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Neuroregenerative Potential of Lion's Mane Mushroom, *Hericium erinaceus* (Bull.: Fr.) Pers. (Higher Basidiomycetes), in the Treatment of Peripheral Nerve Injury (Review)

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ABSTRACT: We present a model case study of the activity of aqueous extract of *Hericium erinaceus* fresh fruit bodies in promoting functional recovery following crush injury to the peroneal nerve in adult female Sprague-Dawley rats. The aim was to explore the possible use of this mushroom in nerve repair. The activities of aqueous extract were compared to activities exhibited by mecobalamin (vitamin B_{12}), which has been widely used in the treatment of peripheral nerve disorders. Analysis of walking track indicated that return of hind limb function and normal toe spreading occurred earlier in treated groups than in the negative control (non-treated) group. Regeneration of axons and reinnervation of motor endplates/neuromuscular junction in extensor digitorum longus muscle of rats in treated groups developed better than in the negative control group. Further, immunofluorescence studies also showed that dorsal root ganglia neurons ipsilateral to the crush injury in rats of treated groups expressed higher immunoreactivities for Akt and MAPK signaling pathways as well as c-Jun and c-Fos genes compared to the negative control group. Akt cascade plays a major role in mediating neurotrophin-promoted cell survival, while MAPK cascade is involved in mediating neurite outgrowth. Immediate early gene expression was also involved in the cascade of events leading to regeneration. Local axonal protein synthetic machinery was also enhanced in the distal segments of crushed nerves in treated groups. Therefore, daily oral administration of *H. erinaceus* could promote the regeneration of injured rat peroneal nerve in the early stage of recovery.

KEY WORDS: medicinal mushrooms, *Hericium erinaceus,* functional recovery, peripheral nerve, crush injury, toe-spreading reflex, extensor digitorum longus muscle, axonal regeneration, axonal reinnervation, dorsal root ganglia

ABBREVIATIONS: Akt: protein kinase B; **Ca^{2+}:** calcium ion; **CREB:** cAMP response element-binding protein; **CES-D:** Center for Epidemiologic Studies Depression Scale; **CNS:** central nervous system; **DRG:** dorsal root ganglia; **EDL:** extensor digitorum longus; **ERK:** extracellular signal-regulated kinases; **HDS-R:** Hasegawa Dementia Scale; **HMG-CoA**: 3-hydroxy-3-methylglutaryl-coenzyme A; **ICI:** Indefinite Complaints Index; **JNK:** c-Jun N-terminal kinase; **KMI:** Kupperman Menopausal Index; **MAPK:** mitogen-activated protein kinase; **MCA:** middle cerebral artery; **MMP-1:** matrix metalloproteinase-1; **mRNA:** messenger RNA; **MRSA:** methicillin-resistant *Staphylococcus aureus*; **NF-κB:** nuclear factor kappa-light-chain-enhancer of activated B cells; **NF-200:** neurofilament heavy; **NGF:** nerve growth factor; **PFI:** peroneal functional index; **PLF:** print length factor; **PNS:** peripheral nervous system; **PSQI:** Pittsburgh Sleep Quality Index; **TIMP-1:** tissue inhibitor of metalloproteinases 1; **TSF:** toe-spread factor; **w/v:** weight per volume

I. INTRODUCTION

Hericium erinaceus (Bull.: Fr.) Pers. (Hericiaceae, higher Basidiomycetes, also known as Lion's Mane, Monkey's Head, Hedgehog Mushroom, Satyr's Beard, Pom Pom Blanc, Igelstachelbart, and Yamabushitake) is one of the edible and medicinal mushrooms distributed in Asia, Europe, and North America.[1] It is a temperate mushroom that requires cool temperatures of 18°C to 24°C to produce fruit bodies. The nutritional and medicinal properties of *H. erinaceus* grown in low temperature conditions are well known and documented in Europe, China, and Japan.[1] Since 2000, it has been successfully domesticated via adaptation to tropical climate in Ma-

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FIGURE 1. *Hericium erinaceus* fresh fruit bodies grown in tropical climate of Malaysia.

laysia. This mushroom grows and produces fruit bodies in lowlands of tropical temperature (Fig. 1).

