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Pyrroloquinoline quinone-loaded coaxial nanofibers prevent oxidative stress after spinal cord injury

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Oxidative stress plays a major role in the secondary injury of the spinal cord tissue due to the high lipid content of nervous tissue. In the present study, coaxial nanofibers were loaded with the natural antioxidant pyrroloquinoline quinone (PQQ) and used as an implantable drug-delivery system and a scaffold post-SCI. The obtained data show that the concentration of NO and the activity of inducible nitric oxide synthase (iNOS) were significantly ($P < 0.05$) increased in the spinal cord injury (SCI) group. These levels were significantly decreased following treatment with nanofibers/PQQ. Implantation of nanofibers/PQQ resulted in a significant ($P < 0.05$) drop in the level of malondialdehyde (MDA) compared to the SCI group. The application of nanofibers loaded with PQQ after SCI caused a significant ($P < 0.05$) elevation of superoxide dismutase (SOD) and catalase (CAT) activity in the spinal cord tissue. The present work shows the protective role of coaxial nanofibers loaded with PQQ against oxidative stress in spinal cord injury. The reversal of oxidative stress with PQQ can lead to better outcomes following spinal cord injury.

1. Introduction

Following the primary mechanical damage to the spinal cord, a series of cellular and biochemical events occur, leading to secondary tissue damage characterizing spinal cord injury (SCI). These secondary events include a significant decline in the activity of antioxidant enzymes and anti-inflammatory cytokines. Therefore, spinal cord injury causes permanent neural dysfunction.[1–4] The spinal cord is highly vulnerable to oxidative injury due to its high lipid content, which is especially susceptible to peroxidation by reactive oxygen species (ROS).[5] Excessive generation of ROS during SCI results in oxidative stress and subsequent increase in free radicals leading to the progression of secondary damage.

Several anti-inflammatory and antioxidant compounds have a neuroprotective effect in SCI.[6] Pyrroloquinoline quinone (PQQ), also known as methoxatin, is a water-soluble, thermo-stable triglyceride-quinone that is widely distributed in nature and characterized as a mammalian vitamin-like redox cofactor.[7–9] It is also considered an essential nutrient that has many useful properties such as cardioprotective,[10] hepatoprotective,[11] and antioxidant properties.[12] PQQ has also been found to elevate the concentration of nerve growth factors in astrocytes and enhance sciatic nerve regeneration in rats.[13] Intraperitoneal administration of PQQ effectively promotes functional recovery after spinal cord injury in rats after hemisection.[14] Therefore PQQ is considered a promising compound with extensive benefits for the nervous system to diminish the secondary injury events that follow the primary mechanical injury.

The main challenge has always been delivering the drug to the target site in an optimal dose and increasing its effectiveness. Nanofibers have become a promising tool for drug delivery in several biomedical fields. The main advantage of the nanofiber coaxial design is that after electrospinning and encapsulating the drugs in the core and during the release, these drugs remain bioactive due to the protection of the sheath.[15,16] The current study investigates the use of PQQ loaded on coaxial nanofibers in an experimental model of spinal cord injury in rats.

2. Experimental section

2.1. Preparation of CS/PVA coaxial nanofibers

Coelectrospinning was used to fabricate CS/PVA coaxial nanofibers (Fig. 1) using an acetic acid/distilled water solution mixture as a spinning solvent as previously described.[15] Briefly, optimum preparation conditions for CS/PVA nanofibers were established as follows: a polymer volume ratio of 30/70 (CS/PVA), a mixture concentration of 50%, an applied voltage of 27 kV, a flow rate of 0.4 mL h^{-1} for the shell and 0.3 mL h^{-1} for

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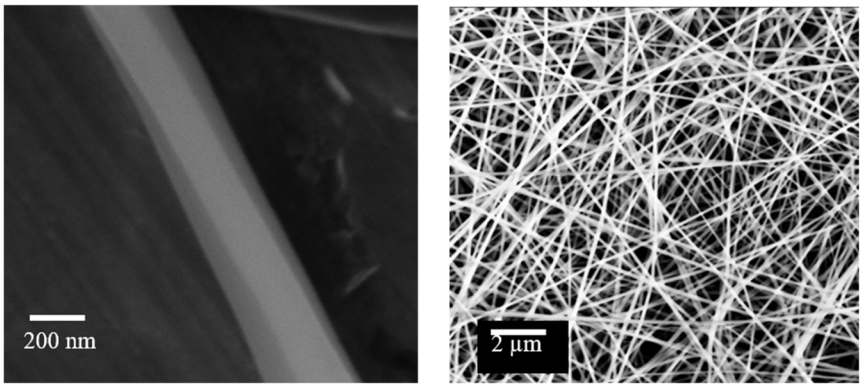

Fig. 1 FESEM images of the synthesized coaxial nanofibers.

the core, and a tip to collector distance (TCD) of 10 cm.[15] For the fabrication of the PQQ-loaded coaxial nanofibers, CS/PVA (shell) and CS/PVA/PQQ (core) solutions, for drug loading, PQQ (15% w/v) was added to the CS/PVA mixture. The CS/PVA solution was loaded in the syringe of the outer needle (shell) and the CS/PVA/PQQ solution was loaded in the syringe of the inner needle (core) under the same previous conditions.

