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Chitosan produces potent neuroprotection and physiological recovery following traumatic spinal cord injury

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SUMMARY

Chitosan, a non-toxic biodegradable polycationic polymer with low immunogenicity, has been extensively investigated in various biomedical applications. In this work, chitosan has been demonstrated to seal compromised nerve cell membranes thus serving as a potent neuroprotector following acute spinal cord trauma. Topical application of chitosan after complete transection or compression of the guinea pig spinal cord facilitated sealing of neuronal membranes in *ex vivo* tests, and restored the conduction of nerve impulses through the length of spinal cords *in vivo*, using somatosensory evoked potential recordings. Moreover, chitosan preferentially targeted damaged tissues, served as a suppressor of reactive oxygen species (free radical) generation, and the resultant lipid peroxidation of membranes, as shown in *ex vivo* spinal cord samples. These findings suggest a novel medical approach to reduce the catastrophic loss of behavior after acute spinal cord and brain injury.

Key words: chitosan, nanoparticle, nerve tissue engineering, membrane, self assembly.

INTRODUCTION

Traumatic spinal cord or brain injury is accompanied by a significant loss of body functions. The acute compromise of intact cell membranes in response to mechanical forces leads to the deterioration of the barrier function of the plasma membrane and leads to cell and tissue necrosis (Borgens, 1988; Borgens, 2001; Povlishock et al., 1997). Compromise of the damaged membrane can be simply a local increase in membrane permeability to a more extensive open breach in the membrane (McNally and Borgens, 2004). Pathological transport of intracellular and extracellular ionic substances across this region leads to a rapid conduction block in nerve fibers, and eventually cell and tissue death resulting from the persistent absence of cellular resealing and the re-establishment of physiological homeostasis (Borgens, 2003). Thus, in an effort to avoid further permeabilization and progressive disruption of neuronal tissue, it is essential to re-establish the barrier function of damaged membranes. If loss of membrane integrity is not particularly severe, natural resealing of the injury site may probably ameliorate ionic derangement. However, in more severe cases, delayed, persistent, and progressive injury processes arising from membrane disruption produces so-called 'secondary injury'. The concept of secondary injury involves the production of highly reactive oxygen species, or free radicals, morphing to the lipid peroxidation of the inner lamella of the membrane (LPO). It ends with the production of potent endogenous toxins such as acrolein (Luo and Shi, 2004; Luo and Shi, 2005) that kill the cell. We have previously shown that water-soluble nonionic polymers such as polyethylene glycol (PEG) or poloxamer 188 (P188), were able to effectively reverse the progressive permeabilization of cell membranes by sealing the membrane (Borgens and Shi, 2000; Borgens et al., 2002; Lee et al., 1992; Merchant et al., 1998; Serbest et al., 2006; Shi and Borgens, 1999). Both *in vivo* and *in vitro* studies reveal that such polymers can induce a rapid (minutes) sealing of permeabilized membrane, restore nerve impulse conduction, and produce a recovery of function in both spinal cord and head injury models (Koob and Borgens, 2006; Koob et al., 2005; Laverty et al., 2004). Despite their promising potential, both the molecular mass, and aqueous concentration of the PEG and PEG-based polymers is somewhat limited – associated as it is with either an unwanted increase in the viscosity of the injectable solution and/or by increasing the levels of dangerous circulating subunits – particularly PEG monomers (Brent, 2001; Caravati et al., 2005). Moreover, nonionic polymers such as PEG are not biodegradable and circulate in the blood vascular system before being eliminated from the body. The smaller the molecular mass of the polymer used relative to the length that it is sustained in the circulation increases the risk of monomer and dimer toxicity (Working et al., 1997).

Chitosan, a positively charged natural biopolymer, is formed by *N*-deacetylation of chitin. Chitin is the second most abundant natural polysaccharide and is primarily obtained from the exoskeletons of many crustaceans and the cell walls of fungi. The abundance of primary amine groups enables chitosan to be covalently coupled to various biomolecules because the amine moieties become protonated and positively charged below pH 6.5 whereas the amines become deprotonated at pH 6.5 and above. Owing to its excellent biocompatibility, biodegradability and low toxicity, chitosan has been extensively investigated as a potential biomaterial in a variety of applications, including drug carriers, wound-healing agents, lung surfactant additives, and tissue engineering scaffolding (Gan and Wang, 2007; Janes et al., 2001; Madihally and Matthew, 1999; Roy et al., 1999; Ueno et al., 1999; Yuan et al., 2004; Zuo et al., 2006). In particular, chitosan is capable of forming large phospholipid aggregates by inducing the fusion of small dipalmitoyl phosphatidylcholine (DPPC) bilayers, which are a major component of the plasma membrane (Pavinatto et al., 2007; Zuo et al., 2006). Thus the use of chitosan as a 'fusogen' might be more advantageous as a potential clinical tool relative to nonionic polymers (e.g. PEG or P188). In this study, we assess whether chitosan is able to rescue

damaged nerve cell membranes following spinal cord trauma, reduce injury-mediated reactive oxygen species (ROS) production; restrict continuing lipid peroxidation (LPO); and permit the recovery of nerve impulse conduction in the spinal cord. These observations are crucial for the development of a novel therapy for acute neurotrauma based on the resealing of damaged cell membranes.

MATERIALS AND METHODS

We first mention that tests on injured spinal cords employed a standardized compression model of injury, as this technique is the most clinically meaningful. However, transection was used in some cases where the maximal loss of a label – or the maximum amount of labeling – was desired in the control procedures. All studies but two were performed on freshly dissected 1 cm long samples of the mid-thoracic region of the guinea pig spinal cord. The exceptions were the targeting study and the *in vivo* conduction study, both described below.

