Possible role of antioxidative capacity of (–)-epigallocatechin-3-gallate treatment in morphological and neurobehavioral recovery after sciatic nerve crush injury

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OBJECTIVE This study examined the capacity of the major polyphenolic green tea extract (–)-epigallocatechin-3-gallate (EGCG) to suppress oxidative stress and stimulate the recovery and prompt the regeneration of sciatic nerve after crush injury.

METHODS Adult male Wistar rats were randomly assigned to one of 4 groups: 1) Naïve, 2) Sham (sham injury, surgical control group), 3) Crush (sciatic nerve crush injury treated with saline), and 4) Crush+EGCG (sciatic nerve crush injury treated with intraperitoneally administered EGCG, 50 mg/kg). All animals were tested for motor and sensory neurobehavioral parameters throughout the study. Sciatic nerve and spinal cord tissues were harvested and processed for morphometric and stereological analysis. For the biochemical assays, the time points were Day 1, Day 7, Day 14, and Day 28 after nerve injury.

RESULTS After sciatic nerve crush injury, the EGCG-treated animals (Crush+EGCG group) showed significantly better recovery of foot position and toe spread and 50% greater improvement in motor recovery than the saline-treated animals (Crush group). The Crush+EGCG group displayed an early hopping response at the beginning of the 3rd week postinjury. Animals in the Crush+EGCG group also showed a significant reduction in mechanical allodynia and hyperalgesia latencies and significant improvement in recovery from nociception deficits in both heat withdrawal and tail flick withdrawal latencies compared with the Crush group. In both the Crush+EGCG and Crush groups, quantitative evaluation revealed significant morphological evidence of neuroregeneration according to the following parameters: mean cross-sectional area of axons, myelin thickness in the sciatic nerve (from Week 4 to Week 8), increase of myelin basic protein concentration and gene expression in both the injured sciatic nerve and spinal cord, and fiber diameter to axon diameter ratio and myelin thickness to axon diameter ratio at Week 2 after sciatic nerve injury. However, the axon area remained much smaller in both the Crush+EGCG and Crush groups compared with the Sham and Naïve groups. The number of axons per unit area was significantly decreased in the Crush+EGCG and Crush groups compared with controls. Sciatic nerve injury produced generalized oxidative stress manifested as a significant increase of isoprostanes in the urine and decrease of the total antioxidant capacity (TAC) of the blood from Day 7 until Day 14. EGCG-treated rats showed significantly less increase of isoprostanes than saline-treated animals and also showed full recovery of TAC levels by Day 14 after nerve injury. In spinal cord tissue analysis, EGCG-treated animals showed induced glutathione reductase and suppressed induction of heme oxygenase 1 gene expression compared with nontreated animals.

CONCLUSIONS EGCG treatment suppressed the crush-induced production of isoprostanes and stimulated the recovery of the TAC and was associated with remarkable alleviation of motor and sensory impairment and significant histomorphological evidence of neuronal regeneration following sciatic nerve crush injury in rats. The findings of this study suggest that EGCG can be used as an adjunctive therapeutic remedy for nerve injury. However, further investigations are needed to establish the antioxidative mechanism involved in the regenerative process after nerve injury. Only upregulation of glutathione reductase supports the idea that EGCG is acting indirectly via induction of enzymes or transcription factors.

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KEY WORDS EGCG; oxidative stress; neuroregeneration; neuroprotection; stereological analysis; rat

ABBREVIATIONS C_T = threshold cycle; CV = coefficient of variation; EGCG = (–)-epigallocatechin-3-gallate; EPT = extensor postural thrust; MBP = myelin basic protein; RT-PCR = real-time-polymerase chain reaction; TAC = total antioxidant capacity; WRL = withdrawal response latency.

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A STRONG association has been shown between the intake of the major polyphenolic green tea extract (–)-epigallocatechin-3-gallate (EGCG) and vital biological mechanisms that expedite regeneration and neuroprotection and improve cognitive function and learning ability.^{21,59,68,69} It has been suggested that the beneficial effects may result from EGCG's antioxidant activity in reducing intracellular reactive oxygen species and their cascading destructive consequences.^{23,38,73} Another study suggested that EGCG has compelling neuroprotective effects in a D-galactose-induced mouse model of aging through antiapoptotic and antioxidative mechanisms.²²

Recently, we have shown that EGCG exerted a neuroprotective effect on the spinal cord by modulating neurological function when intravenously infused into adult rats in both acute and chronic spinal cord injury models.^{48,52} Moreover, EGCG attenuated peripheral nerve degeneration of rat sciatic nerve crush injury, an effect that seemed to be associated with its antiapoptotic properties.^{49,50} Another characteristic of EGCG reported by many investigators is its antiinflammatory activity.^{57,58,72} EGCG pretreatment abolished the cytokine-mediated upregulation of inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) via the JAK/STAT cascade. Via an antiinflammatory mechanism, EGCG has the capacity of protecting against various neurodegenerative diseases, and it has been shown to prevent TNF- α -induced NF- κ B activation, thereby upregulating the ATP-binding membrane cassette transporter A1 (ABCA1) via the Nrf2/Keap1 pathway in macrophage foam cells.²⁸ EGCG acts against infrasound-induced neuronal impairment by inhibiting microglia-mediated inflammation through a potential NF- κ B pathway-related mechanism in the CNS.⁶ However, *in vivo* mechanisms of EGCG with respect to the amelioration of neurobehavioral parameters resulting from peripheral nerve injury are not fully understood. Moreover, no thorough morphometric analysis of the effect of EGCG treatment on the sciatic nerve injury throughout the recovery period (Weeks 2–8) has been previously reported.

In this study, we examined the possible antioxidative and/or antiinflammatory role of EGCG associated with beneficial histopathological changes at different time points following sciatic nerve crush injury. Furthermore, we analyzed the relationship between sensory and motor neurobehavioral parameters and quantitative morphological changes in individual nerve fibers (axon plus myelin sheath), such as fiber diameter, axon diameter, myelin thickness, and g-ratio, during regeneration of the peripheral nerve.

Methods

Animals

Male Wistar rats (weight 250–300 g) were obtained from the Animal Resource Center of the Kuwait University Health Sciences Centre. The animals were kept under conditions of constant temperature (23°C $\pm$ 2°C) and humidity, with a 12-hour light/dark cycle. The rats were housed in pairs and were provided with food and water *ad*

libitum. Approval was obtained for all procedures from the Animal Ethics Committee of the Kuwait University Health Sciences Center, and the study was carried out in accordance with the US guidelines. All efforts were made to minimize the number of animals used in the study and their suffering.

A total of 204 rats were used in this study. The study consisted of 3 separate experiments (neurobehavioral, morphometric, and biochemical). For each experiment, equal numbers of animals were randomly assigned to the following 4 treatment groups: 1) Naïve (no surgery or sciatic nerve injury); 2) Sham (sham-injury surgical control group); 3) Crush (saline-treated, crushed sciatic nerve); or Crush+EGCG (50 mg/kg EGCG-treated, crushed sciatic nerve).

Sixty rats were used in the neurobehavioral experiment (15 per group); all of these rats were killed at the end of the experiment (Week 10). Forty-eight rats were used in the morphometric experiment (12 per group), with 3 rats in each group being killed at 2, 4, 6, and 8 weeks. Ninety-six rats were used in the oxidative pathway biochemical experiment (24 per group), with 6 rats per group being killed at each of the following time points: Day 1, Day 7, Day 14, and Day 28.

