·Review·

Polyethylene glycol repairs membrane damage and enhances functional recovery: a tissue engineering approach to spinal cord injury

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The integrity of the neuronal membrane is crucial for its function and cellular survival; thus, ineffective repair of damaged membranes may be one of the key elements underlying the neuronal degeneration and overall functional loss that occurs after spinal cord injury (SCI). It has been shown that polyethylene glycol (PEG) can reseal axonal membranes following various injuries in multiple *in vitro* and *in vivo* injury models. In addition, PEG may also directly prevent the effects of mitochondria-derived oxidative stress on intracellular components. Thus, PEG repairs mechanically injured cells by at least two distinct pathways: resealing of the disrupted plasma membrane and direct protection of mitochondria. Besides repairing primary membrane damage, PEG treatment also results in significant attenuation of oxidative stress, likely due to its capacity to reseal the membrane, thereby breaking the cycle of cellular damage and free-radical production. Based on this, in addition to the practicality of its application, we expect that PEG may be established as an effective treatment for SCI where membrane disruption and mitochondrial damage are implicated.

Keywords: axolemmal; reseal; fusogen; cutaneous trunci muscle; somatosensory evoked potential; neuroprotection

Introduction

Membrane disruption is common during all mechanical injuries to cells, and repair of the damaged membrane is essential for functional recovery[1-4]. This article reviews the significance of membrane disruption and the development of an effective membrane-repair agent, polyethylene glycol (PEG), in treating mechanically injured neurons, specifically pertaining to spinal cord injury (SCI).

Membrane Disruption Leads to Functional Deficits

The plasma membrane serves many functions, including protecting the cell from extracellular environment and maintaining a functional intracellular environment. Selective permeability and membrane ion pumps maintain an unequal distribution of ions across the membrane and the resulting resting membrane potential. When the integrity of the neuronal membrane is disrupted, ions and other molecules immediately leak into and out of the cell down their electrochemical gradients. Influx of extracellular molecules such as Ca^{2+} can have lethal effects on the cell by activating proteases, generating free oxidative radicals, disrupting mitochondrial metabolism, and activating apoptotic pathways[5-12]. Influx of Na^+ leads to the functional loss of neurons as the membrane can no longer be polarized or excited. Intracellular accumulation of Ca^{2+} and Na^+ also leads to formation of the mitochondrial permeability transition pore, release of cytochrome C, liberation of reactive oxidative species (ROS) from mitochondria, loss of mitochondrial membrane potential, and inhibition of ATP synthesis[6-9].

The effects of mechanical disruption on neuronal membranes have been extensively studied both *in vitro* and *in vivo*[4,11,13-24]. A strong correlation between membrane resealing and functional recovery, as well as neuronal survival, has long been established[4,13,17,18]. It has been shown that enhancing membrane repair in rodent spinal cord axons using various physical, chemical, and biochemical interventions is associated with significant functional recovery[4,18,19,25-27]. Membrane resealing is required for both the long-term survival of the cell as well as the prevention of further degeneration in severed axons or those crushed axons progressing to secondary axotomy. Early repair increases the prospect that the neuron will survive and functionally recover; however, if the axon is severed, recovery may require axonal regrowth or plasticity.

Shi, in previous studies, found that significant membrane damage exists in the spinal cord axons at 1 h, 3 days, and 7 days after controlled compression injury *in vivo*[28]. More importantly, the location of axonal injury expands from the original 2 mm crush site to a distance of 20 mm in each direction at 7 days post-compression[28]. These observations further highlight the importance of axonal membrane disruption in traumatic SCI.

Unfortunately, *in vivo* tissue conditions produced by SCI are not conducive to membrane repair. It is well established that membrane repair is a Ca^{2+}-dependent process. Specifically, membrane resealing is significantly inhibited when Ca^{2+} falls below 0.5 mmol/L[17]. Young and co-workers[29] showed three decades ago that extracellular Ca^{2+} activity ($[Ca^{2+}]_o$) decreases from 1.2 mmol/L before injury, to between 0.01 and 0.1 mmol/L within minutes after SCI in cats. This low level of Ca^{2+} persists for several hours, especially evident in the central region of a compression lesion. Similar $[Ca^{2+}]_o$ changes were reported in a dog SCI model[30]. The reduction of $[Ca^{2+}]_o$ resulting from SCI may prevent damaged axons from resealing. On the other hand, the reduced $[Ca^{2+}]_o$, though unable to trigger membrane repair, is still several magnitudes higher than the level of intracellular Ca^{2+} ($[Ca^{2+}]_i$). This likely supports a prolonged influx of Ca^{2+} into injured cells with disrupted membranes causing consequent axonal degeneration. If axonal injury is physically close to the cell body, this may ultimately lead to the death of the cell itself, as has been shown through *in vitro* studies[31,32]. Therefore, the inability of the membrane to reseal following injury may, in part, contribute to the progressive degeneration that has been reported in mammalian SCI; thus, it is reasonable to suggest that accelerating membrane resealing might prove to be an effective acute treatment for SCI.