2.2. Experimental animals

Adult female Sprague-Dawley rats (*Rattus norvegicus albinus*), weighing 200–250 g, were housed in polypropylene cages in temperature-controlled rooms (23 ± 2 °C) and under a natural day and night cycle. They were fed standard chow pellets and drinking water *ad libitum*. All procedures were performed in compliance with the National Institute of Health (NIH) guidelines for the Care and Use of Laboratory Animals, and according to Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. All procedures were approved by the Cairo University IACUC committee (CUIF9017). Rats were randomized using the block randomization method into normal control, sham control, or experimental groups. Rats were anesthetized with intraperitoneal (i.p.) injection of ketamine/xylazine (ketamine 80–100 mg kg^{-1} and xylazine 10–12.5 mg kg^{-1} IP). The skin was prepped and incised at the back of rats, muscles were split, and laminectomy was performed at T9-10 thoracic vertebrae, and the dura was incised longitudinally. Experimental groups received a right lateral hemisection of the spinal cord at T9-10 using micro iris scissors, followed by the immediate application of (0.5 cm × 0.5 cm) either coaxial nanofibers without PQQ or coaxial nanofibers loaded with PQQ. The dura was sutured, and the muscles and skin were closed in layers. All animals received wound care and analgesia as needed following the surgery (Fig. 2).

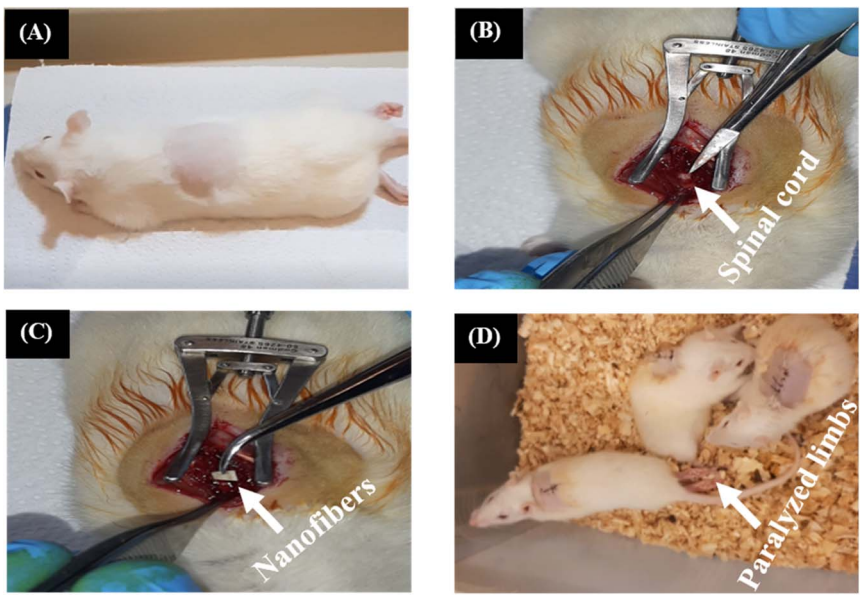

Fig. 2 Surgical procedures of the experimental SCI model in rats. (A) Rat prepared for the surgery, (B) exposure of the spinal cord (arrow shows the spinal cord), (C) immediate application of nanofibers (arrow shows the nanofiber scaffold), and (D) post-surgical procedure (arrow shows paralyzed lower limbs).

2.3. Biochemical analyses

2.3.1. Determination of nitric oxide (NO) concentration. The level of NO was estimated in spinal cord tissue according to the method described by Montgomery & Dymock.[17] In an acidic medium and in the presence of nitrite, the formed nitrous acid diazotizes sulphanilamide. The product is coupled with *N*-(1-naphthyl)ethylenediaminedihydrochloride (NEDA), and the resulting azo dye has a bright reddish-purple color which is measured at 540 nm.

2.3.2. Quantitative determination of inducible nitric oxide synthase (iNOS). The level of iNOS was quantitively determined in the spinal cord tissue of rats by using ELISA according to the manufacturer's protocol (MyBioSource, USA).