Procedures for Control and Surgical Groups

The Naïve group received no surgery or injury. The Sham group was subjected to all the surgical steps (cutting the skin, separating muscle and exposing the sciatic nerve) except crushing the nerve. In the Crush and Crush+EGCG groups, the sciatic nerve crush injury was performed as previously described.^{50–52} Briefly, animals were anesthetized with an intraperitoneal injection of a mixture of ketamine and xylazine. The sciatic nerve was crushed at a distance of 10 mm from the sciatic notch for 60 seconds using micro mosquito forceps (12.5 cm, straight, Item No. 500451, [09F S/N], World Precision Instruments, Inc.). The nerve was examined to ensure that the epineurial sheath was intact but translucent (axotomy). Then it was replaced under the muscle, and the skin incision was sutured.^{5,6,39,50–53,56,70} Animals in the Crush+EGCG group were injected intraperitoneally with 50 mg/kg EGCG (Sigma-Aldrich, product no. E4143, dissolved in saline) daily for 3 days starting 1 hour after the sciatic nerve crush injury.^{12,34,48,52,62,64} The stock solution was made fresh daily, using 50 mg EGCG and 1 ml saline. Animals in the Crush group were administered an equal volume of saline according to the same schedule. Numerous previously described measures were followed in order to minimize animal-animal variation in producing the injury and to induce a standard direct trauma.^{20,40,49–53,56,70,67,71} The crush-lesioned rats were randomly distributed among the experimental groups to minimize group variations as much as possible.

Assessment of Functional Recovery

All animals were evaluated for walking track analysis preoperatively. All surviving animal groups (Naïve, Sham, Crush+Saline, and Crush+EGCG) were evaluated once a week following surgery (zero point) as described ear-

lier.^{49–53} All the tests were repeated 3 times (at 3-minute intervals), and the average of the 3 readings was used for data analysis. Investigators were blinded to all treatments in all experiments. Several tests of reflexive sciatic nerve function (foot position, toe spread, extensor postural thrust [EPT] and hopping tests) were conducted as described in our previous studies.^{49,53} Briefly, EPT was measured by calculating the functional deficit in this feature; thus higher values indicate greater deficit.³⁷ The hopping test was done to test several integrated functions. Each leg of the rat was suspended above a table surface, and then the leg was placed on the table and was slowly moved laterally, with one foot touching the table surface at a time. The scoring was based on whether the rat hopped on the foot that was touching the table surface (1 for hopping, 0 for no hopping).⁶³

Four different neurobehavioral sensory tests were implemented as follows.

1. Tactile allodynia: The von Frey method was used to evaluate the sensitivity of the skin to tactile stimulation as described previously.^{49,50,51} Mechanical allodynia thresholds from the right and left hind limbs were measured in different animal groups and then the withdrawal reflex latencies (mean $\pm$ SD) were calculated.
2. Mechanical hyperalgesia: Paw pressure thresholds expressed in grams were measured using an analgesy meter (Model 37215, UGO Basile). Three consecutive mechanical nociceptive threshold values were measured for each hind limb, and the mean and standard deviation (SD) of the paw pressure latencies were calculated.^{13,37,48–50,53}
3. Thermal nociception: A modified hot-plate test (50°C, Model 39D Hot Plate Analgesia Meter, Columbus Instruments) was performed, with the withdrawal response threshold defined as the time elapsed from the onset of hot-plate contact to the withdrawal of the hind paw (paw flick), recorded to the nearest 0.1 second; a standard cutoff latency of 20 seconds was employed to prevent tissue damage.³² A normal response is considered withdrawal within 4 seconds. The withdrawal response threshold was measured for both hind limbs. Each hind limb was tested 3 times, with 20 minutes between tests to avoid sensitization. The withdrawal response latency (WRL) was calculated, and the 3 WRL values were averaged to obtain a single value for each animal.
4. Tail flick: A UGO Basile tail flick unit (Model 7360) was used to assess spinally mediated nociceptive thresholds, recording the amount of time that it took for an animal to move its tail away from the heat.³⁶ A cutoff time of 20 seconds was used to prevent tissue damage.^{32,49,50}

Statistical Analysis

All behavioral data were analyzed by 2-way repeated measures ANOVA followed by Bonferroni's F-test and Fisher least significant difference (LSD) comparison post hoc test or Student t-test, as appropriate. All statistical analysis was performed using SPSS (version 21.0, IBM Corp.); p values less than 0.05 were considered statistically significant.

Morphometric and Stereological Analyses

Tissue Processing for Morphometric Analysis

At the end of Week 2, Week 4, Week 6, and Week 8, 3 animals from each experimental group were randomly selected for histopathological evaluation as previously described.^{7,8,50} Briefly, animals were killed by decapitation, and the right sciatic nerve from each animal was exposed and removed. The specimen was then fixed by immersion in a mixture of 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, and stored in the fixative overnight at 4°C. On the following day, the specimens were rinsed in phosphate buffer (pH 7.4) twice, postfixed in 1% osmium tetroxide, dehydrated through a graded alcohol series, immersed in propylene oxide, and embedded in Epon resin (Hexion).⁵⁰ Semi-thin cross sections (1 μ m) were cut from each block using an RMC MT-7 ultramicrotome (Research and Manufacturing Co.) and stained with 1% toluidine blue for histopathological examination and morphometric analysis.⁵⁰

Quantification of Myelinated Fibers

Sciatic nerves removed from rats at 2, 4, 6, and 8 weeks postinjury were processed for stereological and histopathological examination. Stereological analyses of these sciatic nerves were performed by an experimenter blinded to the groups according to principles described previously.^{3,17,26,67} A stereological workstation composed of a digital camera (Nikon COOLPIX 4500), a manual dial indicator-controlled specimen stage,²⁹ and a light microscope (Olympus DP71 optical microscope) were used for counting the axons. Sections from each nerve (at least 5 sections from each rat) were examined in a systematic, uniform, and random manner, and nerve fibers were counted (Fig. 1A). Area sampling of nerve sections was done with 100 $\times$ 100- μ m successive, systemic random steps along adjacent areas of each nerve sample, and representative fields were photographed under a 100 $\times$ oil-immersion objective lens (total magnification 1000 $\times$). At least 10 photos per sample were taken randomly at 1000 $\times$ for morphometric analysis. Digital images were captured with an Olympus DP71 digital camera mounted on top of the microscope.⁶⁵ All morphometric parameters were calculated by light microscopy using Image-Pro Plus version 6.0.0.260 for Windows 2000/XP Professional (Media Cybernetics).¹⁴ Figure 1A shows a representative photograph of a cross section of sciatic nerve myelinated axons stained with toluidine blue. The unbiased counting frame method (square sample area 900 μ m²) was used to obtain an estimate of total axon number from nerve cross sections. A counting frame was placed on a monitor, and the sampled area was selected in a systematic, uniform, and random manner.²⁹ A mean sampling of each sectioned nerve profile was done in a 70 $\times$ 70-mm frame size in a systematic random manner. This ensured that all locations within a nerve cross section (1- μ m thickness) were equally represented and that all axon profiles were sampled with equal probability regardless of shape, size, orientation, or location.^{18,19,25,41,43,44,65,71}

Counting of axons was performed using a 100 $\times$ oil objective lens (total magnification 1000 $\times$), which allowed accurate recognition of myelinated nerve fibers. The camera was connected to a 500-MHz Pentium-III personal

FIG. 1. A: Representative photomicrograph of a cross section of sciatic nerve myelinated axons stained with toluidine blue. The unbiased counting frame method with a square sample area was used to count the myelinated axons. The nerve profiles marked with *yellow asterisks* along the *solid yellow lines* (inclusion lines) were included in the counts, but the profiles marked with *red Xs* touching the *broken red lines* (exclusion lines) were excluded. **B–E:** Representative photomicrographs of a cross section of sciatic nerve myelinated axons stained with toluidine blue demonstrating the method of calculating myelin thickness and g-ratios. For calculating the myelin thickness ($m = [D - d]/2$) and g-ratio (d/D), the axon diameter (d) and the nerve fiber diameter (D) were measured automatically using Image-Pro Plus 6.0. Thickness measurements from all sampled axons were averaged to obtain the mean myelin thickness. Magnifications: B and D, 1000 $\times$ (100 $\times$ oil immersion $\times$ 10 $\times$ ocular); C and E, 3000 $\times$. Figure is available in color online only.

were then averaged to obtain the mean myelin thickness. Myelin sheath thickness was calculated as the difference between the fiber diameter and the axon diameter, divided by 2: $(D - d)/2$. The myelin thickness/axon diameter ratio was calculated as $(D - d)/2d$, and the nerve fiber diameter/axon diameter ratio was D/d .