PEG-mediated Membrane Repair

PEG as a Cell Fusogen

PEG, a hydrophilic polymer, is a known fusogen[33]. For example, PEG has been used to fuse PC-12 cells to form giant cells and facilitate electrophysiological recordings[34]. PEG has also been used to fuse the ends of severed invertebrate giant axons *in vitro*[35]. The ability of PEG to fuse cell membranes is attributed to its hydrophilic nature. The mechanism by which it acts is not well understood, but it is thought to dehydrate cell membranes, allowing for protein and lipid structural elements to resolve into each other and therefore eliminating membrane breaches[36-38]. In addition, as a surfactant, PEG may also encourage membrane resealing by reducing membrane surface tension and enhancing membrane fluidity, conditions favorable for membrane resealing[4].

PEG-mediated Membrane Repair of in vitro Primary Spinal Cord Injury

Based on the ability of PEG to fuse the cell membrane, we theorized that PEG is capable of fusing completely severed strips of spinal cord white matter. Although this may not be very clinically relevant, it would be a proof-of-principle demonstrating PEG's ability to repair damaged membranes in a simple injury model. The highly sensitive double sucrose gap recording (DSGR) device has been an indispensable technique for monitoring acute recovery after SCI[17,25,39]. This method has allowed unprecedented resolution in recording compound action potentials (CAPs) using isolated strips of ventral white matter from adult guinea pig spinal cord[25,40].

Here is a brief description of the technique used to evaluate impulse conduction using the DSGR device[40]. A strip of ventral white matter, ~38 mm long, is isolated from guinea pigs weighing ~400 g. Once the cord is mounted on the recording device and the CAP recording has stabilized, the cord is completely transected between the stimulating and recording electrodes, eliminating the propagation of CAPs. The two segments of spinal cord are then carefully aligned and gently pressed together. PEG (MW ~2 000,

50% *w/w* in distilled water) is applied directly to the injury site for ~2 min in a continuous stream. While the transected untreated cord (aligned and pressed together without PEG) never recovers CAP conduction, the PEG-treated cord typically recovers within 5 min of PEG application[40]. An identical procedure has been used to treat the compressed cord. Similar to a transection injury, a severe compression completely eliminates the CAP. A small and variable level of spontaneous recovery occurs within ~1 h in the absence of PEG. However, CAP amplitude in the PEG-treated cord is 7 times that recorded from injured but untreated preparations[25].

In spinal cord transection experiments, the anatomic continuity of fused axons has been examined by injecting intracellular markers (fluorescently labeled dextrans) into each end of the spinal cord strip. In PEG-fused cords, variable numbers of labeled axons are continuous across the original transection plane, while such a phenomenon is never observed in control (injured without PEG) cords. High-resolution light microscopy (1-μm plastic sections) has also revealed axonal continuity across the transection plane in PEG-treated cords[40]. These observations demonstrate that the restoration of physiological function is accompanied by axonal reconnection.

In addition to reconnecting severed axons, PEG has also been shown to repair membrane damage in compressed or crushed axons, a more clinically-relevant injury. Anatomical sealing of PEG-treated compression injuries to spinal cord nerve fibers was assessed using a dye exclusion test[17,18]. When the high-molecular weight label, horseradish peroxidase (HRP; 40K MW), is administered to the extracellular milieu it diffuses into injured axons wherever there is a break in the membrane. The cord crush procedure results in numerous labeled axons, while the density of HRP-labeled axons is significantly reduced in crushed cords treated with PEG[18]. This morphological evidence of axolemmal resealing is associated with significantly enhanced recovery of physiological conduction in compressed cords following PEG treatment[41].

Furthermore, it has been shown that PEG enhances the resealing of the cut end of a completely transected axon, a mechanical injury that produces a much larger membrane breach at the focus of the injury than a compression injury[4]. Specifically, PEG appears to be able to promote resealing of membrane disruptions that are equivalent to the diameters of axons that range from less than 1 mm to several micrometers[4]. It is understandable that severed axons have to first seal the membrane of the proximal stump to stop the degeneration and survive the injury, an indispensable step to allow subsequent axonal regeneration. In light of this, it is reasonable to state that PEG-mediated axolemmal resealing ensures and contributes to axonal regeneration.

