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Prolonged focal application of polyethylene glycol induces conduction block in guinea pig spinal cord white matter

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Abstract

A 2-min focal application of Polyethylene Glycol (PEG) to injured mammalian spinal cords can offer significant yet limited restoration of functional and structural integrity. However, longer application of PEG has not been tested in similar injuries. In this study, isolated spinal cord white matter strips from adult guinea pigs were subjected to a 25 min exposure of PEG (MW: 2000; 50% w/w), with or without prior compression. When applied in a continuous steam, PEG, with a delay of about 6 min, suppressed the compound action potential (CAP) amplitude to 64 ± 4% of the pre-PEG level in uninjured cords and to 64 ± 7% in compressed cord strips. Both recovered to 70 ± 5% (uninjured) and 88 ± 11% (compressed) of the pre-PEG level following wash. When PEG was applied in a pulsatile manner, no significant decrease of CAP amplitude was observed.

In summary, our results show that focal continuous application of PEG has minimal toxicity if applied for less than 5 min. Pulsatile application could extend this duration to at least 25 min with no toxicity. This study could be useful in determining the optimal protocol for the use of PEG in both animal research and human spinal cord victims.

Keywords: Axon; Membrane fusion; Neuronal toxicity; Anoxia; Sucrose gap recording

1. Introduction

Polyethylene glycol (PEG) is a hydrophilic polymer capable of fusing cell membranes (Davidson et al., 1976; O'Lague and Huttner, 1980; Lee and Lentz, 1997). A 2-min application of PEG (MW: ~2000; 50% solution, w/w) to transected or crushed mammalian spinal cords can partially restore functional and structural integrity (Shi and Borgens, 1999a,b, 2000). This beneficial effect has been attributed to its ability to repair damaged axonal membrane following mechanical insult, as evidenced by horsradish peroxidase exclusion (Shi and Borgens, 2000). However, in the case of PEG-induced reconnection of completely severed cord strips, the success rate is only about 5%. Specifically, after complete transection, only 5% of the axons can be reconnected and begin to function again following focal application of PEG (Shi et al., 1999). Therefore, an improved method that will render more axonal reconnection is clearly warranted.

One possibility to potentiate the PEG effect is to increase the duration of PEG exposure. The potential benefit and toxicity of a longer period of application of PEG, however, has not been tested with such injuries. Due to the very nature of a fusogen, it is also logical to speculate that excessive exposure of PEG may lead to derangement of axonal membranes by inducing unwanted membrane fusion. One such scenario is a

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significant lateral membrane fusion between adjacent axons. Therefore, despite possible benefits, prolonged application of PEG may directly cause axonal damage, or mask the beneficial effects of PEG.

The goal of this study was to investigate whether a longer-than-2 min application of PEG to isolated guinea pig spinal cord white matter could inflict functional loss. More specifically, we would like to know whether a 25 min application of PEG, which is approximately 10 times the effective dosage used in our previous studies (Shi and Borgens, 1999a,b), will inflict irreversible CAP reduction. At the completion of this study, we also expect to determine the upper limit of the duration of the non-toxic level of focal PEG application.

2. Materials and methods

2.1. Isolation of the spinal cord

All animals used in this study were handled in strict accordance with the NIH guide lines for the *Care and Use of Laboratory Animals* and the experimental protocol was approved by the Purdue Animal Care and Usage Committee. In these experiments, every effort was made to reduce the number and suffering of the animals used. The technique for isolation of the cord was similar to that described previously (Shi and Blight, 1996, 1997; Shi and Borgens, 1999b; Shi et al., 2000; Shi and Pryor, 2000, 2002). Adult female guinea pigs were anesthetized with a combination of ketamine (80 mg/kg) and xylazine (12 mg/kg), and perfused with oxygenated, cold Krebs' (15 °C) solution to remove blood and to lower the cord temperature. The entire vertebral column was excised rapidly and the spinal cord was removed from the vertebrae and immersed in cold Krebs' solution. The cord was then subdivided to produce ventral white matter strips that were subsequently incubated in fresh Krebs' solution at room temperature for 1 h, and bubbled continuously with 95% oxygen, 5% carbon dioxide (Fig. 1). The composition of the Krebs' solution was as follows (in mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2 $CaCl_2$, 20 glucose, 10 sodium ascorbate, and 26 $NaHCO_3$, equilibrated with 95% O_2–5% CO_2 to produce a pH of 7.2–7.4.