The number of axons in the image (N) was calculated by using the following equation: $N = (A \times n)/a$, where n is the number of axons in small square n , which is identified through Image-Pro Plus; a is the area of the small square, and A is the total area of the image (length $\times$ width). Also, the g-ratio was calculated as the quotient obtained by dividing the axon diameter by the fiber diameter, and the coefficient of variation (CV) was calculated as (standard deviation/mean) $\times$ 100. ANOVA multicomparison test was used, followed by Bonferroni post hoc analysis.

Morphometric Data Analysis

Images of the distal and proximal portions of the right sciatic nerve obtained from 9 rats per group were captured and digitized using Image-Pro Plus software. The distal and proximal portions of the right sciatic nerves were analyzed separately; the average number of the fibers analyzed per nerve segment was 155. A total of 790 images (10 from each section) were digitized and stored in uncompressed tagged image file format (TIFF) with 24-bit RGB class and 640×480 -pixel resolution. All data are presented as the mean $\pm$ SD. All calculations and all statistical procedures were performed using SPSS (version 21.0). The group values were compared using a Mann-Whitney U-test. A p value of less than 0.05 was considered significant.

Biochemical Analyses

Oxidative Stress Measurements

A total of 24 rats (6 from each of the 4 groups) were used for each time point (1, 7, 14, and 28 days). Sciatic nerve, spinal cord, blood, and urine samples were harvested at each time point and stored at -80°C until analyses were performed. The following assays were performed: 1) total antioxidant capacity in blood (Antioxidant Assay Kit, No. 709001, Cayman Chemical); 2) urine isoprostanes (8-Isoprostane EIA Kit, No. 516351, Cayman Chemical); 3) lipid peroxidation (Lipid Hydroperoxide LPO Assay Kit, No. 705003, Cayman Chemical); and 4) glutathione to

computer. The 3 color channels of the camera were balanced by adjusting the light intensity and camera gain and offset, using Olympus software. Images were viewed on a 15-inch Samsung SyncMaster 500S color monitor (Fig. 1B–E). The axon diameter (d) and the nerve fiber diameter (D) were measured automatically using Image-Pro Plus 6.0 image analysis software and were used to calculate myelin thickness ($m = [D - d]/2$) and g-ratio (d/D). The thickness measurements acquired from all sampled axons

TABLE 1. Summary of gene information for oxidation and inflammation pathways

Gene Symbol	Gene Name	Accession No.	Life Technologies Gene Assay ID
<i>Tank</i>	TRAF family member–associated NFKB activator	NM_001164073.1	Rn00595794_m1
<i>Tnf*</i>	Tumor necrosis factor	NM_012675.3	Rn01525859_g1
<i>Il1b</i>	Interleukin 1 beta	NM_031512.2	Rn00580432_m1
<i>Ptgs2†</i>	Prostaglandin-endoperoxide synthase 2	NM_017232.3	Rn01483828_m1
<i>Cyp1a1</i>	Cytochrome P450, family 1, subfamily a, polypeptide 1	NM_012540.2	Rn01418021_g1
<i>Hmox1</i>	Heme oxygenase 1	NM_012580.2	Rn01536933_m1
<i>Nr3c1‡</i>	Nuclear receptor subfamily 3, group C, member 1	NM_012576.2	Rn00561369_m1
<i>Gabpa§</i>	GA binding protein transcription factor, alpha subunit	NM_001108841.1	Rn01767215_m1

ID = identifier.

All genes listed are *Rattus norvegicus* (Norway rat) genes.* Also known as *Tnfa*, *RATTNF*, and *TNF-alpha*.† Also known as *Cox2*.‡ Also known as *GR* (glucocorticoid receptor).§ Also known as *Nrf2a*.

glutathione disulfide ratio (GSH/GSSG-412, Catalog No. 21040, OxisResearch). All tests were performed according to manufacturers' protocols.

Real-Time–Polymerase Chain Reaction Measurements

Total RNA Isolation and Reverse Transcription. To perform real-time–polymerase chain reaction (RT-PCR) measurements, we isolated total RNA from frozen nerve and spinal cord samples using Trizol reagent (Life Technologies Inc.). RNA quantities were evaluated using multivolume analysis of nucleic acids at 260-nm absorption spectroscopy (Epoch Multi-Volume Spectrophotometer System, BioTek Instruments, Inc.). Complementary DNA (cDNA) was synthesized by random priming of 2–5 µg total RNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) at 85°C for 2 hours and stored at –20°C.

For RT-PCR, optimized TaqMan probe/primers kits used for the detection of specific genes and β -actin (housekeeping gene) were purchased from Applied Biosystems. The complete information on the specific genes used in this study is listed in Table 1. RT-PCR was performed in duplicates using the Applied Biosystems 7500 PCR system.^{1,24,25,44,50,52,61} Amplification of the above genes was done in 96-well reaction plates. The reaction mixture contained 3 µl of cDNA, 200 nmol/L of TaqMan probe/primer assays, and 12.5 µl of qPCR master mix (Applied Biosystems) in each well. A nontemplate PCR-negative control and a positive PCR control were included. Quantitative mRNA expression was determined by the comparative threshold cycle (C_T) method. The target gene–specific signal was normalized based on the expression levels of the target gene versus reference gene (β -actin signal) using the formula $2 - \Delta\Delta C_T = 2 - (C_T, \beta\text{-actin} - C_T, \text{target gene})$, resulting in the evaluation of the samples as an n-fold difference relative to that of β -actin mRNA expression.

RT-PCR Statistical Analysis

All biochemical RT-PCR data were analyzed by 1-way

repeated measures ANOVA followed by the Neuman-Keuls test, using SPSS (version 21). Results were considered significant at $p < 0.05$. Data are presented as mean $\pm$ standard error of the mean (SEM).

Results

General Assessment

The hind paws of the Sham animals clinically looked normal following sham surgery. In addition, the skin and muscle wounds did not appear to hinder the animals' gait throughout the progression of the experiment. The toes and foot of the hind limb in the sciatic nerve crush-injured rats without EGCG treatment were intensely flexed. During the first week, the animals were also unable to stand on their operated hind limb. Partial weight bearing was noticed in the crush-injured animals starting from the second week. In contrast, in the EGCG-treated animals the clinical picture and weight bearing were improved over the subsequent weeks compared with the controls.

Behavioral Tests

The mean values for foot position (Fig. 2A) and toe spread (Fig. 2B) were consistently higher in the Crush+EGCG group than in the Crush, Sham, and Naïve groups starting from the end of Week 1 until the end of Week 8 following injury. This difference in progressive improvement of the Crush+EGCG group compared with the other groups was statistically significant ($p < 0.001$). The evaluation of the functional recovery as measured using the extensor postural thrust (EPT) following crush injury and EGCG treatment is shown in Fig. 2C. Crush+EGCG rats displayed more than 50% improvement in motor recovery ($p < 0.001$) compared with the Crush group from Week 1 to the end of the experiment at Week 10 after sciatic nerve injury. Similarly, the results of the hopping test (Fig. 2D), which examines several integrated sensory and motor functions, demonstrated significant ($p < 0.0001$) improvement in the Crush+EGCG group compared with the Crush group. The Crush+EGCG group dis-