2.3.3. Determination of malondialdehyde (MDA) concentration. The lipid peroxidation and oxidative stress marker malondialdehyde (MDA) was determined in the spinal cord tissue according to the method described by Ohkawa *et al.*[18] Briefly, thiobarbituric acid (TBA) reacts with malondialdehyde (MDA) in an acidic medium at a temperature of 95 °C to form the thiobarbituric acid reactive product. The absorbance of the resultant pink product can be measured at 534 nm.

2.3.4. Determination of super oxide dismutase (SOD) activity. The activity of the antioxidative enzyme superoxide dismutase (SOD) was determined in the spinal cord tissue according to the method described by Nishikimi *et al.*[19] This assay relies on the ability of the enzyme to inhibit the phenazine methosulphate-mediated reduction of nitroblue tetrazolium dye.

2.3.5. Determination of catalase (CAT) activity. Catalase was determined in the spinal cord tissue according to the method described in ref. 20 and 21. The method depends on the dissociation of hydrogen peroxide. which is proportional to the activity of the catalase enzyme in the used sample.

2.3.6. Statistical analyses. Statistical analyses were carried out using SPSS® version 15 software. All data were expressed as mean ± standard error of the mean (SEM). The independent variables of individual comparisons were illustrated by using the LSD *post hoc* test of one-way ANOVA to determine significant differences in mean values between the different groups. P values less than 0.05 were considered statistically significant.

3. Results & discussion

3.1. Nitric oxide (NO) concentration in spinal cord tissue

The NO concentration was significantly ($P < 0.05$) increased in the SCI group compared to the control and sham groups. The percentage increase from the control value was +229.59, +164.72, +155.46 and +134.96 at 1, 7, 14 and 21 days post injury, respectively. Statistically, the concentration of NO in the unloaded nanofibers and PQQ groups was significantly ($P < 0.05$) decreased when compared to the SCI group at all time intervals explored.

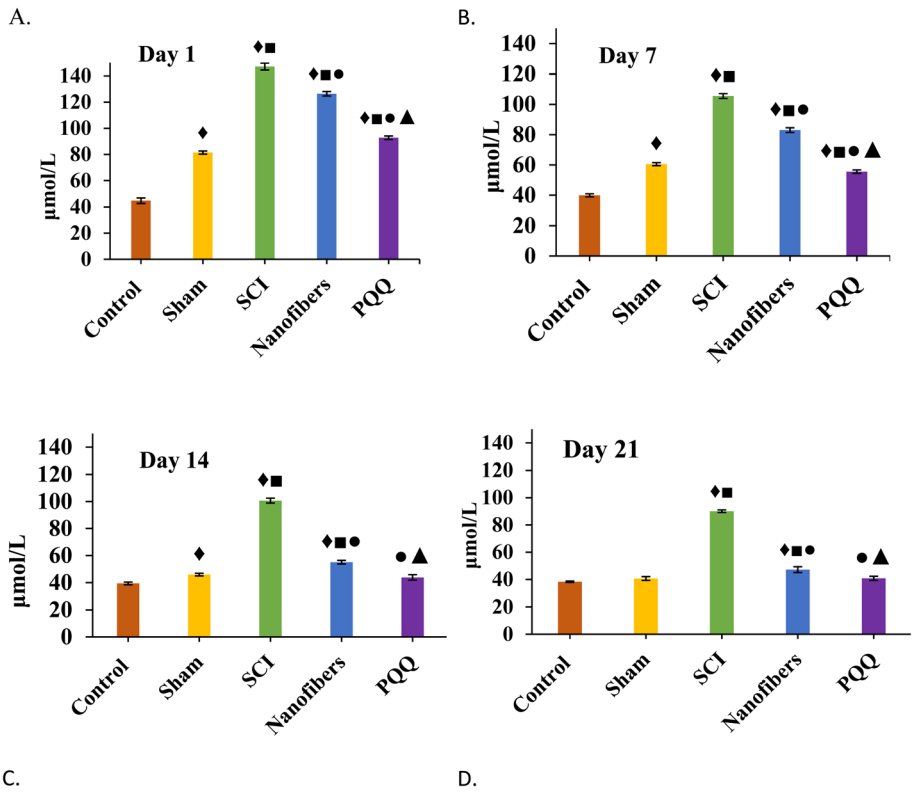

Fig. 3 Nitric oxide levels (µmol L^{-1}) in spinal cord tissue of rats. The NO concentration was significantly ($P < 0.05$) increased in the SCI group compared to the control and sham groups. The levels in unloaded nanofiber and nanofiber/PQQ groups were significantly ($P < 0.05$) decreased compared to the SCI group at all time intervals. After 1 (A), 7 (B), 14 (C), and 21 (D) days, NO concentration of the nanofiber/PQQ group was reduced to reach control values. $N = 5$, $P < 0.05$ (mean ± SEM).