2.2. Recording chamber

Various configurations of the basic recording chamber have been described in previous publications (Shi and Blight, 1996; Shi and Borgens, 1999b; Shi and Pryor, 2002). Briefly, a strip of isolated spinal cord white matter, approximately 35 mm in length was supported in the three-compartment chamber. The central compartment, ~12 mm in diameter, was continuously superfused

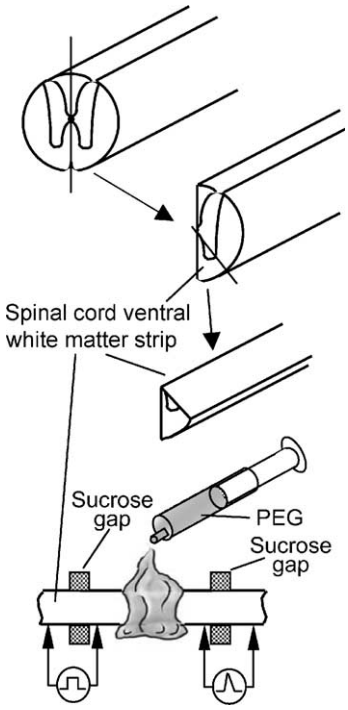

Fig. 1. Diagram showing the isolation of spinal cord white matter strips, the method of PEG application, and recording arrangement. Top panel: Steps to isolate white matter strips from a spinal cord extracted from an adult guinea pig are shown here. Bottom panel: The electrical stimulating and recording arrangement is shown. The electrodes were formed of silver–silver chlorides wires. Action potentials were generated at the left sucrose gap, conducted through the part that is exposed to PEG, and recorded at the right gap with the use of a bridge amplifier. Also shown here is the application of PEG solution using a motor-controlled syringe. The PEG solution was ejected, either continuously or intermittently, directly on the cord segment.

with oxygenated Krebs' solution at 2 ml/min. The ends of the tissue were carried through the sucrose gap channels to side compartments that were filled with isotonic (120 mM) potassium chloride. The white matter strip was sealed on either side of the sucrose gap channels using shaped fragments of glass cover-slips and a small amount of silicone grease to attach the cover slip to the walls of the channel and to block the flow of fluid in the narrow gap between the cover-slip and the tissue surface. An isotonic sucrose solution was run continuously through the channel at a rate of 1 ml/min. The temperature of the central chamber was maintained at 37 °C with an in-line solution warmer (Warner Instruments), and the temperature was separately monitored across the chamber with additional thermocouple probes. The axons were stimulated and compound action potentials were recorded at opposite ends of the strip of white matter by silver–silver chloride wire electrodes positioned within the side chambers and the central bath. The central bath was similarly connected to an instrument ground.

2.3. Compound action potential amplitude

Compound action potentials (CAP) are formed by the spatio-temporal summation of many single unit action potentials fired by individual axons. For the recording of the CAP amplitude, stimuli, in the form of 0.1 ms constant current unipolar pulses, were delivered at a frequency of one stimulus every three seconds. A supramaximal stimulus (110% of the maximal stimulus) intensity was chosen for this test. The digitized profile of each responding CAP was recorded continuously and stored in the computer for later analysis. In addition, a real time plot of CAP amplitude was also displayed during the experiment (Shi and Blight, 1996; Shi and Borgens, 1999b; Shi and Pryor, 2002). All of the on-line recording and subsequent analysis was performed using LabView software (National Instruments) on a Dell computer.