FIG. 2. A: Differences in the mean values for the foot position analysis for the Naive, Sham, Crush, and Crush+EGCG groups. Ψ indicates $p < 0.001$, Crush+EGCG vs Crush, Sham, and Naive; * indicates $p < 0.001$, Crush vs Naive, Sham, and Crush+EGCG; α indicates no significant difference between Crush+EGCG and Crush. **B:** Differences in the mean values for the analysis of toe spread outcome. The Crush+EGCG rats showed statistically significant clinical recovery in toe spread following sciatic nerve injury. As with foot position, recovery of toe spread started at the end of postinjury Week 1 and continued until the end of Week 8. Ψ indicates $p < 0.001$, Crush+EGCG vs Crush, Sham, and Naive; * indicates $p < 0.001$, Crush vs Sham, Naive, and Crush+EGCG; α indicates no significant difference between Crush+EGCG and Crush groups. **C:** Evaluation of functional recovery as measured by EPT following crush injury and EGCG treatment. EPT was measured by calculating the percentage of the functional deficit; thus, a high value indicates a poor outcome. * indicates $p < 0.001$, Crush vs Sham, Naive, and Crush+EGCG; Ψ indicates $p < 0.001$, Crush+EGCG vs Crush, Sham, and Naive; no significant difference between Crush+EGCG and Sham; α indicates $p < 0.01$, Sham vs Naive group. **D:** Differences in the mean values of the hopping test outcome among the experimental animal groups. The Crush+EGCG group displayed an early hopping response (as calculated by the percentage of recovered animals) in the beginning of Week 7 postsurgery. * indicates $p < 0.001$, Naive vs Sham, Crush, and Crush+EGCG; Ψ indicates $p < 0.001$, Naive vs Crush and Crush+EGCG; α indicates $p < 0.0001$, Crush vs Crush+EGCG, Sham, and Naive. Values are expressed as means and SDs (error bars). A = first reading of the week; B = second reading; C = third reading.

played an early recovery of the hopping response in the beginning of Week 7 postsurgery (Fig. 2D).

Several neurobehavioral sensory tests were performed, including assessment of allodynia, mechanical hyperalgesia, thermal hyperalgesia, and tail flick latencies. The time course graph displaying the assessment of allodynia time latency of the different experimental groups before and after sciatic nerve injury is shown in Fig. 3A. The Crush+EGCG group showed a significant ($p < 0.001$) reduction in withdrawal reflex latency time compared with the Crush group from the beginning of Week 2 to the end of the experiment at Week 10. Moreover, it was noticed that the latency times

of the Crush+EGCG animals followed that of the Sham group. Likewise, in the paw pressure latency (mechanical hyperalgesia) test, Crush+EGCG rats showed a significant ($p < 0.001$) decrease in paw withdrawal reflex latency periods compared with Crush group starting from Week 3 (Fig. 3B). This decrease lasted up until the end of the experiment at Week 10. Nerve crush injury produced a severe nociception deficit in the Crush group as demonstrated using the hot plate analgesia meter (Fig. 3C). On the other hand, the Crush+EGCG group showed a significant ($p < 0.001$) nociceptive recovery starting at Week 2, which was maintained throughout until the end of the experiment at Week 10 (Fig.

FIG. 3. A: Time course of tactile allodynia. The Crush+EGCG group showed a significant reduction in withdrawal reflex latency time compared with the Crush group from the beginning of Week 2 until the end of the experiment at Week 10. Moreover, the latency times of the Crush+EGCG animals followed those of the Sham group. * indicates $p < 0.001$, Crush vs Crush+EGCG, Sham, and Naïve; Ψ indicates $p < 0.001$, Crush+EGCG vs Crush, Sham, and Naïve; Φ indicates $p < 0.001$, Crush+EGCG vs Sham and Naïve. Values are expressed as means and SDs (error bars). **B:** Time course of paw pressure latency (mechanical hyperalgesia). The Crush+EGCG group showed a significant decrease in paw withdrawal reflex latency periods compared with the Crush group starting from Week 3 and continuing until the end of the experiment (Week 10). (Note that the paw withdrawal latency rather than the absolute withdrawal reflex was measured, so we expect the values to be higher in the Crush group compared with the controls.) * indicates $p < 0.001$, Crush vs Crush+EGCG, Sham, and Naïve; Ψ indicates $p < 0.001$, Crush+EGCG vs Crush, Sham, and Naïve groups; Φ indicates $p < 0.002$, Crush+EGCG animals compared with Naïve and Sham groups. Values are expressed as means and SDs (error bars). **C:** Time course of hot plate latency. Nerve crush injury produced a severe nociception deficit in the Crush group. The Crush+EGCG group showed a significant nociceptive recovery starting at Week 2 and maintained until the end of the experiment (Week 10). * indicates $p < 0.001$, Crush vs Crush+EGCG, Sham, and Naïve; Ψ indicates $p < 0.001$, Crush+EGCG vs Crush, Sham, and Naïve. Values are expressed as means and SDs (error bars). **D:** Time course of tail flick withdrawal latency. The Crush+EGCG group showed significant nociceptive recovery compared with Crush (saline-treated) group by the end of the 2nd week postsurgery. This nociception improvement was sustained until the end of the experiment (Week 10). Furthermore, the EGCG-treated group displayed a significant decrease in nociception compared with the Sham and Naïve groups. * indicates $p < 0.001$, Crush vs Crush+EGCG, Sham, and Naïve; Ψ indicates $p < 0.001$, Crush+EGCG vs Crush, Sham, and Naïve; α indicates $p < 0.001$, Sham vs Crush+EGCG, Crush, and Naïve. Values are expressed as means and SDs (error bars).

3C). The tail flick withdrawal latency in the Naïve, Sham, Crush, and Crush+EGCG groups was also examined, and the results are depicted in Fig. 3D. The Crush+EGCG group showed significant ($p < 0.001$) nociceptive recovery compared with the saline-treated rats (Crush group) by the end of Week 2 postsurgery. This central nociception improvement was sustained until the end of the experiment at

Week 10. Furthermore, EGCG-treated animals displayed a significant ($p < 0.001$) decrease in their nociception parameter compared with Sham and Naïve groups (Fig. 3D).

Histological and Morphometric Analysis

Light Microscopy

Histological examination of the photomicrographs of

semi-thin transverse sections of sciatic nerves sampled from animals in the Sham group at different time points revealed a normal sciatic nerve appearance. Specimens from these animals showed the regular distribution of small- and large-diameter nerve fibers and normal proportion between myelin sheath thickness, fiber diameter, and intraneural blood vessels (Fig. 4A, D, G, and J). Wallerian degeneration and unmyelinated fibers were evident in the tissue sections of the sciatic nerve 10 mm distal to the lesion site of animals in the Crush group (Fig. 4B, E, H, and K) starting from Week 2 (Fig. 4B) until Week 8 (Fig. 4K) after crush nerve injury. In contrast, nerves of Crush+EGCG (Fig. 4C, F, I, and L) animals showed a remarkable axonal and myelin regeneration and contained a greater number of large newly regenerated nerve fibers that were surrounded by much more myelin compared with the Crush group. At Week 4, Crush+EGCG (Fig. 4F) animals showed remarkable histological improvement in comparison with Crush (Fig. 4E) animals. Crush+EGCG (Fig. 4L) nerves reached almost normal morphology by the end of Week 8 postsurgery in contrast to Crush group rats (Fig. 4K). The histological appearance of nerve specimens from the Crush+EGCG group (Fig. 4L) was similar to that of specimens from the Sham (Fig. 4J) group after 8 weeks postsurgery.

Stereological Analysis

Morphometric analysis data of sciatic nerves after 2, 4, 6, and 8 weeks postsurgery are presented in Figs. 5, 6, and 7. Figure 5A shows the mean total number of myelinated axons that were calculated for each experimental group at Weeks 2, 4, 6, and 8 following sciatic nerve injuries using a square counting frame. At Week 2 after sciatic injury, the Crush+EGCG and Crush animals showed a significant ($p < 0.0001$) decrease in the mean total myelinated axons compared with Naïve and Sham groups. Starting at Week 4, the mean total number of myelinated axons in the Crush+EGCG and Crush animals increased to the same levels as Naïve and Sham groups and became significantly ($p < 0.0001$) higher at Week 6 compared with Naïve and Sham control groups. EGCG treatment significantly decreased the number of myelinated axonal fibers in sciatic nerve crush animals compared with Crush group ($p < 0.0001$) at Week 6 and Week 8; however, it remained significantly higher ($p < 0.0001$) compared with Sham and Naïve control groups (Fig. 5A).