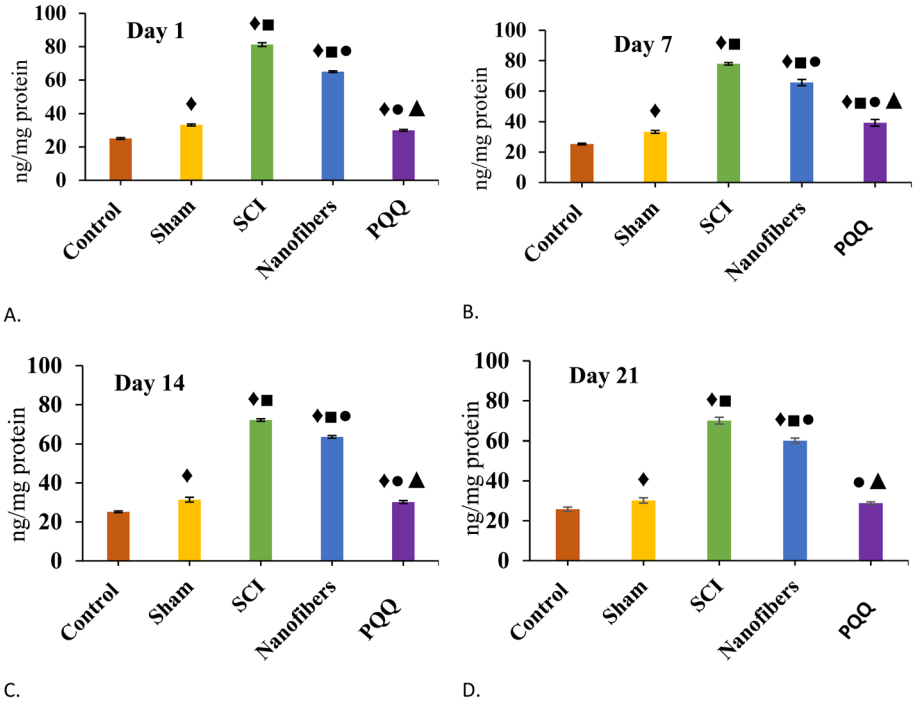

Fig. 4 Inducible nitric oxide synthase levels (ng mg^{-1} protein) in spinal cord tissue. The figure illustrates the activity of iNOS in spinal cord tissue after 1 (A), 7 (B), 14 (C), and 21 (D) days of injury. The results showed that the activity of iNOS was significantly ($P < 0.05$) increased in the SCI group in comparison with control and sham groups at all the time intervals studied. Treatment of injured rats with unloaded nanofibers and nanofibers loaded with PQQ resulted in a significant ($P < 0.05$) decrease in the iNOS activity compared with the activity recorded for the SCI group. $N = 5$, $P < 0.05$ (mean ± SEM).

Moreover, after 14 and 21 days of injury, NO concentration in the PQQ group was reduced to reach control values, Fig. 3.

3.2. Inducible nitric oxide synthase (iNOS) level in spinal cord tissue

The results showed that the activity of iNOS was significantly ($P < 0.05$) increased in the SCI group in comparison with control and sham groups at all the time intervals studied. The increase in the activity of iNOS with respect to control was +223.45%, +207.58%, +186.23% and +171.89% after 1, 7, 14 and 21 days, respectively. However, treatment of injured rats with unloaded nanofibers resulted in a significant ($P < 0.05$) decrease in the iNOS activity if compared with the activity recorded for the SCI group. Additionally, treatment of rats with nanofibers loaded with PQQ caused a more prominent decrease in the enzyme activity compared to the SCI and unloaded nanofiber groups but remained higher than that of the control group, Fig. 4.

3.3. Malondialdehyde (MDA) concentration in spinal cord tissue

Spinal cord injury caused a significant ($P < 0.05$) increase in MDA levels compared to the control and sham groups at all time intervals. Implantation of unloaded and PQQ nanofibers caused a significant ($P < 0.05$) decrease in the level of MDA compared to the SCI group. With respect to control, this increase was +152.07%, +115.99%, +101.51% and +88.97% higher after 1, 7, 14 and 21 days of injury, respectively. However, MDA levels in the unloaded nanofiber group remained significantly ($P < 0.05$) elevated compared to control values throughout the whole experimental period, Fig. 5. Nanofibers loaded with PQQ caused a significant ($P < 0.05$) decrease in MDA levels in the spinal cord tissue to near normal levels after 14 and 21 days of injury as a nonsignificant change, compared with either control or sham groups was observed.