Spinal cord isolation

All guinea pigs (*Cavia porcellus* Erxleben 1777) used in this study were handled in accordance with Purdue Animal Care and Use Committee (PACUC) guidelines. Adult female guinea pigs were anesthetized with intramuscular injection of ketamine (60 mg kg^{-1}) and xylazine (10 mg kg^{-1}). Then, they were perfused through the heart with approximately 500 ml of sterile lactated Ringer's (SLR) solution to remove the blood. The vertebral column was then rapidly removed and a complete dorsal laminectomy performed along the length of the vertebral column, exposing the spinal cord. The spinal cord was carefully removed from vertebrae and immersed in cold, oxygenated Krebs solution (124 mmol l^{-1} NaCl, 2 mmol l^{-1} KCl, 1.24 mmol l^{-1} KH_2PO_4, 1.3 mmol l^{-1} $MgSO_4$, 1.2 mmol l^{-1} $CaCl_2$, 10 mmol l^{-1} glucose, 26 mmol l^{-1} $NaHCO_3$, and 10 mmol l^{-1} ascorbic acid). In the *ex vivo* targeting, ROS and LPO, and *in vivo* experiments, a standardized compression injury was induced using a modified forceps by constant mechanical force for 15 s (Fig. 1). However, transection of the cord was employed in the tetramethyl Rhodamine dextran (TMR) dye exclusion tests and lactate dehydrogenase (LDH) leakage experiments described below. The transection injury was performed using a miniature scalpel-like cutting tool (#10315-12 microscalpel, Fine science tools, Foster City, CA, USA). The segments of spinal cord were immediately immersed in oxygenated Krebs solution following severe compression or transection of the cord. In all *ex vivo* experiments but one, the injury was created in the center of a 1 cm strip of cord after dissection of the entire spinal cord from the guinea pig as discussed above, and five such samples from five separate animals were used for each experiment. These 1 cm strips were all mid thoracic in location, and were further weighed and measured to be consistent one with another. All chemicals were purchased from Sigma-Aldrich (Milwaukee, WI, USA) unless otherwise specified.

Measurement of membrane permeability using tetramethyl Rhodamine dextran

Membrane breaches induced by transection injury were quantified after exposure to the hydrophilic dye TMR (10 kDa; Invitrogen, Carlsbad, CA, USA). TMR is a common dye used for intracellular uptake and tract-tracing with a high internalization index (Kumari and Mayor, 2008). Briefly, 1 cm segments of spinal cord were allocated to one of the following treatments groups: control (intact), injured, and injured and chitosan (injured–chitosan) which was treated for 15 min in the chitosan solution. Subsequently, all spinal cord segments were transferred to 0.1% TMR dextran and incubated for 15 min. Spinal cord segments were then fixed in 4% paraformaldehyde in 0.1 mol l^{-1} phosphate-buffered saline (PBS) for 4 h and sectioned at 50 µm thickness using a freezing microtome (Thermo Electron, Waltham, MA, USA). Sections were visualized by epifluorescence on an Olympus BX61 microscope with a standard Rhodamine cube (545 and 590 nm of excitation filter and emission filter; Olympus), quantified using ImageJ™ (NIH), and averaged for five sections selected randomly from the center of each spinal cord segment.

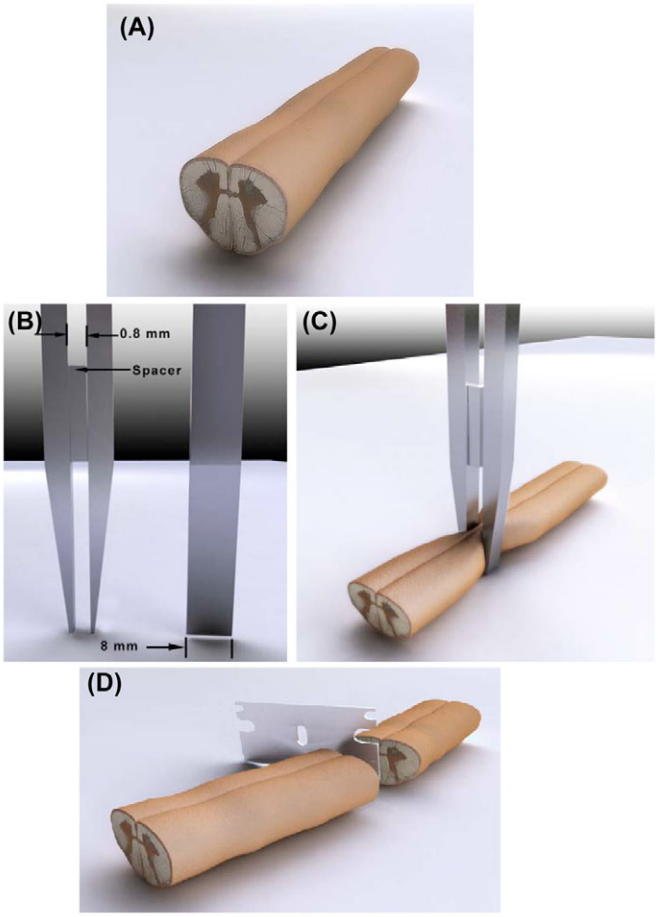

Fig. 1. Illustration of the isolated spinal cord and the types of injury. (A) Complete spinal cords (~40 mm length) were isolated from guinea pigs. (B) The front and side view of the modified forceps used to compress the cord. These have a 0.8 mm wide spacer (détente), which produced a standard displacement injury. (C) Spinal cord samples were placed between the tips of the forceps and squeezed for 5 s against the spacer to induce compression injury. (D) In some experiments, the isolated spinal cord was cut in two to produce the transection injury.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) leakage from the cell can be an indicator of the intactness of the plasma membrane. LDH, an intracellular enzyme (140 kDa), moves from the cytosol to the extracellular compartment subsequent to membrane collapse. We have previously reported this technique as one of several probes of membrane integrity (Luo et al., 2004; Luo et al., 2002). Briefly, 1 cm segments of mid-thoracic spinal cord were removed from the whole excised spinal cords of each animal and incubated in Krebs solution for at least 1 h to allow axonal membranes to repair from

injury that occurred as a result of tissue isolation and handling. These 1 cm samples were then incubated for 15 min in Krebs solution, in one of the following treatment groups: injured (by transection), injured and PEG (injured–PEG; 50% w/w in distilled water) treated, and injured and chitosan (injured–chitosan) treated (at different pH and concentrations for comparison). Samples were finally incubated for an additional 1 h in modified Krebs solution to allow LDH to leak out of any membrane breaches that were produced in each of the treatment groups. 200 µl samples of the solution bathing the segments were then removed. Levels of LDH were assayed using the TOX-7 kit (Sigma) and normalized for control values from the same animal to control for variation between animals.