The result of the mean cross-sectional areas of myelinated axons obtained from 10 different and randomly selected samples from each animal are depicted in Fig. 5B. The averaged areas of myelinated axons measured in square micrometers (μm^2) were calculated for each experimental group at Weeks 2, 4, 6, and 8 following sciatic nerve injuries. The Crush and Crush+EGCG rats showed significant ($p < 0.0001$) decrease in the average areas of myelinated axons compared with Sham and Naïve control groups throughout the experimental period. However, the Crush+EGCG animals showed a significant gradual increase in the mean areas of the myelinated axons at Week 6 ($p < 0.03$), and Week 8 ($p < 0.0001$) compared with Crush groups following nerve injury (Fig. 5B).

Myelinated axon diameters (d) (Fig. 6A), myelinated

nerve fiber diameter (D) (Fig. 6B), and myelin thickness (Fig. 6C) were stereologically estimated from 10 different random samples. These samples were selected from each animal for each experimental group at Weeks 2, 4, 6, and 8 following sciatic nerve injuries using a square counting frame. The Crush and Crush+EGCG groups displayed significant ($p < 0.0001$) decrease in axon diameter size at all the time points assessed following nerve injury compared with Sham and Naïve animals. However, Crush+EGCG groups showed no significant change compared with Crush animals even at Week 8 after nerve injury. Similarly, the Crush and Crush+EGCG groups displayed a significant ($p < 0.0001$) decrease in nerve fiber diameter sizes at all the time points assessed following nerve injury compared with Sham and Naïve animals. However, the Crush+EGCG group showed significant ($p < 0.008$) nerve fiber diameter increase by the end of Week 8 postinjury (Fig. 6B). The calculated myelin thickness for the Crush and Crush+EGCG groups at different time points following sciatic nerve injury showed a significant decrease ($p < 0.0001$) throughout the experiment starting from Week 2 until Week 8 (Fig. 6C). However, the myelin thickness in Crush+EGCG animals started to increase significantly at Week 6 ($p < 0.05$) and became much greater at Week 8 ($p < 0.0001$) compared with values obtained in the Crush group.

Stereological analysis of the g-ratios described as the axon diameter to myelinated fiber diameter ratio (Fig. 7A) and myelin thickness to axon diameter ratio (Fig. 7B) were calculated from the data measurements obtained from each animal. The Crush and Crush+EGCG groups showed a significant ($p < 0.001$) increase in the axon diameter to myelinated fiber diameter ratio at Week 2 and Week 4 postinjury. The Crush+EGCG animals displayed significantly ($p < 0.05$) lower axon diameter to myelinated fiber diameter ratio compared with Crush group only at Week 2 following injury (Fig. 7A). The Crush and Crush+EGCG groups showed a significant ($p < 0.0001$) decrease in the myelin thickness to axon diameter ratio at Week 2 and Week 4 compared with all other groups (Fig. 7B). Conversely, the Crush+EGCG animals exhibited significantly ($p < 0.01$) higher myelin thickness to axon diameter ratio throughout at Week 6 and Week 8 following injuries compared with Crush group (Fig. 7B).

In support of the stereological data analysis, the myelin basic protein (MBP) concentration and gene expression analyses of the sciatic nerve and spinal cord samples showed a similar pattern at different time points following nerve injury (Fig. 8). EGCG treatment significantly ($p < 0.002$) increased the MBP concentration in the sciatic nerve samples starting from Day 1 until Day 14 postinjury compared with Crush group (Fig. 8A and B). RT-PCR analysis of MBP from sciatic nerve tissues revealed a gradual increase in MBP gene expression at Day 3 and Day 7 following EGCG treatment. At Day 14, there was significant ($p < 0.01$) increase in the MBP gene expression in the sciatic nerves of the EGCG-treated group compared with Crush animals (Fig. 8C). Similarly, analysis of MBP concentration from the spinal cords at different time points following nerve injury showed a significant ($p < 0.005$) increase in the MBP concentration of the EGCG-treated animals at

FIG. 4. Photomicrographs of semi-thin transverse sections of sciatic nerves obtained from animals in the Sham (**A, D, G, and J**), Crush (**B, E, H, and K**), and Crush+EGCG (**C, F, I, and L**) groups at different time points. Sciatic nerves from Crush and Crush+EGCG animals show the presence of smaller minifascicle nerve fibers as time progressed following crush injury. Nonetheless, sciatic nerve sections of Crush+EGCG (**C, F, I, and L**) animals showed remarkable nerve regenerations compared with those from the Crush (**B, E, H, and K**) group starting from Week 2. Figure is available in color online only.

FIG. 5. A: The mean totals of myelinated axons counted from 10 different random samples from each animal used in the morphometric study. The mean total number of myelinated axons was calculated for each experimental group at Weeks 2, 4, 6, and 8 following sciatic nerve injury using a 1-mm² square counting frame. * indicates $p < 0.0001$, Crush vs Naïve, Sham, and Crush+EGCG; Φ indicates $p < 0.0001$, Crush+EGCG vs Naïve, Sham, and Crush; α indicates $p < 0.01$, Crush+EGCG vs Crush. **B:** The mean cross-sectional areas of myelinated axons were obtained from 10 different and randomly selected samples from each animal (3 animals/group). The averaged cross-sectional areas of myelinated axons measured in square micrometers (μm^2) were calculated for each experimental group at Weeks 2, 4, 6, and 8 following sciatic nerve injuries. The Crush+EGCG animals showed a significant increase in the mean cross-sectional areas of the myelinated axons at Week 6 and Week 8 compared with the Crush group. * indicates $p < 0.0001$, Crush vs Naïve and Sham; Φ indicates $p < 0.0001$, Crush+EGCG vs Naïve, Sham, and Crush; α indicates $p < 0.03$, Crush+EGCG vs Crush; Ψ indicates $p < 0.0001$, Crush+EGCG vs Crush; ** indicates $p < 0.005$, Sham vs Naïve. Values are expressed as means and SDs (error bars).

FIG. 6. Stereological estimation of the myelinated axon diameter (A), myelinated nerve fiber diameter (B), and myelin thickness (C) obtained from 10 different random samples from each of 3 animals per group. The mean values of the axon diameters (d) and nerve fiber diameters (D) were calculated using an image analysis software package (Image-Pro Plus 6.0) for each experimental group at Weeks 2, 4, 6, and 8 following sciatic nerve injuries using a square counting frame. **A:** The Crush and Crush+EGCG groups displayed a significant decrease in axon diameter at all the time points assessed following nerve injury compared with Sham and Naïve animals. However, Crush+EGCG animals showed no significant change in comparison with Crush animals even at Week 8. * indicates $p < 0.0001$, Crush vs Naïve and Sham; Φ indicates $p < 0.0001$, Crush+EGCG vs Naïve and Sham; α indicates $p < 0.0001$, Sham vs Naïve. **B:** Likewise, the Crush and Crush+EGCG groups displayed a significant decrease in nerve fiber diameter at all the time points assessed following nerve injury compared with Sham and Naïve groups. The Crush+EGCG group showed a significant nerve fiber diameter increase by the end of Week 8. * indicates $p < 0.0001$, Crush vs Naïve and Sham; Φ indicates $p < 0.0001$, Crush+EGCG vs Naïve and Sham; Ψ indicates $p < 0.008$, Crush+EGCG vs Crush; α indicates $p < 0.001$, Sham vs Naïve. **C:** The calculated myelin thickness for the Crush and Crush+EGCG groups at different time points following sciatic nerve injury shows a significant decrease in myelin thickness throughout the experiment starting from Week 2 until Week 8. However, the myelin thickness in Crush+EGCG animals started to increase significantly at Week 6 and became more so at Week 8 compared with Crush group. * indicates $p < 0.0001$, Crush vs Naïve and Sham groups; Φ indicates $p < 0.0001$, Crush+EGCG animals compared with Naïve and Sham groups; Ψ indicates $p < 0.0001$, Crush+EGCG animals compared with Crush group; α indicates $p < 0.05$, Crush+EGCG group compared with Crush group. Values are expressed as means and SDs (error bars).