3.4. Superoxide dismutase (SOD) activity in spinal cord tissue

The activity of SOD was significantly ($P < 0.05$) inhibited following SCI. However, applying unloaded nanofibers after SCI caused a significant ($P < 0.05$) elevation of SOD activity compared with the SCI group throughout the whole experiment (Fig. 6). The inhibition in enzyme activity with respect to control was −65.23%, −39.29%, −33.98% and −25.39% after 1, 7, 14 and 21 days, respectively. Treatment with nanofibers loaded with PQQ induced a significant ($P < 0.05$) increase in SOD activity in spinal cord tissue of rats in comparison with both SCI and unloaded nanofiber groups at all the time intervals studied. Moreover, nonsignificant changes in SOD activity were noticed after 1, 7, 14, and 21 days with respect to control and after 21 days only with respect to the sham group, Fig. 6.

3.5. Catalase (CAT) activity in spinal cord tissue

Catalase activity in the spinal cord tissue showed a significant ($P < 0.05$) decrease following SCI. Treatment with unloaded

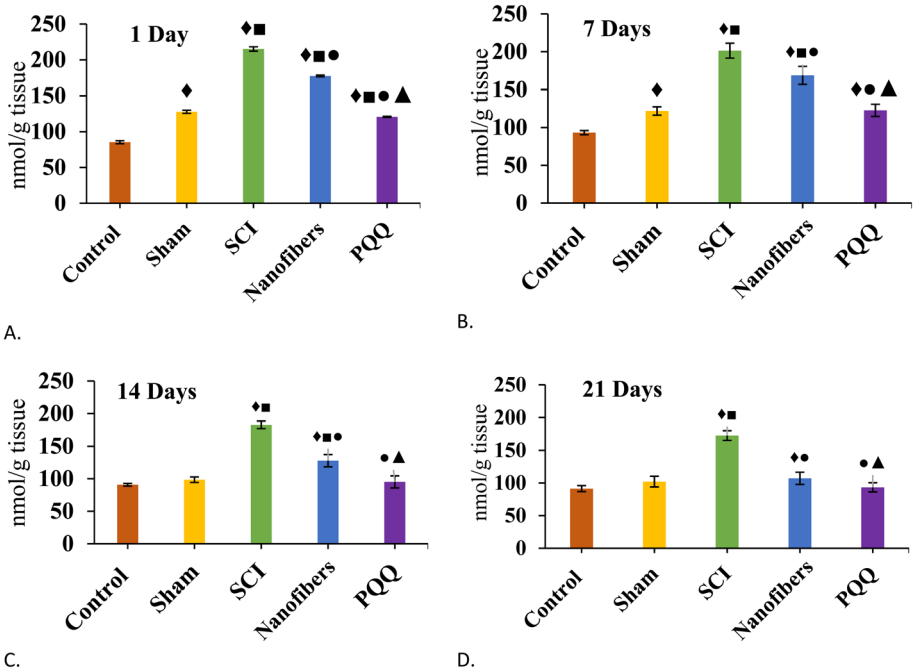

Fig. 5 Malondialdehyde concentration (nmol g^{-1} tissue) in spinal cord tissue. Spinal cord injury caused a significant ($P < 0.05$) increase in MDA levels compared to the control and sham groups at all time intervals studied. Implantation of both types of nanofibers resulted in a significant ($P < 0.05$) drop in the level of MDA compared to the SCI group. However, in the unloaded nanofibers, MDA levels remained significantly higher ($P < 0.05$). Nanofibers loaded with PQQ caused a significant ($P < 0.05$) decrease in MDA levels to normal levels after 1 (A), 7 (B), 14 (C), and 21 (D) days of injury. $N = 5$, $P < 0.05$ (mean ± SEM).

nanofibers caused a significant ($P < 0.05$) increase in the level of CAT activity in spinal cord tissue of rats in comparison with the SCI group (Fig. 7). With respect to control, the decrease was −61.04%, −58.41%, −50.66% and 45.25% at 1, 7, 14 and 21 days post injury, respectively. CAT activity in spinal cord tissue of animals treated with PQQ-loaded nanofibers was significantly