Measurement of superoxide, reactive oxygen species and lipid peroxidation

The detection of superoxide anion (O_2^-) was performed as follows. Hydroethidine (HE), an oxidant fluorescent dye, is rapidly converted to ethidium (E^+) in the presence of intracellular O_2^-. The 1 cm samples removed from the excised spinal cord strips were injured by compression in their approximate center and were immersed in 1 ml of PBS with HE at a final concentration of 1 µmol l^{-1} for 10 min in the dark. Subsequently, samples were fixed, sectioned, analyzed with epifluorescence on an Olympus BX61 microscope with a standard Rhodamine cube (545/590 nm of excitation/emission filter, Olympus), and quantified using ImageJ (NIH) by averaging five sections randomly selected from the center of each spinal cord segment, to measure the amount of ethidium bromide uptake. Additionally, the monitoring of LPO can also be an indicator of the stability of the lipid components of plasma membranes. Once compression-injured spinal cords were weighed, homogenized and centrifuged at 2000 ***g*** for 5 min, a lipid hydroperoxide assay (Cayman Chemical Company, Ann Arbor, MI, USA) was carried out. After the preparation of the chromogen in sample solutions, the absorbance of each sample was read at 500 nm using a spectrophotometer (SLT spectra plate reader, Salzburg, Austria). Tissue lipid peroxidation was calculated and expressed as nanomoles per 70 mg of wet tissue.

MTT assay

Based on a colorimetric assay system, the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay can be used to estimate of mitochondrial function. Briefly, 1 cm segments of only mid-thoracic spinal cord were used for these experiments. Acrolein was prepared fresh daily in PBS solution. Following the application of acrolein (100 µmol l^{-1}), spinal cord segments were divided into the following treatment groups: control, injured, injured and hydralazine (injured–hydralazine; 500 µmol l^{-1}) treated, and injured and chitosan (injured–chitosan) treated. The last two were incubated for 15 min in the test solution. According to the synaptosomal mitochondria isolation method previously described (Luo and Shi, 2004), spinal cord segments were homogenized in 3 ml isolation buffer (0.25 mol l^{-1} sucrose, 0.5 mmol l^{-1} di-potassium EDTA, 10 mmol l^{-1} Tris–HCl), and centrifuged twice for 3 min at 4°C and 2000 ***g***. The supernatant was then centrifuged at 16,000 ***g*** for 10 min at 4°C. The crude mitochondrial pellet was resuspended in 1 ml PBS (0.9 mmol l^{-1} $CaCl_2$, 2.7 mmol l^{-1} KCl, 1.5 mmol l^{-1} KH_2PO_4, 0.5 mmol l^{-1} $MgCl_2$, 137 mmol l^{-1} NaCl, 6.5 mmol l^{-1} Na_2HPO_4, pH 7.2–7.4). This procedure yielded a suspension containing approximately 8–10 mg ml^{-1} protein, as determined by the BCA protein assay (Pierce, Rockford, IL, USA; data not shown). 100 µl of MTT solution (100 mmol l^{-1} pyruvate, 12 mmol l^{-1} MTT) was then added to the mitochondrial suspension and incubated for 1 h at 37°C. Formazan crystals were pelleted by centrifugation and dissolved in 1 ml of a 50/50 (v/v) solution of ethanol and DMSO. The absorbance of 200 µl was read at 550 nm minus the background at 660 nm. An ethanol–DMSO solution was used as a blank. Five spinal cord samples originating from five animals were used for each group.

Tracing the distribution of chitosan

FITC-labeled chitosan was prepared according to the method of Qaqish et al. (Qaqish and Amiji, 1999) and used to determine the targeting ability of chitosan in injured spinal cord. Three samples of the whole intact spinal cord and another three that had received compression injuries were isolated from six individual animals. Each cord was immersed in FITC-labeled chitosan solution for 15 min and subsequently incubated in modified Krebs solution for another 30 min. The spinal cords were immediately fixed in 4% paraformaldehyde, sectioned with a freezing microtome at 50 µm thickness, and evaluated with an epifluorescence microscope using excitation and barrier wavelengths of 490 and 520 nm, respectively. Labeling with a FITC dye was quantified using NIH Image™ software by statistical comparisons of fluorescence (minus background) intensity. Note these were whole cord samples, and not the typical 1 cm segments used in previous experiments.

The *in vivo* examination of spinal cord conduction recovery

A total of 14 spinal injured guinea pigs were divided into two groups for *in vivo* conduction studies (six controls and eight chitosan treated). The constant displacement (compression) injury was performed using modified forceps in the mid-thoracic region of the spinal cord using previously reported techniques (Borgens et al., 2004; Borgens and Shi, 2000; Borgens et al., 2002). The sham-treated control group received a single subcutaneous injection of lactated Ringer's while the experimental group received a single subcutaneous injection of a chitosan solution. Injections were made in the loose skin at the back of the neck, after a baseline somatosensory evoked potential (SSEP) was obtained, and within 30 min of the spinal cord injury. SSEP recordings were carried out on all animals before and after (1 day, 1 week and 2 weeks) spinal cord injury.