Days 1, 3, and 14 postinjury compared with Crush group (Figs. 8D and 10E). RT-PCR analysis of MBP from spinal cord tissues revealed a significant ($p < 0.005$) increase in

MBP gene expression at Days 7 and 14 following EGCG treatment compared with Crush animals (Fig. 8F).

The CV represents the ratio of the standard deviation

FIG. 7. Stereological analysis of the axon diameter to myelinated fiber diameter (g-ratio) (A), myelinated nerve fiber diameter to axon diameter ratio (B), and myelin thickness to axon diameter ratio (C) calculated from the data measurements obtained in 3 animals per group. The calculated data were averaged for each experimental group at Weeks 2, 4, 6, and 8 following sciatic nerve injuries. **A:** The Crush and Crush+EGCG groups showed a significant increase in the g-ratio at Week 2 and Week 4 postinjury. The Crush+EGCG animals displayed significantly lower g-ratio compared with Crush group only at Week 2 following injury. * indicates $p < 0.001$, Crush vs Naive and Sham; Φ indicates $p < 0.01$, Crush+EGCG vs Naive and Sham; Ψ indicates $p < 0.05$, Crush vs Crush+EGCG. **B:** The Crush group showed a significant decrease in the myelin thickness to axon diameter ratio throughout the experiment period compared with all other groups, while the Crush+EGCG animals displayed a significantly lower fiber diameter to axon diameter ratio only at Week 2 and Week 8 compared with the Naive and Sham groups. Also, the Crush+EGCG group significantly exhibited a higher fiber diameter to axon diameter ratio throughout the experimental period following injuries compared with the Crush group. * indicates $p < 0.0001$, Crush vs Naive and Sham; Φ indicates $p < 0.0001$, Crush+EGCG vs Naive and Sham; α indicates $p < 0.001$, Crush vs Crush+EGCG group. Ψ indicates $p < 0.01$, Crush vs Crush+EGCG, Naive, and Sham. Values are expressed as means and SDs (error bars).

of the mean, and it is a useful statistic for comparing the degree of variation from one data series to another, even if the means are drastically different from each other. Analysis of the mean CVs for myelin thickness, axon diameter (d), and fiber diameter (D) for all the experimental groups at the different time points (Weeks 2, 4, 6, and 8) following

sciatic nerve injury are given in Table 2. At the end of the experiment, the CVs for myelin thickness, axon diameter (d), and fiber diameter (D) of Crush+EGCG group were lower in comparison with Crush animals. Simply put, the lower the ratio of standard deviation to mean return, the better the risk-return trade-off.

FIG. 8. Graphs of MBP Western blotting and RT-PCR gene expression analysis of the sciatic nerves and spinal cords at different time points following sciatic nerve crush injury. **A and B:** Western blot analysis of MBP in the sciatic nerves from all the experimental groups at different time points following nerve injury showing a significant increase in MBP concentration in the EGCG-treated animals from Day 1 until Day 14 postinjury compared with Crush group. # indicates $p < 0.01$, Sham vs Naive; α indicates $p < 0.0001$, Crush vs Sham and Naive; * indicates $p < 0.0001$, Crush+EGCG vs Naive; Φ indicates $p < 0.02$, Crush+EGCG vs Crush; \$ indicates $p < 0.002$, Crush+EGCG vs Crush; ¥ indicates $p < 0.03$, Crush+EGCG vs Naive and Sham. **C:** RT-PCR analysis of MBP from sciatic nerve tissues revealed a gradual increase in MBP gene expression at Day 3 and Day 7 following EGCG treatment. At Day 14, there was a significant increase in the MBP gene expression in the sciatic nerves of the EGCG-treated group compared with Crush animals. α indicates $p < 0.03$, Crush vs Sham and Naive; * indicates $p < 0.01$, Crush+EGCG vs Naive and Sham; Φ indicates $p < 0.001$, Naive vs Crush+EGCG, Crush, and Sham; # indicates $p < 0.01$, Crush vs Crush+EGCG and Sham. **D and E:** Western blot analysis of MBP in the spinal cords from all the experimental groups at different time points following nerve injury showing a significant increase in MBP concentration in the EGCG-treated animals at Day 1, Day 3, and Day 14 postinjury compared with the Crush group. α indicates $p < 0.0001$, Crush vs Sham and Naive; * indicates $p < 0.005$, Crush+EGCG vs Crush; Φ indicates $p < 0.004$, Crush vs Naive; # indicates $p < 0.007$, Sham vs Naive; \$ indicates $p < 0.04$, Sham vs Naive. **F:** RT-PCR analysis of MBP from spinal cord tissues revealed a significant increase in MBP gene expression (exp) at Day 7 and Day 14 following EGCG treatment compared with Crush animals. * indicates $p < 0.005$, Crush vs Crush+EGCG, Sham, and Naive; α indicates $p < 0.02$, Crush vs Sham; Φ indicates $p < 0.002$, Crush vs Crush+EGCG. Values are expressed as means and SDs (error bars). C = Crush; N = Naive; S = Sham; T = Crush+EGCG; 1 = Day 1; 3 = Day 3; 7 = Day 7; 14 = Day 14.

TABLE 2. Coefficients of variance for myelin thickness, axon diameter, and fiber diameter

Variable & Time Point	Group			
	Naïve	Sham	Crush	Crush + EGCG
Myelin thickness				
Week 2	15.555	9.161	34.757	28.044
Week 4	15.555	7.468	14.761	20.543
Week 6	15.555	8.232	16.173	12.977
Week 8	15.555	10.038	17.093	12.586
Axon diameter				
Week 2	5.13	7.232	13.875	17.901
Week 4	5.13	12.628	7.763	12.177
Week 6	5.13	13.951	8.273	9.728
Week 8	4.591	9.572	10.508	5.13
Fiber diameter				
Week 2	7.878	5.652	12.046	14.698
Week 4	7.878	7.484	5.404	10.458
Week 6	7.878	9.234	6.584	6.354
Week 8	7.878	5.061	10.33	8.509

Oxidative Stress

Attempts to determine lipid peroxidation products, the level of reduced glutathione, and activities of antioxidant enzymes in sciatic nerve specimen did not produce reliable data. The sample size measuring up to 10–15 mm in length and a few milligrams in weight was too small, and values were too low even when applying ultra-microassays.

Isoprostanes

Isoprostanes in urine were measured as an integrative marker of oxidative stress. Figure 9A shows isoprostane concentration in the urine of rats from different experimental groups. Samples were taken at Day 1, Day 7, Day 14, and Day 28 after the sciatic nerve injury. Statistically higher levels of isoprostanes were detected in the urine of sciatic nerve crushed rats at Day 7 after the injury, reaching a maximum at Day 14. Treatment with EGCG prevented the increase of isoprostanes.

Total Antioxidant Capacity

Changes in total antioxidant capacity (TAC) in the blood of different experimental groups at Day 1, Day 7, Day 14, and Day 28 after induction of the sciatic nerve injury are shown in Fig. 9B. Assessment of TAC, irrespective of its drawbacks, provides indirect information about the consumption of antioxidants as a result of oxidative stress. Figure 9B shows that the TAC level was significantly decreased in all groups except the control, irrespective of the treatment. TAC levels gradually recovered, and Sham and Crush groups reached control levels at Day 28 after the surgery. EGCG treatment accelerated recovery, and only in the EGCG-treated group was recovery complete at Day 14.