Peripheral nerve problems are common and encompass a large spectrum of traumatic injuries, diseases, tumors, and iatrogenic lesions. The incidence of traumatic injuries is estimated as more than 500,000 new patients annually.[2] Injuries to peripheral nerves result in partial or total loss of motor, sensory, and autonomic functions in the involved segments of the body. Nerve crush injury lends itself to investigation of the intrinsic cellular and molecular events that intervene in peripheral nerve regeneration, and to assessment of factors such as drugs that might enhance the speed of regeneration and the effectiveness of reinnervation.[2] It is known that after the initial injury, free oxygen radicals increase and cause further tissue damage.[3]

Traditionally, functional nerve defects have been remedied by many methods, including nerve transfer, nerve grafts, artificial nerve conduit bridging, and end-to-side neurorrhaphy.[4] However, these methods only provide a regenerative environment for injured nerves. Recovery of function depends on various local and systemic factors. Regeneration of axons from the proximal stump of an injured nerve to the distal nerve stump is one of the most important factors in reinnervation of peripheral tissue.

Studies have shown that locally applied neurotrophins can enhance survival of damaged neurons and regrowth of lesioned axons in the central and peripheral nervous systems in rats.[5] However, in situ treatment is not an ideal treatment pattern. The beneficial effect of systemically administered neurotrophins on axonal regeneration is largely limited by enzymatic degradation. In addition, systemically delivered neurotrophins show unexpected side effects such as the toxicity of the circulating protein.[6] Therefore, it is important to explore substances that can produce neurotrophin-like effects on axonal regeneration without toxicity problems.

Mushrooms can be good candidates to induce

neuronal differentiation and promote neuronal survival.[1,7] Recently, much attention has been given to medicinal mushrooms' neurotrophic effects[8] and stimulation of nerve growth factor (NGF) synthesis in the brain.[9] This raises the possibility that medicinal mushrooms may promote peripheral nerve regeneration after injury through the enhancement of NGF production. They are expected to have a regenerative action on the injured tissues in peripheral nerve disorders.

Research on the medicinal value of *H. erinaceus* grown in Malaysia, a tropical country, is minimal and has not been explored. In an earlier study, Wong et al.[10] had reported that aqueous extract of *H. erinaceus* grown in a tropical environment could stimulate neurite outgrowth of neural hybrid clone NG108-15 cells. However, there is a paucity of information on the nerve regeneration and repair properties of the mushroom.

II. NATURAL HABITAT AND ORIGIN OF *H. ERINACEUS*

H. erinaceus is a saprophytic inhabitant on dead trunks of hardwoods, including oak, walnut, beech, maple, sycamore, elm, and other broadleaf trees. It is found most frequently on logs or stumps, and is one of the wood-destroying fungi that cause white rot.[11] The first report on the cultivation of *H. erinaceus* was published in China in 1988.[12] It has been cultivated by artificial log using bottles and polypropylene bags, making it possible to constantly put this mushroom into market year round.[12]

H. erinaceus has been well-known for hundreds of years and treasured in traditional Chinese and Japanese cookery and herbal medicine. In China, it is called Houtou, as its fruit bodies look like the head of a baby monkey, and Shishigashira (Lion's Head). It is one of the famous four dishes in China (the other three are sea cucumber, bear palm, and bird's nest).[13] In Japan, it is called Yamabushitake because it resembles the ornamental cloth worn by Yamabushi—Buddhist monks practicing asceticism in the mountains. It is also called Jokotake (funnel-like), Usagitake (rabbit-like), and Harisenbontake (porcupine fish-like) according to its appearance. Japanese scientists have studied and confirmed the biological activities of *H. erinaceus* as a highly prized medicinal mushroom.[13]

III. MEDICINAL PROPERTIES OF *H. ERINACEUS*

The health benefits of *H. erinaceus* as a curative for problems of digestive tract such as stomach and duodenal ulcers are widely known among Chinese doctors. The effectiveness of *H. erinaceus* tablets in the treatment of ulcers, inflammations, and tumors of the alimentary canal was proven in the clinical trial subjects of Shanghai Third People's Hospital.[14] Ingestion of this mushroom was reported to have a remarkable effect in extending the life of cancer patients. Pills were used in the treatment of gastric and esophageal carcinoma.[15] Further, sandwich biscuits supplemented with the fruit bodies were used in the prevention and treatment of nutritional anemia of preschool children.[16]