Evoked potentials from the extremities of guinea pigs arriving at the contralateral sensorimotor cortex were recorded using subdermal electrodes with a reference electrode situated in the ipsilateral pinna of the ear. Pairs of subdermal stimulating electrodes directly fired the tibial nerve of the hindlimb, or the median nerve of the forelimb (at stimuli trains of 200 repetitive stimulations at 3 Hz; 3 mA square wave at 200 µs duration). The recording of tibial-nerve-evoked potentials at the brain was completely extinguished by mid-thoracic damage to the spinal cord, but the transmission of medial nerve potentials was not affected because the neural circuit is 'above' the level of the injury. The latter stimulation paradigm was a control for false negative tibial stimulation. Stimulation/recording and management of the electrical recordings were carried out on a Nihon Kohden (Foothill Ranch, CA, USA) Neuropak 4.

Statistical analysis

Unless otherwise specified, unpaired Student's *t*-test (for comparison of two groups) or one-way ANOVA and *post-hoc* Newman–Keul tests (for more than two groups) were used for statistical analyses (InStat™ software). Normality was tested for by Shapiro–Wilk test (STATA). Equal variances were tested by the method of Barlett for $N \geq 5$ (InStat), and by less than twofold difference in standard

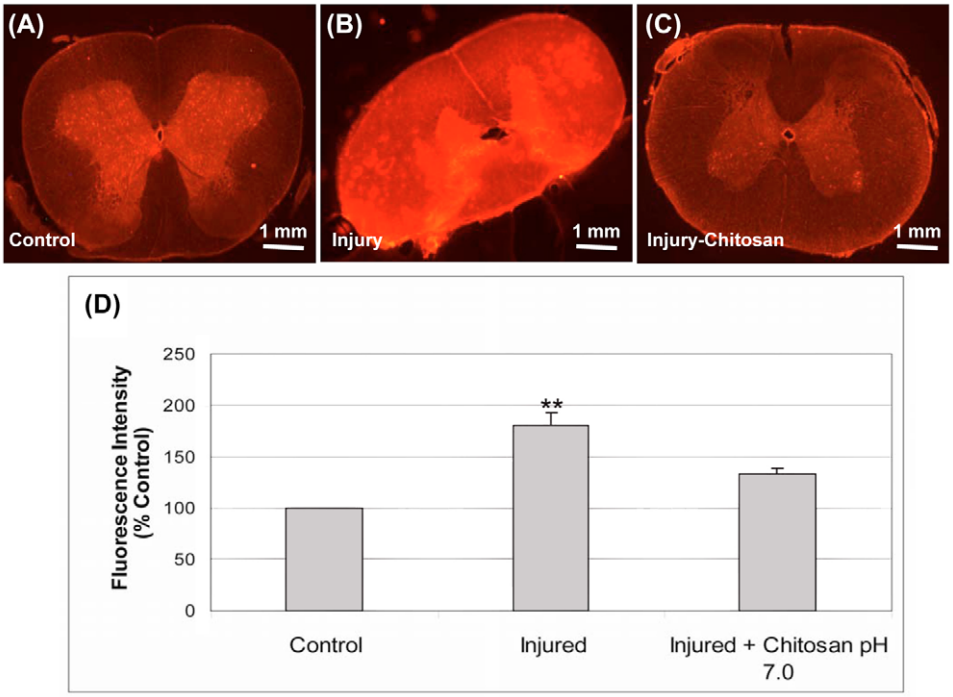

Fig. 2. TMR fluorescence test in spinal cord tissues to assess anatomical membrane sealing. Membrane sealing was confirmed by the application of tetramethyl Rhodamine dextran (TMR) after complete transection of the isolated spinal cord. TMR is a hydrophilic dye that is imbibed into damaged neurons. (A) Uninjured cord (Control), (B) completely transected but untreated spinal cord (Injury), and (C) completely transected spinal cord subsequently exposed to a 0.05% (w/w) chitosan solution at pH 7.0 for 10 min (Injury–Chitosan). Injured cords showed an increase in fluorescence intensity, especially in the gray matter, and around the edges of the tissue, whereas chitosan-treated spinal cords showed a reduction in the intensity of labeling. (D) Labeling was quantified using NIH ImageJ software to measure the fluorescence (minus background) intensity. $^{**}P<0.001$.

deviation (s.d.) for $N<5$. Results are expressed as mean ± s.d. $P<0.05$ was considered statistically significant.

RESULTS

Resealing of disrupted plasma membrane by the application of chitosan

The *ex vivo* injury (compression/transection) is shown in (Fig. 1). Spinal cords were assessed by the TMR dye exclusion test and the LDH assay. Cord samples were immersed in TMR (10 kDa). Note that transections were used in this experiment to produce the maximum amount of labeling possible in control preparations. In transected spinal cords, damaged neurons were labeled and revealed a significant increase in TMR uptake compared with control (uninjured) groups (Fig. 2). The fluorescence intensity following transection injury was significantly increased to 175±14% of control values ($P<0.001$), indicating permeability of the membrane to the entry of the hydrophilic TMR dye. However, TMR uptake in tissues immediately treated with chitosan after transection was reduced to 133.5±6% of control values. This reduction was not significantly different from the un-injured controls ($P>0.05$). The LDH leakage assay was used to further support the results of the TMR dye exclusion test. Similarly, complete transection induced significant release of intracellular LDH (144 kDa; Fig. 3). However, the loss of LDH was substantially reduced (to normal control levels) following chitosan treatment in a pH-dependent way. As shown in Fig. 3A, protonated chitosan, at pH 3.0, significantly inhibited the release of LDH (90±16% of control values) whereas even deprotonated chitosan, at pH 12.0, served as a sealant by reducing LDH efflux (97.5±15% of control values). We also evaluated membrane permeability as a function of the concentration of chitosan (Fig. 3B).