It is worth pointing out that although PEG is capable of reconnecting severed axons in *ex vivo* preparations, it is unlikely that this mechanism plays a major role during *in vivo* SCI. This is because, as described by Shi and colleagues[26], such axonal reconnection after transection requires manipulation (aligning and gently pressing together at the two cut ends), which is not possible *in vivo*. Therefore, PEG-mediated repair of membrane breaches, as well as resealing of the proximal cut end is likely to be the main mechanisms underlying the beneficial effects seen in PEG treatment *in vivo*. Since compression and transection usually coexist following physical insults in SCI, these findings further underscore the effectiveness of PEG in repairing membrane damage and promoting structural and functional recovery in CNS trauma.

PEG Reduces Secondary Spinal Cord Injury
We have shown that PEG not only repairs membrane disruption, primary damage, but also strikingly reduces the elevation of ROS secondary to mechanical injury. Specifically, application of PEG significantly reduces superoxide production at 1 h post-injury. The level of lipid peroxidation is also significantly decreased as a result of PEG treatment[42-44]. This is important since oxidative injury is a key component of secondary cascades in the pathogenesis of traumatic spinal cord and brain injury[45-48].

We have shown that PEG has no intrinsic free radical-scavenging abilities[42]. Therefore it is probable that PEG-mediated reduction in oxidative stress is dependent on its capacity to seal the membrane, thereby breaking the cycle of cellular damage and free-radical production. In this interpretation, PEG repairs membrane damage, reducing Ca^{2+} influx, inhibiting the formation of the mitochondrial permeability transition pore and associated oxidative stress, thereby preventing further increased membrane permeability that would be linked to the production of O_2^- and H_2O_2[7, 49].

PEG Repairs Spinal Cord Injury *in vivo*

The effects of PEG have also been examined after *in vivo* SCI[50-52]. *In vivo* testing allows for the assessment of recovery from behavioral deficits, as well as long-term studies. These studies were carried out using a well-established guinea-pig SCI model[50-53]. Specifically, PEG was applied topically for 2 min to completely exposed compression injuries in the mid-thoracic region, either within 30 min or ~8 h following injury. A standardized compression injury to the cord completely abolished the cutaneous trunci muscle (CTM) reflex in all animals on both sides below the level of the crush. The somatosensory evoked potential (SSEP) was also completely eliminated in all animals. Recovery of SSEP conduction was not observed in any control injured animals in up to one month of monitoring. All of the guinea-pigs treated with PEG, either within 30 min or 8 h post-SCI, showed measurable SSEP conduction. Less than 17% of the control animals recovered any level of CTM reflex after SCI while 100% of the PEG-treated animals recovered variable levels of CTM function, regardless of when the PEG was applied[50]. In addition to behavioral recovery, PEG treatment also resulted in significant reduction of injury size[53]. The fact that the delayed application of PEG (8 h post-injury) still leads to significant functional and anatomical recovery is consistent with an earlier study which showed that membrane damage continues to exist up to 7 days after spinal cord compression *in vivo*[28]. Thus, PEG-mediated axonal repair would be possible for an extended period of time post-injury. In addition to guinea-pig, significant beneficial behavioral and anatomical results of PEG have been found in several rat SCI models[54-56] which further highlights the effectiveness of PEG in treating traumatic SCI in different animal models.

In addition to the beneficial effect in the acute stage described above, we have found that apoptosis, a major component of secondary injury, is also suppressed after PEG treatment. Specifically, when applied locally and immediately following compression injury, PEG significantly reduces the number of apoptotic cells at 1 and 7 days following injury and treatment. Consistent with these findings, caspase-3 activity and cytochrome C release are also markedly reduced in PEG-treated compared to untreated groups[57]. This is consistent with our findings that PEG reduces oxidative stress, which is known to cause apoptosis after traumatic SCI[42-44]. This indicates that cells rescued by PEG at the acute stage do not later die by apoptosis. Since continuing cellular destruction through apoptotic cell death may play a critical role in the pathophysiology of SCI[58-60], these findings emphasize the importance of early intervention in modifying long-term biochemical and functional recovery after SCI.