Hericenones A and B (cytotoxic phenols)[17] and a novel fatty acid[18,19] isolated from fruit bodies, as well as erinapyrones A and B (γ-pyrones),[20] hericenes A, B, and C (phenolic derivatives),[21] and erinapyrone C (γ-dihydropyrone)[21] isolated from mycelium, exhibited cytotoxicity against HeLa cells. Mori et al.[22] found that hericenone B had strong antiplatelet activity, and it might be a novel compound for antithrombotic therapy possessing a novel mechanism. Hericenone B has been shown to specifically inhibit collagen-induced platelet aggregation through the inhibition of upstream of arachidonic acid liberation in integrin α2/β1 signaling.

Fifteen polysaccharides were isolated from hot-water extracts of *H. erinaceus* fruit bodies. Five of them showed antitumor activity and prolonged the longevity of the hosts. These high-molecular-weight compounds were identified as xylan, glucoxylan, heteroxyloglucan, and galactoxyloglucan.[1] Lectin, which inhibits erythrocyte aggregation,[23] and hericerins,[24] with an inhibitory effect on pine pollen germination and tea pollen growth, were also isolated from *H. erinaceus* fruit bodies. A rhamnoglucogalactan fraction known as (1→3)-β-D-glucan, which inhibits the growth of tumor, was isolated from alkaline extract of fruit bodies.[25] Xu et al.[26] showed that polysaccharides of *H. erinaceus* possess anti-skin-aging activities by enhancing skin antioxidant enzymes, MMP-1 and TIMP-1 activities, and collagen protein levels in aged rats.

An ethanol extract of *H. erinaceus* promoted an antimutagenic effect, as examined by the Ames

test.[27] Methanol extract of fruit bodies was found to have a hypoglycemic effect and reduce elevation rates of serum triglyceride and total cholesterol levels when administered to streptozotocin-induced diabetic rats.[28] An exo-biopolymer produced from a submerged culture of *H. erinaceus* reduced the level of plasma total cholesterol, low-density lipoprotein cholesterol, triglyceride, phospholipids, atherogenic index, and hepatic HMG-CoA reductase activity. It also preserved the high-density lipoprotein at a relatively high level in dietary-induced hyperlipidemic rats. These could help reduce the risk of atherosclerosis.[29]

Chemotherapeutic resistance to drugs is a major obstacle to the successful treatment of human hepatocellular carcinoma. Lee and Hong[30] demonstrated that purified components of *H. erinaceus* act as enhancers to sensitize doxorubicin (Dox)-mediated apoptotic signaling, and this sensitization can be achieved by reducing c-FLIP expression via JNK activation and enhancing intracellular Dox accumulation via the inhibition of NF-κB activity. These findings suggest that *H. erinaceus* in combination with Dox serves as an effective tool for treating drug-resistant human hepatocellular carcinoma.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is currently one of the most prevalent pathogens in nosocomial infections. Erinacines A, B,[31] and K[32] were isolated as anti-MRSA compounds from the mycelium. A clinical trial in Japan showed that MRSA in some patients disappeared after they consumed the mushroom. On top of that, two novel and one known chlorinated orcinol derivatives were also isolated from mycelium that exhibited antimicrobial activities against *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Verticillium dahliae*, and *Aspergillus niger*.[33]

It has been shown that cultivation conditions did not affect selected bioactive properties of *H. erinaceus* grown in tropical Malaysia.[10,34–36] The total phenolic content and total antioxidant activity in the oven-dried fruit bodies was higher compared to the freeze-dried or fresh fruit bodies.[34] This may be due to generation and accumulation of Maillard's reaction products during the heating process, which are known to have antioxidant properties. Various extracts of *H. erinaceus* also inhibited the growth of pathogenic bacteria but not of the tested fungi.[34] Besides that, freeze-dried fruit bodies exhibited a cytoprotective effect against ethanol-induced gastric mucosal injury in rats,[35] and topical application of aqueous extract of fruit bodies accelerated the rate of wound healing enclosure in rats.[36]

Two of our articles on *H. erinaceus*[10,37] were published in the *International Journal of Medicinal Mushrooms* and one[38] was published in *Evidence-based Complementary and Alternative Medicine*. These articles highlighted the ability of *H. erinaceus* to stimulate neurite outgrowth and promote regeneration after peripheral nerve injury.