Chitosan-induced neuroprotection of disrupted plasma membrane

As acute damage to spinal cord resulted in significant oxidative stress, we determined the levels of ROS and LPO generation as a result of the injury and after the application of chitosan. As shown in Fig. 4A–E, superoxide production, measured by oxidized HE fluorescence, was increased to 205±45% of control values after compression injury, showing prominent fluorescence in both grey and white matter. However, the application of chitosan to plasma membranes reduced the level of ROS to 105±15% of control values at certain concentrations, which was not significantly different from that of the control group. In a similar way, LPO induced by disruption of spinal cord cell membranes was monitored as a function of various concentrations of chitosan. The level of LPO was significantly increased to 37.5±4.5 nmol 70 mg^{-1} after spinal compression, compared with that of the control (uninjured) group (10.5±2 nmol 70 mg^{-1}), which is an almost fourfold increase. The application of 0.2% chitosan following compression injury reduced the level of LPO to 5.5±2.5 nmol 70 mg^{-1}, which was even below that of the control group – an approximate decrease of 80% (Fig. 4F).

The targeting ability of chitosan following traumatic spinal cord injury

The intact and crushed spinal cords removed from six animals ($N=3$, each group) were incubated in the chitosan-FITC solution for 15 min followed by immersion in oxygenated Krebs solution for an additional 30 min. Note, the ~40 mm length of cord was preferable to the 1 cm sections due to the greater distance between the cut ends (from the dissection) and the central injury. According to the fluorescent intensity, crushed regions showed intense labeling in both gray and white matter (Fig. 5). This represented an almost four-fold increase in fluorescent intensity compared with intact cords. By contrast, uninjured regions obtained from at least three or four vertebral segments away from the injury site exhibited faint labeling, confined to the lesion but not undamaged adjacent regions.

Chitosan, hydralazine and acrolein challenged mitochondria

The effect of chitosan treatment on acrolein-mediated toxicity is shown in Fig. 6. The MTT assay was conducted with synaptosomal mitochondria isolated from spinal cord segments that were treated with 100 µmol l^{-1} acrolein followed by hydralazine or chitosan, to

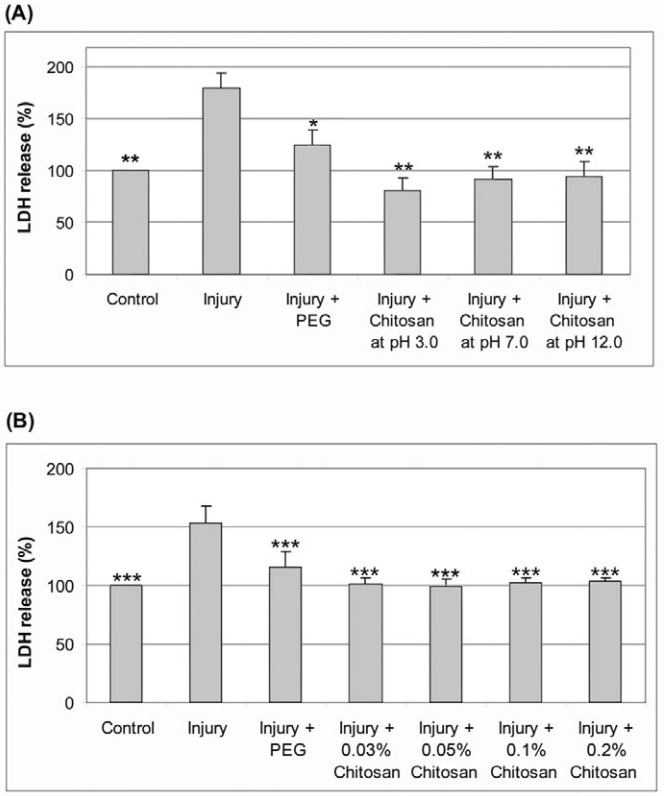

Fig. 3. LDH assay to evaluate membrane integrity. Membrane damage results in the escape of a large enzyme, lactate dehydrogenase (LDH, 160 KDa) from the cytosol to the extracellular medium. (A) LDH release as a function of pH of chitosan solutions. Medium from spinal cord tissues of control, injured, injured and immediately treated with PEG (50% w/w), and injured and immediately treated with chitosan solution (0.05% w/w) at different pHs were spectrophotometrically evaluated for LDH activity. Chitosan-treated cords showed significant lower LDH release compared with PEG-treated cords. In addition, the level of LDH release was dependent on the pH of the solution. Especially at low pH, protonated amines caused chitosan molecules to integrate toward damaged, negatively charged areas of cell membrane. (B) LDH release as a function of concentrations of chitosan solution at pH 7.0. The LDH release was not influenced by the concentration of chitosan. Results are expressed as percentage of the control values ± s.d. (N=5). One-way paired ANOVA and *post-hoc* Newman–Keul's test were used for statistical analysis. *P<0.05, **P<0.001, ***P<0.005.