Mechanisms of EGCG Action

To study the beneficial effects of EGCG, the levels of

expression of TNF- α , IL-1 β , Nrf2, HO-1, and glutathione reductase were examined. We found that sciatic nerve crush induced the proinflammatory cytokine TNF- α in the spinal cord. Induction of TNF- α was first detected at 8 hours and reached a maximum at 16 hours after the injury. After that the level of the cytokine gradually decreased but remained significantly higher than in controls (Fig. 10A). Treatment with EGCG delayed the induction of TNF- α , and at 8 hours postinjury the TNF- α level in the EGCG-treated animals was similar to that in controls. At 16 hours, however, no difference between the treated and the nontreated animals was observed, and at 24 hours the level of cytokine expression in the EGCG-treated group was higher than in the nontreated animals (Fig. 10A). The TNF- α level gradually decreased after reaching a maximum at 16 hours after the injury and was even much higher in controls at 72 hours.

In our experiments, sciatic nerve damage caused induction of Nrf2 mRNA in the spinal cord, reaching a maximum 8 hours after the injury and then returning to normal (Fig. 10B). A similar pattern was observed in the EGCG-treated animals, but the level of induction was much lower than in the Crush group (Fig. 10B).

Nrf2 regulates proteins/enzymes involved in protection against oxidative stress and inflammation. Among the proteins expressed by activated Nrf2 is HO-1. It catalyzes the oxidation of heme to biliverdin and also acts as a heat-shock protein (HSP-32).⁵⁵ Our data show that HO-1 was induced as a result of sciatic nerve damage. The induction was statistically significant 1 hour after the damage and gradually increased, reaching a maximum at 16 hours. Induction of HO-1 mRNA followed the same pattern in the EGCG-treated animals, but mRNA levels remained lower at all time points (Fig. 10C).

Levels of IL1- β mRNA dropped in the 1st hour in all groups that underwent surgery and then progressively increased, reaching a maximum for the Crush group at 8 hours after the injury (Fig. 10D). A similar pattern was observed for the Crush+EGCG group, but EGCG treatment delayed the expression of IL1- β ; the maximum was reached at 16 hours postinjury. Also, at all time points, the level of IL1- β remained lower in the Crush+EGCG than in the Crush group.

Nerve crush caused mild induction of the enzyme glutathione reductase, presumably as a physiological response to increased glutathione oxidation (Fig. 10E). Dramatic induction of glutathione reductase was observed at 24 and 72 hours after injury in animals treated with EGCG (Fig. 10E).

Discussion

The results of our study support the hypothesis that EGCG treatment is a potent stimulator of neuroregeneration after crush injury of the sciatic nerve. EGCG treatment substantively improved several sensory and motor parameters as well as mended the morphological alterations after nerve crush injury. The results of the current work are consistent with our previous studies and other investigations that examined the neuroprotective properties of EGCG on peripheral neuropathies,^{36,44,51,63} Parkin-

FIG. 9. Concentration of isoprostanes in urine and blood TAC. Samples from Naïve, Sham, Crush, and Crush+EGCG experimental groups were collected at Days 1, 7, 14, and 28 after the induction of the sciatic nerve injury followed by treatment as indicated in the methodology protocol. **A:** Urine concentrations of isoprostanes. Data are presented as mean $\pm$ SEM. * indicates $p < 0.05$, Crush vs Naïve and Sham; Φ indicates $p < 0.0001$, Crush vs Naïve, Sham, and Crush+EGCG. **B:** TAC in the blood. * indicates $p < 0.003$, Naïve vs Sham, Crush, and Crush+EGCG; Φ indicates $p < 0.05$, Naïve and Crush+EGCG vs Sham and Crush.

son's disease, Alzheimer's disease, and traumatic brain injury.^{26,36,54}

Our results provide further evidence for injury-related pain-relieving and motor-repair effects of EGCG in a post-traumatic sciatic nerve injury rat model. The functional motor recovery of the affected limb showed a progressive improvement in foot position, toe spread, EPT, and hopping tests starting from Week 1 and continuing up until the

end of Week 8 after nerve injury; these results are in complete agreement with our recent studies using rat models of sciatic crush nerve injury and spinal cord injury.^{48,50,51}

EGCG neurobehavioral recovery was well supported by the current and previous morphological improvements in the histological architecture of the crush-injured nerves and the increased survival of neurons in both the dorsal and ventral horns of spinal cords of EGCG-treated rats on

FIG. 10. Expression of mRNA in the spinal cord. **A:** TNF- α . **B:** Nrf2. **C:** HO-1. **D:** IL1- β . **E:** Glutathione reductase. Results are presented as mean $\pm$ SEM. * indicates $p < 0.004$, Crush+EGCG vs Crush; Φ indicates $p < 0.004$, Crush+EGCG vs Naive and Sham.

histological examination.^{50,51} Recently, it has been shown that intraperitoneal injection of EGCG (60 mg/kg) improved the sensory allodynia and thermal thresholds in animals with oxaliplatin-induced peripheral neuropathy.³³ Similarly, chronic oral administration of epigallocatechin-gallate alleviates streptozotocin-induced diabetic neuropathic hyperalgesia in rats with a concomitant decrease of 3,4-methylenedioxymphetamine (MDA) and nitrite content and an increase of erythrocyte superoxide dismutase activity as blood markers of oxidative stress.^{4,55}

Our neurobehavioral pain assessment results are consistent with many recent studies displaying EGCG modulation of pain parameters in different models of neuropathy. A recent study showed that an aqueous solution of EGCG in the drinking water ameliorated mechanical hypersensitivity in a model of streptozotocin-induced diabetic neuropathic pain⁴⁷ and in the posttraumatic osteoarthritis mouse model.³⁵ Moreover, mice treated with EGCG showed reduced osteoarthritis-associated pain, as indicated by higher locomotor behavior (i.e., distance traveled) with a significant concurrent reduction in the expression levels of the chemokine receptor, and proinflammatory cytokines IL-1 β and TNF-1 α in the dorsal root ganglia similar to sham-operated animals.³⁵ Likewise, intrathecal EGCG injection modulated the nociceptive progression and the reactive oxygen species,² the nNOS protein expression and the pronociceptive effects of NO¹¹ in rats with spinal nerve ligation neuropathic pain model. Further, intrathecal injection of EGCG in an animal model of sciatic nerve chronic constriction injury was associated with marked improvement in pain behaviors and decreased expressions of Toll-like receptor TLR4, High Mobility Group 1 protein (HMGB1), NF- κ B, TNF- α , and IL-1 β along with a significant increase in IL-10 content.³¹

Histological examination of semi-thin transverse sections of sciatic nerves from EGCG-treated animals revealed a remarkable axonal and myelin regeneration. Compared with sciatic nerve injury group, these sections contained a greater number of large newly regenerated nerve fibers that were surrounded by much more myelin. Sciatic nerves of the EGCG-treated group reached almost normal morphology by the end of Week 8 postsurgery in contrast to Crush sciatic nerve rats. These results are consistent with the early light and ultrastructural evaluation of EGCG-treated sciatic nerves that showed normal unmyelinated and myelinated axons with regular myelin sheath thickness and normalized appearance of Schmidt-Lantermann clefts.⁵⁰ Furthermore, these results are in line with studies showing EGCG being a neuroprotective and neuroregenerative agent in an experimental model of optic nerve crush injury.^{46,74} Quantitative estimation and stereological analysis of myelinated nerve fiber number, fiber size parameters, and myelin and axonal thickness are essential tools for nerve regeneration investigation. The Crush and Crush+EGCG groups showed a significant decrease in the mean cross-sectional areas of myelinated axons compared with control groups throughout the experiment period. However, the EGCG-treated group showed a significant increase in nerve fiber diameter size, mean areas of the myelinated axons, and myelin thickness starting at Week 6 compared with the Crush group. Biochemically,

this was also confirmed by the increase of MBP concentration and gene expression in both the injured sciatic nerve and spinal cord following EGCG treatment. Recently, we have shown a significant decrease in the number of neurons at the L4–L5 spinal cord levels (corresponding to the sciatic nerve origin) in the Crush group, compared with the Naïve and Sham groups.⁵¹ Further, EGCG treatment increased the number of motor neurons by reducing the expression of the glial fibrillary acidic protein and increased the expression of growth-associated protein-43, a marker of regenerating axons. EGCG treatment also reduced the ratio of B-cell lymphoma 2 (Bcl-2)-associated X protein/Bcl-2 and increased the expression of survivin gene indicating a possible role of EGCG in modulating the apoptotic pathway to protect the neurons.⁵¹ Therefore, it seems reasonable that MBP is downregulated centrally in the Crush animals, due to loss of neurons and their axons while the EGCG treatment upregulated it to restore the damaged myelin or to protect the myelin from disintegrations following a neuronal loss in the spinal cord after nerve injury.

