and 16.32 ± 6.3 % ($p < 0.05$, $n = 5$) for PEG400, PEG1000 and PEG2000, respectively.

The extent of CAP amplitude recovery in the presence of PEG400 at 40 °C (65.46 ± 5 %, $n = 6$) was significantly ($p < 0.001$) enhanced compared with PEG1000 (41.49 ± 2.4 %, $n = 5$) and PEG2000 (37.36 ± 1.6 %, $n = 5$) at the same temperature.

The differences between recoveries at 37 and 40 °C in the PEG400 and PEG1000 groups were not significant. However, increasing the temperature from 37 to 40 °C affected CAP amplitude recovery in the PEG2000 group ($p < 0.05$) and increased the recovery from 33.28 ± 2 % ($n = 5$) to 37.36 ± 1.6 % ($n = 5$).

Spinal cord conduction was quantified using CAP area (area under the curve). The results showed a basic pattern similar to that of CAP amplitude recovery measurements (Fig. 4). The CAP area decreased to 9.12 ± 3.6 % ($n = 6$) of the preinjury level as a result of compression injury and recovered following PEG treatment. CAP area recoveries were compared with the control (injured/untreated) group and depicted as a percentage of preinjury (Fig. 4). As shown in this figure, CAP area recoveries decreased with decreasing temperature in all three molecular weight groups. In the PEG400-treated group, CAP area recovery at 40 °C was higher (49.64 ± 5.2 %, $n = 5$) than the recoveries at 37 °C (38.27 ± 4.5 %, $n = 5$) and 25 °C (16.97 ± 6.8 %, $n = 5$). In the PEG1000- and PEG2000-treated groups, significant differences were observed between recoveries at 40 and 25 °C. In addition, the highest CAP area recovery was observed in PEG400 at 40 °C (49.64 ± 5.2 %, $n = 5$), which was significantly higher than PEG1000 (26.3 ± 7.1 %, $n = 5$) and PEG2000 (17.82 ± 5.9 %, $n = 5$) at 40 °C. Further, in the PEG1000 and PEG2000 groups, no significant differences were observed between 25 and 37 °C.

In our experiment, membrane resealing was indexed by the release of LDH from cytosol to the extracellular medium. Results were expressed as a percentage of control (injured/untreated) values ± SD. Decreased LDH release in the damaged spinal cord indicates membrane repair as a result of PEG treatment in all treated groups (Table 1). This reduction of LDH release is most prominent in the PEG400-treated group at 40 °C, resulting in a reduction of 14.51 ± 3.1 versus 29.06 ± 2.3 % and 31.44 ± 5.6 % in the PEG1000- and PEG2000-treated groups, respectively.

However, the LDH assay results showed that there were no significant differences in resealing effects between the PEG1000 and PEG2000 groups at different temperatures.

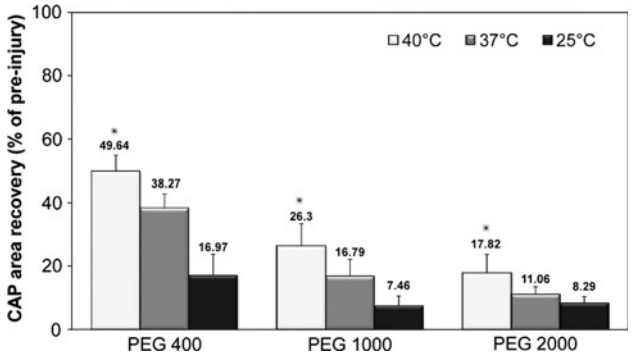

Fig. 4 CAP area recoveries after PEG treatment at different temperatures. CAP area compared with control was presented as a percentage of preinjury value. Note the significant differences between the PEG400 treatment group and the PEG1000 and PEG2000 groups at 40 °C. *$p < 0.05$

Discussion

PEG is a hydrophilic and fusogenic polymer that has been shown to be effective in the sealing of damaged membranes and ceasing of apoptosis and oxidative stress (Luo et al. 2002a; Luo and Shi 2007). Small PEGs form a clear, viscous liquid; but larger ones make waxy solids at room temperature. In addition, PEG solubility in water is inversely proportional to molecular weight. Due to its amphipathic nature, the properties of PEG depend on the temperature and the amount of accessible water; the kinematic viscosity of PEG decreases as temperature increases (Zwirbla et al. 2005). On the other hand, it has been reported that PEG increases membrane component mobility (Nehrt et al. 2010).

Changing the physical parameters, such as temperature, curvature and surface tension, is also known to promote membrane fusion. Temperature acts as a fusion inducer by increasing the density of defect in certain parts of the membranes and, under specific conditions, by decreasing the intermembrane separation (Cevc and Richardsen 1999). In addition, it can affect membrane sealing by changing the fluidity and mobility of membrane constituents, thereby affecting the activity of certain membrane enzymes, such as phospholipase A_2, which triggers the sealing process (Shi and Pryor 2000; Yawo and Kuno 1985).

In this study, CAP amplitude and area recovery of isolated, injured spinal cord treated with PEG in three sizes and at three different temperatures were investigated using the double sucrose gap technique. Our data showed that CAP recovery at 25 °C in all treated groups was lower than recovery with incubation at 37 and 40 °C. This is consistent with the findings of Shi and Pryor (2000), who showed that hypothermia decreases membrane resealing in guinea pig spinal cord.