Several additional findings have emerged from *in vivo* studies: (1) Although the original experiments used local application, it is now clear that intravenous and subcutaneous injection of PEG are also effective, greatly enhancing the practicality of the treatment approach[51,52]. (2) When applied systemically, either by intravenous, intraperitoneal, or subcutaneous injection, fluorescein-conjugated PEG specifically localizes to the site of injury in the guinea-pig spinal cord[52]. We hypothesize that there are probably at least two mechanisms for this phenomenon. First, PEG can gain access to the injury site through the ruptured blood vessels. Second, PEG may have a higher affinity for injured cells because the ruptured membrane may create an environment that attracts PEG. Regardless of the mechanisms, this phenomenon highlights several potential advantages in the use of PEG. One is that PEG can be used as a drug-carrier to deliver effective agents to the injury site in both SCI and traumatic brain injury. This can be accomplished by conjugating PEG to the desired compounds. Furthermore, a PEG-conjugated compound may retain its ability to reseal membranes in addition to the effect exerted by the compound to which it is conjugated.

Veterinary clinical trials using naturally injured and neurologically complete paraplegic dogs have been completed[61]. Intravenous injection of PEG (30% *w/w*) demonstrated noticeable improvements in voluntary ambulation, deep and superficial pain perception, proprioceptive placing of hindlimbs, and SSEP conduction compared to historical controls. The outcome measurements were monitored for 6–8 weeks. The results of this pilot trial provide evidence consistent with the notion that the injection of polymers in acute neurotrauma may be a simple and effective intervention during the acute phase of the injury.

Two points related to the breadth of application of PEG in various nervous injuries are worth mentioning here. First, though most of our published work is geared

towards spinal cord axons, PEG has also been shown to repair peripheral nerves both *in vitro* and *in vivo*[62]. Specifically, PEG treatment greatly enhances functional recovery following compression injury of sciatic nerves *in vivo*. Second, since many similarities exist between brain and spinal cord traumatic injury, it is perhaps not surprising that PEG has been recently shown to significantly alleviate anatomical and functional loss in traumatic brain injury[63]. This suggests that PEG may be an effective agent for many other mechanical injuries where membrane disruption is implicated.

Multiple Targets of PEG Treatment

In addition to repairing the plasma membrane, we have recently shown that PEG is also capable of directly repairing damaged mitochondria[43,64]. This conclusion is based on the following results. First, confocal microscopy using fluorescein-conjugated PEG revealed that PEG enters the cells of the injured spinal cord, placing the polymer in a position to directly interact with cellular organelles such as mitochondria[43]. Second, PEG attenuates the Ca^{2+}-induced functional compromise of isolated normal spinal cord mitochondria *in vitro*[64,65]. The PEG-mediated effects include preventing oxidation of glutathione and mitochondrial swelling[43]. These results indicate that PEG may exert its neuroprotective effect through direct interaction with mitochondria in addition to its ability to rescue neurons and their axons by repairing the plasma membranes.

In summary, based on our findings, we propose a hypothetical model for the neuroprotective action of PEG in the spinal cord after traumatic injury. Traumatic injury to the cord causes breaches in the neuronal membrane, allowing extracellular ions (such as Ca^{2+} and Na^+) to enter the injured cells. This results in rapid organelle damage and possible acute cell death. The primary action of PEG is to immediately seal and progressively eliminate membrane disruptions. In the meantime, some PEG molecules enter the cell through openings in the membrane and interact with mitochondria, preventing formation of the mitochondrial permeability transition pore. This in turn prevents or reduces mitochondrial swelling, allows for maintenance of the mitochondrial membrane potential, and inhibits cytochrome C release and the consequent depletion of

antioxidant systems. Thus, PEG repairs mechanically-injured cells by at least two distinct pathways: resealing of the disrupted plasma membrane and direct protection of mitochondria.

Concluding Remarks and Suggestions of Future Research

Membrane disruption has serious downstream structural and functional consequences in the mechanical injury of neuronal tissue. PEG-mediated resealing of cell membranes is a practical method to repair or reverse the continuous cellular damage subsequent to injury and enhance functional recovery. A brief application of PEG not only reduces acute necrosis, but also apoptosis. In addition to the plasma membrane, PEG also directly protects mitochondria and preserves their function. Based on this, as well as the practicality of application, we expect that PEG may be established as an effective treatment not only for SCI, but also for mechanical injuries in other tissue types where membrane disruption and mitochondrial damage are implicated.

Perhaps other beneficial therapies applied to the early injury may also enhance PEG's benefits through separate and synergistic mechanisms of action[25]. Since the loss of the plasma membrane is not the only pathology present during traumatic injuries, future development of combination therapy, such as PEG combined with other recovery-promoting agents would prove beneficial for therapeutic efficacy. For example, PEG could be combined with agents that combat known secondary injuries such as demyelination, oxidative stress, and glutamate excitotoxicity.

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