The higher number of small fibers observed, especially in EGCG-treated and untreated regenerated crushed nerve, is in agreement with the sprouting phenomenon, which is considered a hallmark of regeneration during and after axonal degeneration.^{41,55} Keilhoff et al.³⁰ reported an increase in the number of immature and newly generated myelin fibers at 8 weeks after nerve injury signifying persistent uncontrolled (futile) sprouting. Injured axons produced dozens of collateral nerve sprouts at the injury site during the initial stage of peripheral nerve regeneration. However, few enter the distal nerve stump due to the limitation of the number of pathfinding structures (bands of Büngner). Many small nerve fibers with a healthy histomorphology, though still ensheathed with a thin myelin layer, may be designated as regenerated nerve fibers that have already begun the myelination process. The healthy unmyelinated nerve fiber bundles found in the crushed nerve can be categorized as either regenerated unmyelinated fibers or regenerated nerve fibers that did not reach the myelination processes.⁷⁰

Analysis of the g-ratios in our study revealed that in the Crush+EGCG group the treatment significantly lowered the axon diameter to myelinated fiber diameter ratio and myelin thickness to axon diameter ratio compared with Crush group values at Week 2 and Week 4 postinjury before normalization with control values. Our morphometric data analysis supports our hypothesis that EGCG has a profound positive effect on the myelinated axons. As the g-ratio is determined by the ratio of the axon to fiber diameter, a higher g-ratio can be expected in a demyelinating neuropathy, as remyelination leads to thinner myelin sheaths while the axon diameter remains the same. This lower axon diameter to myelinated fiber diameter ratio in our study can be due to either the presence of regenerated fibers with a thin myelin sheath in injured nerves, resulting in higher g-ratios or the loss of small myelinated nerve fibers in injured nerves, as small myelinated nerve fibers have higher g-ratios than large myelinated nerve fibers.^{15,27} Our g-ratio analysis is consistent with those of many studies, and it provides verification of the efficacy of the

EGCG treatment in improving and speeding the regeneration process in the crush-injured sciatic nerves.

Tissue damage and disruption of homeostasis are among the known inducers of oxidative stress. In our study, sciatic nerve crush injury produced generalized oxidative stress, manifested as an increase in isoprostane levels in urine and a decrease of TAC of the blood. A rapid increase of isoprostane levels in the brain tissue as the result of traumatic brain injury has been previously reported⁶⁶ and was accompanied by a concomitant decrease of brain antioxidant reserves and increased production of proinflammatory mediators. Induction of a proinflammatory cytokine, TNF- α , was detected in our experiments and was attributable to traumatic damage to the sciatic nerve. A statistically significant increase in urinary isoprostanes was detected at Day 7 postinjury and reached a maximum at Day 14. Such dynamics followed the decrease of myelinated axons. Judging by the urinary excretion of isoprostanes, treatment with EGCG prevented crush-induced generalized oxidative stress and accelerated the restoration of the antioxidant capacity, which coincided with morphological improvement of the nerve. Based on the data for induction of TNF- α , the beneficial effect of EGCG does not seem to be related to the decrease in proinflammatory cytokines. It is known, however, that polyphenols, including EGCG, can suppress oxidative stress by affecting transcription factors regulating the expression of antioxidant enzymes. Among such transcription factors is nuclear factor erythroid 2-related factor 2 (Nrf2).⁴² The redox-sensitive Nrf2-antioxidant response element (ARE) pathway is considered the main sensor and major suppressor of oxidative stress through upregulation of the expression of a large number of genes coding for antioxidant enzymes.¹⁶ We found that Nrf2 in the spinal cord was induced very early after sciatic nerve damage and that EGCG treatment suppressed the induction. Nrf2 is supposedly activated by oxidation of cysteine residues or by phosphorylation. Recent findings indicate that protective effects of EGCG can be mediated via activation of Nrf2, and as a consequence, induction of antioxidant enzymes, including superoxide dismutase (SOD), catalase, glutathione-S-transferase, glutathione peroxidase, etc.¹⁸ Our results suggest that the beneficial effect of EGCG is not mediated via Nrf2 induction in the spinal cord. On the other hand, EGCG treatment induced glutathione reductase, which plays a key role in maintaining glutathione in its functional reduced state.

Another enzyme that is controlled by redox-sensitive transcription factors is the inducible form of heme oxygenase, HO-1. Its induction is considered an adaptive response to oxidative stress and inflammation.⁴⁵ HO-1 was induced very early in the spinal cords of animals with crushed sciatic nerves, and EGCG treatment suppressed its induction. EGCG treatment of sciatic nerve-injured rats was also recently shown to enhance the survival of spinal cord neurons by modulating the expression of neuronal growth genes along with apoptotic and antiapoptotic genes, thus conserving the spinal cord neurons.^{49,51}

Recent in vitro studies provide evidence for the antioxidative and neuroprotective activity of EGCG in aging-associated oxidative damage in rat brain by decreasing the production of hydroxynonenal in the aged brain,

upregulating the antioxidant system, and augmenting the activities of Krebs's cycle enzymes and electron transport chain complexes in aged brain mitochondria.⁶⁰ Likewise, oral administration of EGCG was associated with significant improvement in cognitive deficits in mice and elevated total superoxide dismutase and glutathione peroxidase activities, decreased 3,4-methylenedioxymphetamine contents in the hippocampus, and reduced the cell apoptosis index in the mouse hippocampus. Further, oral administration of EGCG in mice has been shown to enhance cell division and increase the number of neuroblasts in the dentate gyrus⁷⁵ and improve cognitive dysfunction induced by psychological stress through suppressing lipid peroxidation and production of reactive oxidative species and increasing total antioxidative capacity.⁹ This green tea modulation may also result from changes in 5-hydroxytryptamine, cytokines, and catecholamines, and expressions of metallothioneins.¹⁰

Conclusions

In summary, EGCG-treated animals displayed a remarkable decrease in nociception parameters compared with Crush group starting at Week 2 and maintained until the end of the experiment at Week 10. The results of our sensory neurobehavioral tests—allodynia time latency, paw pressure latency (mechanical hyperalgesia), WRL using a hot plate analgesia meter, and central nociception measured as tail flick withdrawal latency—are consistent with our previous studies using the same crush injury of the sciatic nerve rat model as well as with other investigations using EGCG treatment in different models of neuropathy. Also, our quantitative stereological evaluation results reveal significant histomorphological evidence of neuronal regeneration in the EGCG-treated injured animals compared with control groups throughout the duration of the experiment. Only upregulation of glutathione reductase supports the idea that EGCG is acting indirectly via induction of enzymes or transcription factors. Although our study clearly supports the beneficial role of EGCG as a neuroprotective agent and a facilitator of neuronal regeneration, further investigations are needed to establish whether antioxidative mechanisms are involved in the regenerative process after nerve crush injury.

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Disclosures

The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions

Conception and design: all authors. Acquisition of data: all authors. Analysis and interpretation of data: all authors. Drafting the article: Renno. Critically revising the article: all authors. Reviewed submitted version of manuscript: all authors. Approved the final version of the manuscript on behalf of all authors: Renno. Statistical analysis: Renno. Administrative/technical/material support: Renno. Study supervision: Renno.

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