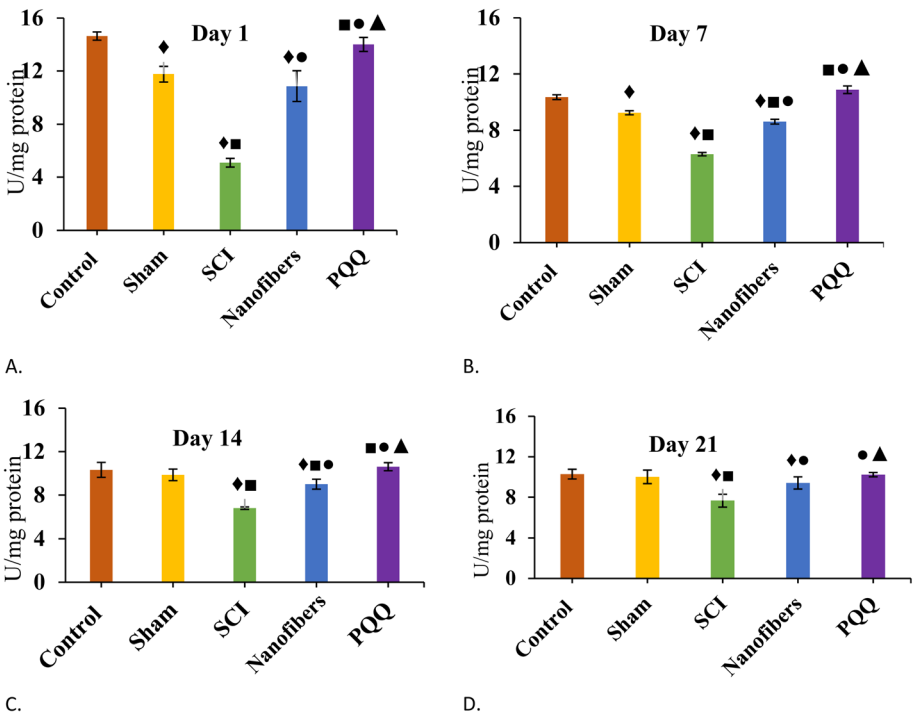

Fig. 6 Superoxide dismutase activity (U mg^{-1} protein) in spinal cord tissue. The activity of SOD was significantly ($P < 0.05$) inhibited following SCI. The application of unloaded nanofibers and nanofibers loaded with PQQ after SCI caused a significant ($P < 0.05$) elevation of SOD activity. $N = 5$, $P < 0.05$ (mean ± SEM) after 1 (A), 7 (B), 14 (C), and 21 (D) days of injury.

increased compared with that of SCI and unloaded nanofiber groups. However, after 14 and 21 days of injury, there was no significant difference in the enzyme activity in the PQQ-loaded nanofiber group compared with the control group, Fig. 7.

Traumatic injuries of the brain and spinal cord cause tissue damage through primary and secondary mechanisms. The primary injury results in damage of neurons and glial cells and disruption of the blood supply. The secondary injury causes further dysfunction.[22] The importance of ROS and lipid peroxidation in SCI is supported by several experimental studies showing the potential neuroprotective efficacy of several bioactive agents with antioxidant properties.[23] The main objective of the present work was to evaluate the effect of coaxial nanofibers loaded with PQQ as an implantable drug-delivery system (scaffold) on SCI in rats. Previous reports show that PQQ reduced the expression of iNOS mRNA in injured spinal cord tissue, and consequently reduced the level of NO.[24,25] Several properties of PQQ[24] could be involved in its neuroprotective activity. First, it may function as an effective antioxidant, and it is known to protect the mitochondrial functions from oxidative damage. Second, PQQ may suppress peroxynitrite ($ONOO^-$) formation because it is a free radical scavenger and a cofactor for quinoprotein enzymes.

Many drugs require repeated administration to accomplish and maintain therapeutic concentration. In the nervous system, physiological barriers such as the blood–brain and blood–spinal cord barriers prevent medications from reaching effective therapeutic concentrations. Implantable drug-delivery systems provide new methods to overcome these hurdles and achieve effective therapeutic concentrations. These drug-delivery systems are enabled by micro and nano-fabrication technologies.[26,27] In the present study, the coaxial electrospinning technique was used to fabricate core–sheath coaxial nanofibers based on the co-electrospinning of CS/PVA blends. They were used as an implantable drug-delivery system and a scaffold post-SCI. Chitosan and PVA are non-toxic water-soluble biocompatible and biodegradable polymers widely used in the biochemical field.[28,29] Biocompatible and biodegradable polymers have been recently utilized in the synthesis, preparation, and functionalization of sustainable nanofiber materials including chitosan for energy, environmental, and biomedical applications. The importance of using biodegradable fibers in recent research is because these materials are environmentally friendly and renewable. Thereby, they can undergo a cyclic process involving regenerative resources and production, and hence can be used in a sustainable recycling loop. Particularly, PVA is easily mixed with chitosan and the final polymer is easily biodegraded by microorganisms. The CS/PVA nanofibers provide three-dimensional scaffolds as temporary supporting structures for growing cells and tissues and support the native extracellular matrix in the healing phase.[30,31] Chitosan also provides mechanical and trophic support for the spinal cord and discourages scar formation through the bridging of the lesion site.[27]