We have shown the repairing effect of PEGs at different temperatures (25, 37 and 40 °C). Nehrt et al. (2010) showed that PEG2000 was effective under the clinically relevant conditions of low Ca^{2+} and temperature at enhancing axolemmal resealing in strips of white mater in guinea pig.

According to our CAP amplitude recovery results, increasing temperature enhances the sealing ability of PEG in all studied groups, especially in PEG2000 ($p < 0.05$). Increased membrane fluidity and the resulting higher lateral diffusion rates facilitate optimal packing of membrane lipids and PEG molecules. The physical properties, hydration and orientation of PEG at different temperatures should have played a certain role in the sealing ability monitored here. One possible explanation for significantly better sealing at 40 °C in the PEG2000-treated group is that PEG solubility in water increases at higher temperatures and, consequently, accessibility of polymer to the damaged site of the membrane is facilitated. Hence, more PEGs diffuse into the spinal cord core, where they seal the damaged membranes of internal fibers, leading to higher recovery in CAP amplitude. However, further study is needed to elucidate the exact diffusion rate and the temperature gradients set toward the core of the spinal cord, to address the role of PEG in sealing core fibers.

On the other hand the sensitivity of different neural tissues to temperature is different and depends on their membrane lipid compositions and their corresponding phase transition temperature, myelination level, cytoskeleton distribution degree as well as its constituent molecules and their interactions with membrane, membrane proteins and so on that altogether shape the heat capacity of cells. Consequently, the extent of intercellular spaces and membrane integrity, an intrinsic characteristic of every

Table 1 Membrane sealing ability of PEG at different temperatures, revealed by the LDH assay and CAP recovery

	PEG400			PEG1000			PEG2000		
Temperature (°C)	25	37	40	25	37	40	25	37	40
LDH release (% of control)	24.68 ± 3.4	18.26 ± 2.5	14.51 ± 3.1*	37.13 ± 6.6	32.63 ± 7.9	29.06 ± 2.3	40.18 ± 5.9	35.31 ± 1.5	31.44 ± 5.6
CAP amplitude recovery (%)	31.16 ± 9.2	50.65 ± 11.6	65.46 ± 5**	18.14 ± 5.9	37.76 ± 2.5	41.49 ± 2.4**	16.32 ± 6.3	33.28 ± 2	37.36 ± 1.6**
CAP area recovery (%)	16.97 ± 6.8	38.27 ± 4.5	49.64 ± 5.2*	7.46 ± 3.1	16.79 ± 5.3	26.3 ± 7.1*	8.29 ± 2.1	11.06 ± 2.4	17.82 ± 5.9*

The amount of released LDH was expressed as a percentage of the control (injured/untreated) group (*$p < 0.05$; **$p < 0.001$)

tissue that is partially manifested by heat capacity, is varied at different temperatures. Our studies show that larger PEGs (PEG2000) require a higher temperature to repair the damaged sites in whole spinal cord samples, while repair of white matter strips of spinal cord in guinea pig took place at low temperature (Nehrt et al. 2010). The difference between the extent of recovery after application of PEG2000 in our study and data reported before (Nehrt et al. 2010) might reflect the importance of the structure of the spinal cord used in these experiments and the accessibility of large polymers to the damaged fibers. In our experiment we used whole spinal cord in an ex vivo preparation. Thus, the solubility and accessibility of PEG to the core of damaged spinal cord fibers played a critical role compared with spinal cord white matter strip preparations used in the mentioned studies. This solubility and accessibility were affected by increasing temperature, as we show in Fig. 3.

Moreover, our results showed that there is a significant difference ($p < 0.001$) between PEG400 and groups treated with PEG1000 and PEG2000 at 40 °C, which highlights the effect of PEG size on CAP amplitude recovery. This phenomenon is probably due to the fact that physical properties of PEGs differ according to their sizes, which can influence their membrane sealing ability. C-wave, the slower conducting component of the CAP profile, disappeared after compression injury, which reflects injury to nonmyelinated axons. Recovery of this component was not observed in any of the PEG treatment groups, which may reflect the ineffectiveness of PEG treatment in our experimental conditions on these kinds of axons.

Assessment of CAP areas in different treatment groups showed a pattern similar to the CAP amplitude results, which is consistent with a previous report that showed the complementary role of these two characteristics of the CAP profile and the equivalency of these two methods of CAP assessment (Pryor and Shi 2006). Thus, we used the CAP area to show that the increasing trend of membrane sealing is in line with increasing temperature and decreasing the size of PEGs used.

Based on the LDH assay, we found significant leakage of LDH after SCI, due to membrane rupture. Following PEG treatment, the decreased level of extracellular LDH (Table 1) demonstrated PEG-mediated membrane sealing, especially in the PEG400-treated group, which reduced the released LDH to 14.51 ± 3.1 % of the injured/untreated level. These results confirmed the obtained CAP recovery patterns in the different treatment groups.

Conclusions

In conclusion, treating injured spinal cord with PEG at higher temperatures (up to mild hyperthermia) can enhance CAP recovery and membrane resealing. We noticed that the low-molecular weight PEG (PEG400) at 40 °C displayed a greater sealing effect than did the larger PEGs (1,000 and 2,000 Da). Consequently, treatment of damaged spinal cord with small PEGs at 40 °C is considered an effective means in our SCI model, and it should be elaborated further in order to set criteria for treating SCIs in the future.

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Conflict of interest The authors report no conflicts of interest.

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