The hot-water extract of dried fruit bodies is used as healthy drink. It has been a practice to extract the mushroom with water or pickle it in brewed wine. A sport drink named Houtou was employed in the 11th Asia Sport Festival (1990) in China.[13] Alcohol-based extracts prepared from pure mycelium cultured on organic brown rice are produced by Fungi Perfecti Co. (Olympia, WA, USA) and tablets of polysaccharide are manufactured by Shanghai Baixin Edible and Medicinal Fungi of the Edible Fungi Institute (Shanghai, China). In Malaysia, capsules containing 100% pure powder of *H. erinaceus* are manufactured by Reishilab (Selangor, Malaysia) and marketed under ANI *Hericium* 450 mg, and an essence is produced by Vita Agrotech (Selangor, Malaysia) by combining three types of mushrooms, namely *Agaricus brasiliensis*, *H. erinaceus*, and *Auricularia auricula-judae*.

IV. NEUROPROTECTION AFTER PERIPHERAL NERVE INJURY

Nerve regeneration is a complex phenomenon that has gained growing interest among scientists for many years. Neurons can be separated into central nervous system (CNS) and peripheral nervous system (PNS), which have different anatomical structures and regenerative ability. In mammals, the central neurons without myelin sheaths are difficult to regenerate. In contrast to CNS, PNS with myelin sheaths is capable of regeneration following injury.[39]

The characterization of neurite formation, maturation, and collapse/resorption is an area of interest because these cellular processes are essential for the connection between sensory and motor neurons. Neurites are particularly interesting in relation to neuropathological disorders, neuronal injury/regeneration, and neuropharmacological

research and screening.[40] Damage due to nerve transection was once believed to be irreversible. However, it has been proved that the regeneration of damaged nerve fibers is an active process under the control of molecules able to inhibit and repulse growing neurites.[41] Therefore, major efforts in nervous system drug discovery research are focused on the identification of compounds that affect the growth of neurites.

Peripheral nerve injuries are encountered in clinical practice due to accidental trauma, acute compression, or surgery. Traffic crashes usually induce traumatic nerve injury resulting in the disruption of intraneural circulation.[42] This condition in turn induces demyelinization, remyelinization, axonal degeneration, axonal regeneration, focal, multifocal, or diffuse nerve fiber loss, and endoneural edema.[43] After injury, free radicals are elevated, producing more tissue damage and retarding the recovery process.[3]

Studies of neurotrophic factors are aimed at finding new and more effective treatments for nerve disorders. These substances, which are produced naturally by the body, protect neurons from injury and encourage their survival. Neurotrophic factors also maintain normal function in mature nerve cells and stimulate axonal regeneration. The effects of these powerful chemicals on the PNS may eventually lead to treatments that can reverse nerve damage and cure peripheral nerve disorders.[44]

Drug therapy is commonly used to promote axonal regeneration in the treatment of nerve injuries,[45] including crush injury, transected injury, or large nerve gap. Therefore, searching for effective drugs, especially those of natural origin, has gained extensive attention. Immunosuppressant and anti-inflammatory drugs may accelerate the rate of nerve regeneration following injury. However, they are associated with severe side effects such as high blood pressure, kidney problems, and liver disorders.[46] Therefore, it is important to search for natural substances and possible new drug treatments that could affect nerve regeneration.

V. NEUROLOGICAL ACTIVITIES OF MUSHROOMS INCLUDING *H. ERINACEUS*

Much interest has been focused on the potential of using medicinal mushrooms as neuroprotective agents. Compounds that stimulate neurite outgrowth and act as substitutes for NGF or exhibit NGF-like activities that cause neurons and myelin to regrow have been extensively studied.