Compared with traditional (uniaxial) nanofibers, core–sheath (coaxial) nanofibers have many advantages. First, the sheath phase can safely protect the bioactive drugs from the harsh environment. Second, the structure of coaxial nanofibers

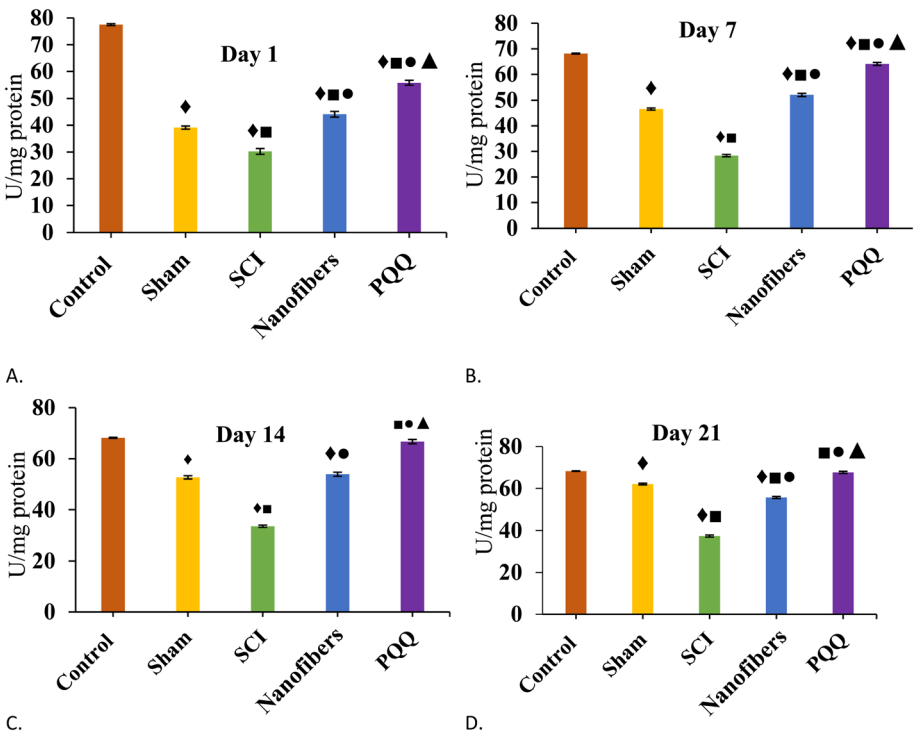

Fig. 7 Catalase activity (U mg^{-1} protein) in spinal cord tissue of rats. Catalase activity in spinal cord tissue showed a significant ($P < 0.05$) decrease following SCI. Treatment with unloaded nanofibers and PQQ-loaded fibers caused a significant ($P < 0.05$) increase in the level of CAT activity in the spinal cord tissue. $N = 5$, $P < 0.05$ (mean ± SEM) after 1 (A), 7 (B), 14 (C), and 21 (D) days of injury.

resembles the extracellular matrix. Third, after the process of electrospinning and release, these drugs remain bioactive due to the protection of the sheath. Finally, the coaxial encapsulation of drugs within nanofibrous materials protects the drugs and can be used as a scaffold in drug delivery.[16,32]

Nitric oxide (NO) is a small chemical messenger involved in several physiological processes in the nervous system. Strong evidence suggests that NO is involved in the mechanisms of neurotoxicity after SCI.[33] It is involved in the control of cerebral blood flow, interneuron communication, intracellular signal transmission, and release of neurotransmitters. It has been demonstrated that NO can exert both protective and detrimental effects in several disease states of the CNS, including SCI.[34] The levels of NO in the spinal cord after acute traumatic injury in rats were markedly increased immediately after injury. The second wave of increase in NO levels was observed at 24 hours and three days after injury.[35]

The present data show that the concentration of NO was significantly increased in the SCI group as compared with the control and sham groups. This increase in the level of NO was accompanied by a simultaneous elevation in the activity of the enzyme iNOS. NO is considered one of the major regulators of spinal cord damage and is involved in developing post-traumatic spinal cord cavitation.[36] NO is not highly toxic.[37] However, it reacts with O_2^- to form the potent oxidant peroxynitrite that can directly oxidize lipids, DNA, and proteins.[38] NO, after reaction with superoxide to form $ONOO^-$, induces neuronal necrosis if the insult is intense. If the insult is mild, however, NO induces neuronal apoptosis.[38] Detection of the biomarkers of ROS and oxidative stress is important for assessing pathogenesis and progression of SCI. Catalase (CAT) is also a very important enzyme in protecting cells from oxidative damage by ROS. MDA levels are elevated as early as 1 h and up to 1 week after SCI.[39] However, SOD and catalase activities are decreased.[40,41]