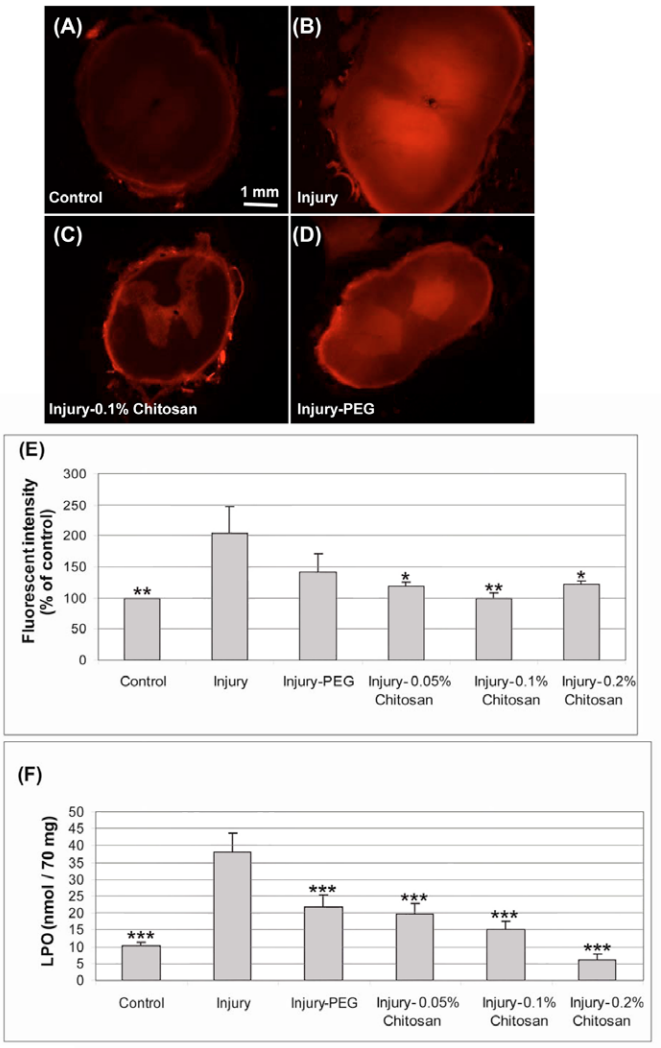

Fig. 4. Effects of chitosan treatment on reactive oxygen species (ROS) and lipid peroxidation (LPO) generation in crushed spinal cords. (A–D) Representative fluorescence microscopy images show the intensity of oxidized hydroethidine (HE) in the control, uninjured (A), injured, untreated (B), injured–0.1% (w/w) chitosan (pH 7.0)-treated (C), and injured–50% (w/w) PEG-treated spinal cords (D). Treatment with chitosan and PEG was conducted immediately after complete compression. The effect of PEG, a known inhibitor of free radical production, was used to compare the level of ROS and LPO with those after application of chitosan. The compression injury enhanced the generation of oxidized HE, but chitosan treatment inhibited it. (E) Quantification of oxidized HE fluorescence intensity as a function of the concentration of chitosan (N=5, *P<0.05, **P<0.001). Note chitosan significantly reduced the production of oxidized HE when compared with PEG. (F) The level of LPO after spinal cord compression followed by the application of chitosan at different concentrations compared to PEG treatment (N=5, ***P<0.005). Compression injury induced a significant elevation in the level of LPO, but chitosan application reduced it.

assess mitochondrial function after acrolein-mediated attack. Hydralazine is an 'antidote' to the endogenous toxin acrolein as it forms a nontoxic hydrazone adjunct. Thus a significant rescue of acrolein-poisoned oxidative metabolism was seen after exposure to hydralazine. Chitosan at 0.05%, 0.1% and 0.2% showed a trend of improving the MTT after acrolein exposure, but this was not statistically significant as it was when hydralazine was the experimental treatment (Fig. 6).

Somatosensory evoked potential recordings

Stimulation of the tibial nerve of the hind limb evoked early and late arriving potentials (latency ~35–50 ms) recorded in the contralateral sensorimotor cortex in every control and experimental animal (Fig. 7). In every case, the standardized crush of the spinal cord eliminated these evoked potentials. All eight chitosan-treated animals recovered the SSEP within 30 min of the injection. The restored ability to propagate SSEPs through the lesion to the brain was stable in six of the animals that were routinely tested from 5 days to 1 week post injury (the other two animals that had recovered the SSEP, died after 2 days of recording). None of the six control animals recovered SSEPs over the 1 week of observation and measurement. This difference in recovery was highly statistically significant (P=0.003; Fischer's exact test).

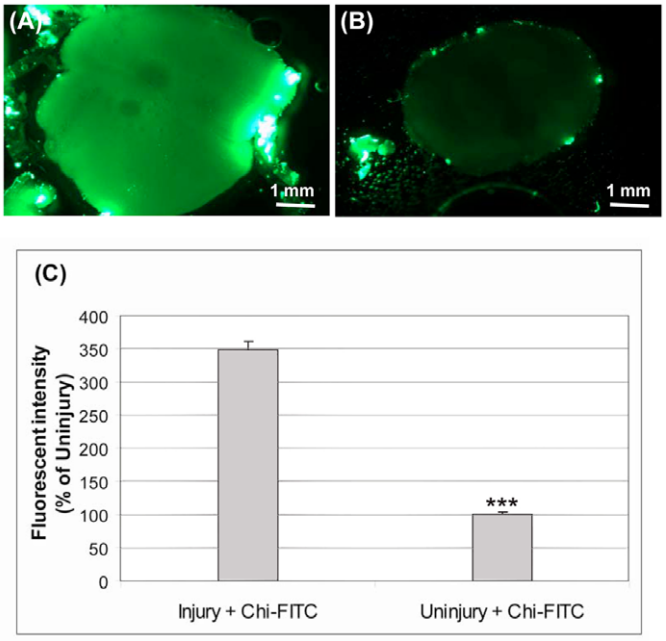

Fig. 5. Targeting ability of chitosan in spinal cord injury. (A,B) The distribution of chitosan-FITC in compressed spinal cord. Chitosan-FITC was applied to the area of compression injury for 15 min and the spinal cord was subsequently immersed in Krebs solution for 30 min. Fluorescence microscopy was used to reveal the labeling of both injured and uninjured cord in 50 μm thick frozen sections. The crushed region (A) shows strong labeling with chitosan-FITC whereas the control site (B; three to four vertebral segments distant) is faintly labeled, indicating that chitosan specifically labeled damaged tissues but did not label undamaged tissues of the adjacent regions. (C) Quantification of fluorescent intensity normalized after subtraction of background. Results are expressed as percentages of the pre-injury values (± s.d.; N=3). ***P<0.005. Chi, chitosan.