2.4. Compression

The compression injury was induced by a constant-displacement of 0.5-s compression of the spinal cord using a modified forceps possessing a spacer that is 0.3 mm thick. Therefore, the forceps are 0.3 mm apart when closed by finger pressure against the spacer. The average diameter of the white matter strips was 1.5 mm. Using such a method, we produced an average compression of ~80%. Under this mechanical insult, most of the cord lost the CAP conduction immediately following crush. However, the CAP slowly recovered in the next 10–15 min. By the time of 30 min post crush, a time point when we usually applied PEG, the CAP amplitude had reached a plateau at a value of 19.2 ± 3.9% ($N = 6$) of the pre-crush value.

2.5. PEG application

A solution of PEG (2000 MW; 50% by weight in distilled water) was applied through a micropipette directly on to the surface of the cord strip (Fig. 1). The length of the cord that was covered by the stream of PEG solution was about 3–4 mm long. The PEG solution was quickly removed by separate suction. The total application time was 25 min. The total volume applied was estimated to be 2.5 ml. The two groups of cord strips, uninjured or compressed group, received the PEG exposure in a similar manner. The solution was applied in two modes: continuous stream or pulsatile (7 ± 2 drops/min.). Specifically, the amount and the duration of PEG applied to the cord were similar in both continuous stream and pulsatile modes. The major difference is that PEG was applied discontinuously at a rate of about 7 drops every minute in pulsatile mode. The other difference is that the tip of the micropipette that delivers the PEG to cords was immersed in the solution in continuous mode, while placed slightly above the surface of the perfusion solution in pulsatile mode. A vital dye was added to the PEG solution to monitor its flow path and to endure its contact to the cord.

2.6. Statistic analysis

Data are expressed as mean ± SD. Statistical analysis was performed by unpaired Student's t-test with $P < 0.05$ considered as statistically significant.

3. Results

3.1. Effect of focal PEG application to uninjured white matter strips

We examined the effect of PEG on the amplitude of the compound action potential, which is an indication of the proportion of the axons conducting action potential. Specifically, the PEG solution was applied on to the cord strips for 25 min, equaling approximately a total of 2.5 ml of the solution. The PEG was largely removed by suction soon after it flowed 5–6 mm away from the cord strips. Based on our experience, removal of PEG by suction in an area inside the recording chamber that is too close to cord (<3–4 mm) could interfere with electrical recording and inadvertently damage the sample. On the other hand, removal of PEG beyond 5–6 mm from the cord could leave too much residual PEG floating around the cord in the chamber. Such accumulation of PEG may also cause uncontrolled damage of the cord sample. Using the methods described in the manuscript, we removed the PEG fairly completely and no obvious accumulation of PEG was noticed in the middle chamber of the sucrose gap recording device.

We first examined the possibility that the presence of PEG in the chamber has a non-specific effect on the recording of the CAP, which is unrelated to the change of the property of the cord strip itself. We performed a control experiment in which PEG was applied continuously for 25 min to an area within the chamber adjacent to the cord strips, and then the PEG was removed in an area that is 5–6 mm away from the cord strips. Therefore, in such a control experiment, the PEG was never in direct contact with cord strips. We have found that such treatment did not significantly affect the amplitude of the CAP (95 ± 5% of pre-PEG, $P > 0.05$).