Following the spinal injury, a significant increase in the MDA level was seen in the SCI group. Oxidative stress, as evidenced by the increased MDA, supports the generation of reactive oxygen species in SCI.[42,43] Oxygen-free radicals and lipid peroxidation induce oxidative stress and contribute to the pathogenesis of secondary SCI. Although free radicals mediate damage in all biomolecules, lipid peroxidation appears to have a dominant role. MDA, an end metabolic product of unsaturated fatty acid peroxidation induced by oxygen free radicals, reflects the level of lipid peroxidation and the degree of injury after free radical exposure.[44,45] Superoxide dismutase (SOD) prevents endothelial and mitochondrial dysfunction by inactivating nitric oxide and inhibiting $ONOO^-$ formation. It also clears oxygen radicals produced in the respiratory chain.[46,47]

In the present study, the data show that the increase in the level of MDA post-SCI was also accompanied by a decrease in the activities of SOD and CAT compared to the control and sham groups. SOD is an enzyme that neutralizes oxygen-free radicals and protects the cell from being oxidized by superoxide toxicity. SOD is consumed during oxidative stress under a variety of conditions, which explains our current data showing a significant decrease in the antioxidant enzyme SOD level in SCI rats.[48,49] Previous studies also reported similar results.[38,50] Meanwhile, catalase catalyzes the breakdown of hydrogen peroxide into water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species.[51] Catalase and SOD are considered the first-line defense antioxidants.

In the current study, the treatment of SCI in rats with PQQ reversed oxidative stress parameters. This agrees with previous reports that show PQQ's anti-inflammatory and antioxidant properties.[52,53] PQQ prevented oxidative changes when fed to animals as a supplement (Stites *et al.*, 2006).[54] The current data also indicated that the NO, iNOS, and MDA levels in the PQQ-loaded nanofibers exhibited a significant decrease compared to the SCI group. PQQ also caused a significant increase in the activity of both antioxidant enzymes, SOD and CAT, compared to other groups. This agrees with previous reports[14] that the intraperitoneal administration of PQQ effectively promotes the functional recovery of SCI in rats, decreases lesion size and increases axon density associated with the lesion area, as well as decreases the activity of iNOS. PQQ treatment also suppressed peroxynitrite formation,[8] which is a potential byproduct of abnormally high nitric oxide or hydrogen peroxide levels.[25]

PQQ has anti-inflammatory and antioxidant effects and improves mitochondrial function *in vitro* and *in vivo*. The increase in the levels of the two antioxidant enzymes SOD and CAT after PQQ treatment could be an attempt to restore the redox balance in the spinal cord, even though the activity of the two enzymes was still lower than the pre-injury values. The present data confirmed previous reports that PQQ could suppress the oxidative stress and inflammatory response and, it has been shown to be a powerful antioxidant that scavenges highly toxic hydroxyl radicals and other ROS that initiate lipid peroxidation, DNA damage and protein oxidation.[52–54] This is in agreement with previous studies that proved the neuroprotective effect of PQQ in the CNS in several models of brain injury and disease.[55,56] Furthermore, a recent study revealed that PQQ treatment elicited antioxidant effects, leading to a reduction in ROS levels observed *in vitro* beside its significant improvements in cognitive function in neurological disorders.[57] PQQ treatment suppressed several pro-inflammatory and oxidative stress mediators (*i.e.*, iNOS, IL-1β, and IL-6), in LPS-treated microglia cells.[58] Furthermore, PQQ prompted antioxidant effects by reducing MDA and ROS levels, with increases in SOD2 gene expression in a mouse D-galactose model.[59] Zhou *et al.*[60] added that PQQ as a redox cofactor has an anti-inflammatory effect and improve the motor function of hind limbs and the pathological changes of neurons and injured spinal cord after SCI.

4. Conclusion

The present work shows the protective role of PQQ loaded on CS/PVA coaxial nanofibers against oxidative stress in spinal cord injury. The removal of oxidative stress by antioxidants (*e.g.* PQQ) can lead to better physiological and clinical results. Further work is needed to evaluate functional recovery and histological changes in the spinal cord in rats following spinal cord injury treatment with coaxial nanofibers. However, this study limits these findings to biochemical evaluation only.

Therefore, the effect of PQQ should be assessed histologically and mechanistically in future studies to evaluate its functional recovery.

Data availability

The data of this article are included within the article itself as part of the results and discussion section.

Conflicts of interest

There are no conflicts to declare.

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