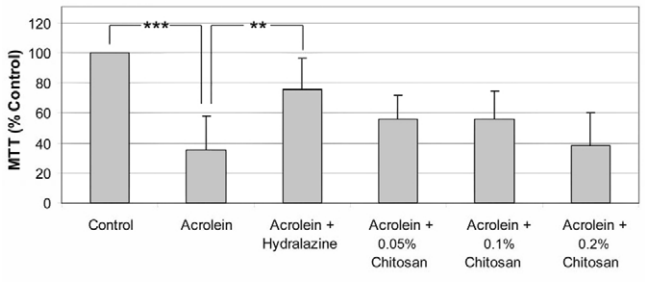

Fig. 6. Chitosan is not an acrolein scavenger. Approximately 15 min after acrolein challenge (100 μM), spinal cords were exposed to 500 μmol l^{-1} hydralazine or chitosan. The enhancement of MTT level after chitosan treatment could be attributed to its sealing effect on damaged cell membranes, but not to it acting as an acrolein scavenger. Results are expressed as percentages of control values (± s.d.; N=5). **P<0.001, ***P<0.005.

DISCUSSION

Our experiments indicated that the polysaccharide chitosan has the same capability to seal damaged cell membranes as polymeric based fusogens.

Chitosan reduces the disruption of cell membrane following traumatic spinal cord injury

This is the first report showing that chitosan can fuse and seal disrupted plasma membranes following traumatic spinal cord injury. Chitosan probably induced the formation of phospholipid aggregates that become a basis for plasma membrane fusion and sealing. The inherent non-toxicity and biocompatible nature of chitosan has received much attention as a functional biopolymer in medical and pharmaceutical areas (Bodmeier et al., 1989; Illum et al., 1994), and the extension of its capabilities as a vehicle for cell membrane fusion can be included in its possible medical applications. The recognition properties of chitosan with membrane phospholipids are mainly governed by electrostatic interactions, hydrogen bonding and hydrophobic forces (Fang et al., 2001; Pavinatto et al., 2007).

The improvement in membrane permeability was examined by the commonly used, but effective, label, TMR. The internalization of the dye into the cytoplasm of damaged cells is dependent on the severity of the disruption. Typically, severely compromised tissues, both white and gray matter, are intensely fluorescent, whereas undamaged cells and tissues are not (as their membranes prevent the internalization of dye) (Cho et al., 2008). The large surfactant aggregates produced by the application of chitosan significantly reduced membrane permeability to the hydrophilic TMR dye, indicating its capacity to seal membranes. Moreover, chitosan application resulted in the inhibition of leakage of the intracellular enzyme LDH in a pH-dependent manner (there was always some leakage of LDH, even in control preparations because of the handling and manipulation of the samples). Generally, the negatively charged cell membrane exhibits higher affinity for positively charged chitosan as a result of an electrostatic interaction between chitosan's primary amine groups and the negative cell surface charge. These results are also consistent with a previous study in which chitosan was adsorbed onto bacterial cells at low pH rather than high pH (Chung et al., 2004). In addition, chitosan, as a gene delivery vector, shows a higher transfection effect at low pH rather than that of high pH by the activity of protonated amine group below pH 7.0 (Mislick and Baldeschwieler, 1996). We examined the chitosan-induced LDH reduction in a concentration-dependent way, but did not discover a correlation. This suggested the base concentration of chitosan probably saturated the regions of damage to the membrane. However, we have shown that chitosan effectively decreased the level of LDH leakage more significantly than PEG, independent of pH and concentration. Although the molecular mechanisms of membrane fusion produced by chitosan has yet to be completely understood, Zuo et al. suggested that unique hydrophilicity and charge-dependent properties of chitosan induce phospholipid aggregates through the phase transition from a gel to liquid crystalline caused by electrostatic interaction (Zuo et al., 2006). Such membrane integrity assays with a different molecular mass compound emphasized that chitosan was capable of sealing permeabilized membranes by repairing breaches through direct interaction with lipid bilayers.

Chitosan targets disrupted membrane

The activity of chitosan on phopholipid-based membranes is very interesting. Pavinatto et al. have discussed the interaction of chitosan with cell membrane models (Fang et al., 2001; Pavinatto et al., 2007). At larger surface areas per molecule, which represents transected or crushed spinal cord membrane in our case, chitosan tends to be accumulated among phospholipid chains by hydrophobic interactions, whereas when the area per molecule is small, as in intact membranes, high surface densities of lipid moieties excludes the penetration of chitosan, resulting in only a surface interaction.