We then applied the PEG solution, in a continuous stream, directly on to the spinal cord strips for 25 min. After a delay of about 6 min (6.25 ± 0.5 min, $N = 6$), the CAP began to decline. By the end of 25 min of continuous exposure of PEG, the amplitude of the CAP reduced significantly to 64 ± 4% of the pre-PEG level ($P < 0.01$, $N = 6$). Following a 30 min wash with regular Krebs' solution, the CAP only partially recovered to

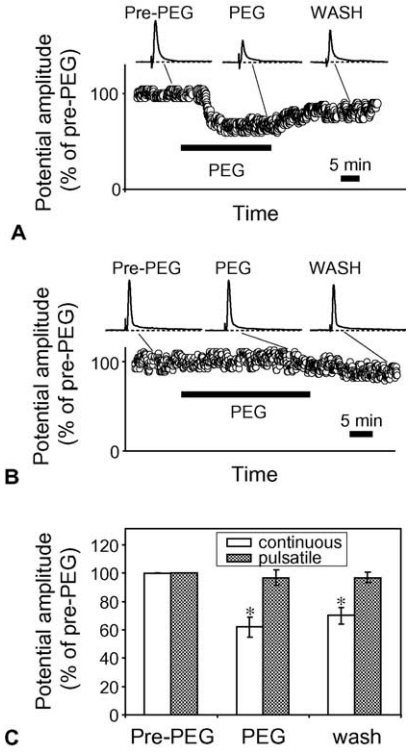

Fig. 2. The effect of focal application of 50% PEG on CAP conduction in *uninjured* cord strips, either in continuous mode (A) or pulsatile (B) mode. (A) The graph is a CAP recording over a period of time plotted against the CAP amplitude. The 25-min PEG exposure is indicated by the bar. Note the CAP amplitude decreased significantly in the presence of PEG and recovered partially upon wash. The three waves set above the graph represent a CAP recoding before, during, and after PEG exposure. (B) This graph is similar to (A), with the exception that the PEG was applied in a pulsatile manner. Note there was no significant change in CAP amplitude following 25-min PEG exposure. Shown in the inset are examples of the CAP waveforms recorded at the time points indicated in the graph. (C) This bar graph shows the quantification of CAP changes in response to 25-min PEG application. Each bar represents the final CAP, after the exposure of PEG, as a percentage of the initial (pre-PEG). $^*P < 0.01$, when compared to pre-PEG. Student *t* test.

70 ± 5% of the pre-PEG level (Fig. 2). ($P < 0.05$ when compared to pre-PEG, $N = 6$).

Interestingly, when we applied the PEG in a discontinuous pulsatile style (7 ± 2 drops/min), while maintaining the similar overall amount of PEG solution as well as the duration of exposure applied in the continuous manner, the CAP amplitude was not significantly affected by the PEG exposure. Specifically, the CAP amplitude following PEG application (in a pulsatile manner) was 96 ± 5% of the pre-PEG level ($P > 0.05$, $N = 6$) (Fig. 2).

3.2. Effect of focal PEG application to injured white matter strip

It is possible that mechanically injured spinal cord axons, though they benefit from a short exposure of PEG

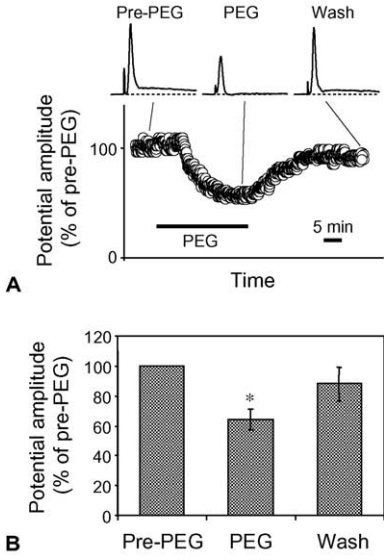

Fig. 3. The effect of focal application of 50% PEG on CAP conduction in *injured* cord strips. (A) The graph represents CAP recording over a period of time plotted against the CAP amplitude. Note the CAP amplitude decreased significantly in the presence of PEG and recovered partially upon wash. Shown in the inset are examples of the CAP waveforms recorded at the time points indicated in the graph. (B) Graphical representation of the effects of PEG on CAP conduction. Each bar represents the final CAP, after the exposure of PEG, as a percentage of the initial (pre-PEG). $^*P < 0.01$, when compared to pre-PEG. Student *t* test.