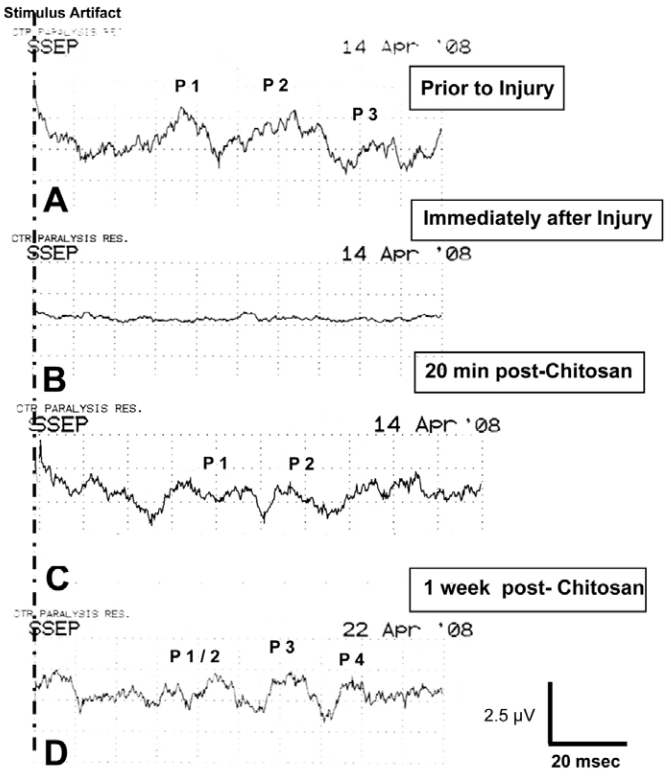

Fig. 7. The loss and recovery of the somatosensory evoked potential. (A) A typical SSEP recording of all animals in this study. The initial peak is a stimulus artifact (marked by the hatched line). This record was signal averaged from three separate sets of complete records taken sequentially (see Materials and methods). Note the typical three 'peaks' (evoked potentials, or EPs, P1–P3). The earliest SSEP was usually recorded at the contralateral sensorimotor cortex, approximately 25–45 ms after stimulation of the tibial nerve of the hind leg. Note the later EPs, P2 and P3. (B) The complete loss of EP conduction to the brain after a midthoracic crush of the spinal cord in response to the continuing identical stimulation regimen shown in A. This record was also typical of every animal in this study subsequent to spinal cord injury. (C) The very rapid recovery of the SSEP in the same animal shown in A and B after a subcutaneous injection of chitosan. This recovery was measured approximately 20 min after the injection. P1 appears extended in duration, and eventually splits into two discernable EPs. (D) The stability of the recovered SSEP is shown at 1-week post-treatment. Note the first EP appears to have segregated into two smaller peaks about 2 ms apart. As discussed, none of the control animals recovered the SSEP, whereas all of the chitosan-treated animals did.

This supports our hypothesis that chitosan preferentially and specifically targets injured tissues. Fig. 5 shows chitosan-FITC distribution at the lesioned and relatively intact sites on the same spinal cord, which indicated the ability of chitosan to achieve specific localization to permeabilized membrane. The fluorescence intensity in the white matter of crushed spinal cord was almost 3.5 times higher compared with that of uninjured cord. In addition, faint labeling of gray matter also supported the targeting ability of chitosan. This remarkable observation was consistent with several chitosan–phospholipid membrane models. As demonstrated by the atomic force microscopy (AFM) study of Fang et al., *in situ* AFM measurements indicated that significant deposition of chitosan initially occurred around areas of defect in phospholipid bilayers (Fang and Chan, 2003). This strengthens the hypothesis that highly packed phospholipids in well organized membrane exclude chitosan, whereas defective areas characterized by disorganized phospholipids are likely to be penetrated by phospholipid chains, thus resulting in a hydrophobic and electrostatic interaction. Even though such observations have been confirmed at the molecular level, we extend these models to include our chitosan-induced membrane sealing and targeting in CNS tissue. Furthermore, this mechanism is not the same as, but may overlap, the mechanisms of polymer-based sealants such as PEG (Borgens, 2001; Maskarinec et al., 2002).

Chitosan blocks the generation of ROS and LPO

As discussed previously, disruption of cell membranes not only affects the structural integrity of cell membranes – but also mitochondrial activity and overall cell vitality through a cascade of pathophysiological events. Progressive catabolism of the membrane and the integrity of the entire cell probably begins with the increased concentration of cytosolic calcium (running down its electrochemical gradient) which destabilizes mitochondria (as well as other organelles and cytoarchitecture) shifting oxygen metabolism to the formation of reactive oxygen species or ROS. Thus, the production of ROS or so-called free radicals (such as superoxide (O_2^-), hydroxyl ions (OH), and hydrogen peroxide (H_2O_2), is a direct result leading to skyrocketing, secondary oxidative stress. Understandably, such endogenous and deleterious substances continue to result in progressive cell destruction *via* LPO and its derivatives, toxic intracellular aldehydes (Burcham et al., 2004; Burcham and Pyke, 2006; Luo et al., 2005; Luo and Shi, 2004; Luo and Shi, 2005; Shi et al., 2002). Since these toxins, like acrolein, can pass through intact healthy membranes, their intracellular concentration increases in the microenvironment permitting the poisoning of nearby undamaged cells (called 'bystander damage'). Considerable studies have been carried out showing PEG- and P188-induced membrane sealing reduces oxidative stress (Cadichon et al., 2007; Frim et al., 2004; Luo et al., 2002; Luo and Shi, 2004). Furthermore, electroporation-induced oxidative injury and LPO has been ameliorated by the application of P188 (Collins et al., 2006). Similar to such nonionic polymers, our observations and measurements indicate that the application of chitosan not only contributed to the sealing of membrane permeability but also induced neuroprotection by interfering with progressive generation of ROS and LPO. However, we should point out chitosan, like PEG, is not an antioxidant in the chemical sense, as it does not have any intrinsic scavenging capability (Liu-Snyder et al., 2007). Chitosan application induces the suppression of reactive oxygen species, apparently by the restructuring of the plasma membrane – and even the mitochondrial membrane if the fusogen moves into the cytosol (Luo et al., 2004; Luo and Shi, 2005). This hypothesis is further supported by the reduction of acrolein-mediated oxidative stress subsequent to chitosan application (Fig. 6). However, this reduction was only a trend and not statistically significant. Thus, as with reactive oxygen species, chitosan does not show any ability to scavenge acrolein, and even the application of highly concentrated chitosan does not repair injury associated with acrolein toxicity (Fig. 6). On the contrary immediate treatment with hydralazine, a well-known acrolein scavenger, permitted recovery from mitochondrial injury and abnormal oxidative metabolism (Burcham and Pyke, 2006; Liu-Snyder et al., 2006). In summary, chitosan enabled the reduction of ROS and LPO production by the restoration of disrupted plasma membrane.

CONCLUSIONS

Through our *ex vivo* and *in vivo* spinal cord injury models, our data demonstrated that (1) the application of chitosan was able to

immediately restore compromised membrane integrity; (2) chitosan was a potent neuroprotective agent, even though it did not show any ROS, or acrolein, scavenging ability; (3) chitosan clearly targeted the area of tissue damage, where uninjured spinal cord exhibited a very weak affinity for chitosan. Finally, the chitosan approach for damaged membranes provides novel therapeutic potential through site-specific delivery following traumatic spinal cord and head injury.

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