(~2 min), are more sensitive to prolonged PEG exposure (>5 min) which could result in injurious effect. We therefore examined whether a 25 min exposure of PEG could reduce the CAP amplitude of a mechanically compressed cord. Following compression and a period of approximately 30 min of recovery, the resurging CAP usually reached a plateau. The PEG was then applied onto the injury site for 25 min in a continuous steam. Following a delay of 5.62 ± 0.98 min, the CAP amplitude began to decrease. By the end of 25 min of continuous exposure of PEG, the amplitude of the CAP was reduced significantly to 64 ± 7% of pre-PEG level ($P < 0.01$, $N = 6$). Following 30 min of wash with regular Krebs' solution, the CAP only partially recovered to 88 ± 11% of pre-PEG level. ($P < 0.05$, compared to pre-PEG level, $N = 6$) (Fig. 3).

4. Discussion

4.1. The use of PEG in membrane fusion and repair

PEG has been used as a membrane fusogen to introduce genetic materials from one cell to another for several decades (Davidson et al., 1976; O'Lague and Huttner, 1980; Lee and Lentz, 1997). Specifically, PEG, at a molecular weight ranging from 400 to 6000 and at a concentration of 50–55% can fuse two or more

cells together for an incubation time of 24 h in a culture dish (Davidson et al., 1976). Recently, PEG, at a molecular weight of ~2000 and a concentration of 50% (w/w), has been shown to be able to reconnect completely severed spinal cord and sciatic nerve when applied topically for only 2 min (Shi et al., 1999; Donaldson et al., 2002). Although systemic application, such as intravenous or introperitoneal application of PEG, has been shown to be able to repair bruised yet continuous spinal cord axons (Borgens and Bohnert, 2001; Borgens et al., 2002), it is unlikely that such application will be effective in reconnecting completely severed central and peripheral axons. So far, focal application at a concentration of 50% (w/w) is the only method known to be effective in reconnecting severed axons (Shi et al., 1999; Donaldson et al., 2002). Based on the this finding, coupled with the fact that a 2 min-application yields a modest 5% functional reconnection (Shi et al., 1999), we chose to examine the possibility of extending the duration of the focal application and at the same time to determine the toxicity as result of such prolonged application of PEG.

4.2. The toxicities of PEG with 25 min focal exposure of PEG

As expected, a 25 min focal application of PEG (50% w/w) induced an irreversible CAP reduction in this study. The CAP reduction was observed in both uninjured and acutely compressed cord strips, with an approximate 6-min delay of CAP reduction upon PEG exposure. This finding is consistent with the study carried out by Benzon and his colleagues showing the toxicity of PEG in the rabbit vagus nerve (Benzon et al., 1987). In their study, following a reduction of CAP upon a 1 h application of PEG (MW: 3350, 40%), the CAP recovered to at least 80% of the pre-PEG level after wash. In our study, the recovered CAP was about 70% of pre-PEG level following 25 min exposure of 50% PEG and 30 min wash. Since the experimental condition, such as the size of PEG, PEG concentration, and exposure period, is different, it is difficult to compare the sensitivity of peripheral nerve to central nerve system as to PEG toxicity.

It is unclear so far about the mechanisms that underly the PEG-toxicity in the central nervous system. One logical assumption to account for irreversible damage is the incidence of lateral fusion of axolemma among neighboring axons. Such non-physiological bonding will obviously disrupt axonal conduction. From our previous studies using spinal cord axons, we have shown that the beneficial effect of PEG exposure (2 min) is accompanied by longitudinal axolemmal fusion between severed axons (Shi et al., 1999), and induced membrane repair in damaged yet continuous axons (Shi and Borgens, 2000). Morphological examinations indicated little lateral fusion in severed and then PEG-fused axons when PEG was applied for only 2 min (Shi et al., 1999) (Shi, unpublished observations). Therefore, it is likely that the onset of lateral fusion is at a time beyond 2 min from the beginning of the PEG application. Further morphological examination is needed to critically examine the onset and the degree of lateral fusion following prolonged exposure of PEG. Another possibility is the disturbance of the Node of Ranvier by the perfused PEG solution and the consequent decrease of CAP amplitude. This may represent the spontaneous reversible component of conduction deficit, which may result from a transient increase in membrane permeability and consequent disturbance of ionic distribution (Shi and Blight, 1996).

One aspect of the PEG toxicity worth mentioning in this study is the effect of local oxygen and glucose deprivation. In the current study, the PEG solution lacked oxygen and glucose. Therefore, in the area that was covered by PEG solution (3–4 mm along the cord), oxygen and glucose deprivation more than likely played an important role in CAP depression. However, based on our experience using the same preparation (Peasley and Shi, 2002), oxygen and glucose deprivation up to 60 min only resulted in transient (or reversible) depression of CAP. Therefore, it is unlikely that 25 min of ischemic treatment would produce irreversible damage. In summary, the function deficits due to ionic disturbance and oxygen deprivation is likely responsible for reversible component of the CAP depression while the irreversible component is likely due to anatomical changes, such as lateral fusion of axonal membrane.

In this study, though no significant level was reached, the compressed cord recovered slightly better than uninjured cords following PEG application (88 ± 11% vs. 70 ± 5%). The possible explanation for this phenomenon may lie in the following factors. In compressed cords, in addition to toxic effects, the PEG likely had beneficial effects in the earlier stage (<2 min) of application (Shi and Borgens, 1999b; Donaldson et al., 2002). Therefore the overall net effect of prolonged exposure of PEG in compressed cords depends on the balance between its toxic and beneficial effects. The uninjured cords, on the other hand, are not expected to receive any beneficial effect upon PEG exposure.

4.3. Possible enhancement of PEG-mediated membrane repair with longer exposure time

Based on our functional analysis in the current investigation, we have shown that there is a roughly 6-min delay of CAP reduction upon PEG exposure. There is no significant difference between the latency of PEG-induced CAP reduction in compressed cords and that of uninjured cord strips (5.62 ± 0.98 min vs. 6.25 ± 0.5 min, $P > 0.05$). This indicates that there is little acute functional toxicity for focal PEG application to the

compressed cord when applied for less than 5 min. This suggests that it is possible to increase the PEG-application time from 2 min, a proven effective dosage (Shi and Borgens, 1999b; Shi et al., 1999), to possibly 4–5 min. Furthermore, the safety duration may be beyond 6 min with more beneficial effect, due to the fact that some degree of recovery is obtainable upon wash. In other words, a complete recovery of CAP is still possible following a slight reduction CAP after PEG application. It is likely, yet unconfirmed, that this increase of the duration of PEG-application will translate to the enhancement of PEG-mediated membrane repair and functional recovery.

4.4. Pulsatile application of PEG

It is interesting to note that when a similar amount of PEG was applied through a pulsatile manner, instead of a continuous steam, no CAP reduction was developed. One possible explanation for the lack of CAP reduction in such mode is that axons were able to quickly recover from conduction deficits, due to the local ionic disturbance and anoxia, between the periods of PEG exposure. The significance of this finding is not clear at this point. It will be interesting to assess the efficiency of pulsatile application on PEG-mediated membrane repair following mechanical insults in cord tissues. Since the beneficial as well as toxic effects of PEG on axolemma are both likely related to membrane fusion, the value of pulsatile application will be dictated by the balance of membrane repair through longitudinal fusion vs. unwanted lateral axonal membrane fusion. Future experiments are needed to determine the effectiveness of pulsatile PEG application in repair of transected as well as compressed spinal cord axons.

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