Sarcodon scabrosus, a bitter mushroom, contains diterpenoid compounds with a unique chemical structure, such as the sarcodonins A-H.[47] Further investigations on the mushroom had led to the isolation of new cyathane diterpenoids—scabronines A,[48] B, C, D, E, F,[49] G,[50] K, and L.[51] They have been tested for their ability to induce NGF secretion from 1321N1 human astrocytoma cells and induce neuritogenesis in PC12 cells (rat pheochromocytoma cells). Scabronines increased the expression of mRNA for NGF, and the secretion of NGF from 1321N1 cells in a concentration-dependent mechanism.[50] Among different scabronines, scabronine G methyl ester (SG-ME) was found to be the most active, and the mechanism of action was studied extensively. Cyrneines A, B,[52] C, and D[53] from *S. cyrneus* and glaucopine C from *S. glaucopus*[54] were tested for their ability to induce NGF production from 1321N1 cells, but they were found to be much less active than scabronine G.[53]

Additionally, extract of *Grifola frondosa* was found to induce neuronal differentiation of PC12 cells by phosphorylation of ERK1/2.[55] An active component was isolated from the extract and identified as lysophosphatidylethanolamine. Lysophosphatidylethanolamine is a phosphate-dylethanol amine molecule lacking a fatty acid. Polysaccharides in aqueous extract of *Ganoderma lucidum* have been reported to induce neuronal differentiation and prevent NGF-dependent apoptosis of rat pheochromocytoma PC12 neuronal cells.[56]

Cordyceps is one of a growing number of traditional Chinese medicines being considered as cures for modern human diseases. Nucleosides, especially adenosine, have been found to stimulate axon growth *in vitro* and in the adult CNS.[57] Cerebrosides known as termitomycesphins A-D isolated from *Termitomyces albuminosus*[58] and aqueous extract of *Lignosus rhinoceros* sclerotium[59] were shown to stimulate neurite outgrowth and induce neuronal differentiation of PC12 cells. *L. rhinocerus* is a unique national treasure and is also known as "cendawan susu rimau" in the Malay language, or tiger's milk mushroom in English.

The effect on the nervous system of some compounds isolated from mushrooms, such as muscarine, muscimol, and ibotenic acid, has been

studied.[60] Neurotrophic effects of extracts of *H. erinaceus*,[61,62] *Amanita* sp.,[63,64] *Lentinus edodes*,[65,66] hallucinogenic mushroom *Psilocybe cubensis*,[67] and other higher Basidiomycetes[68,69] have also been reported.

Preliminary studies suggest that *H. erinaceus* may be useful in the treatment of a number of neurological disorders. Kawagishi et al.[9] reported that the most promising activity of *H. erinaceus* is the stimulation of NGF synthesis by hericenones from fruit bodies and by erinacines from mycelium. Phenol derivatives identified as hericenones C, D, E,[70] F, G, and H[71] were isolated from fruit bodies of *H. erinaceus*. Diterpene-xyloside possessing cyathan skeletons derivatives identified as erinacines A, B, C,[72] D,[73] E, F, G,[74] H, I,[75] P,[76] Q,[77] J, K,[32] and R,[78] as well as erinacol,[79] were isolated from mycelium of *H. erinaceus*. These compounds accelerated the synthesis of NGF and are observed to stimulate neurons to regrow. Shimbo et al.[80] showed that erinacine A increased catecholamine and NGF content in the CNS of rats. Rats treated with this compound had increased levels of both noradrenaline and homovanillic acid in the locus coeruleus at 4 weeks of age and increased levels of NGF in both LC and hippocampus at 5 weeks of age.

It has been reported that NGF has a protective or inducible effect on neuronal cell death through the Trk and p75 pathway.[81] An exo-polysaccharide of *H. erinaceus* could induce neuronal differentiation and promote neuronal survival.[82] An endoplasmic reticulum stress-attenuating compound known as 3-hydroxyhericenone F[83] and dilinoleoyl phosphatidylethanolamine[84] purified from an extract of *H. erinaceus* dried fruit bodies were shown to reduce endoplasmic reticulum stress-induced cell death, which might reduce the risk of neurodegenerative disease–induced cell death. Extracts of *H. erinaceus* also induced phosphorylation of c-Jun N-terminal kinase (JNK) and its downstream substrate c-Jun, and increased c-Fos expression, suggesting that *H. erinaceus* promotes NGF gene expression via JNK signaling.[85]

Furthermore, the efficacy of *H. erinaceus in vivo* has been examined. Mice that were given feed containing 5% dry powder for 7 days showed an increase in the level of NGF mRNA expression in the hippocampus.[85] Hazekawa et al.[86] investigated the neuroprotective effect of *H. erinaceus* on ischemic brain damage in a middle cerebral artery (MCA) occlusion model in mice. Infarct volumes were markedly reduced in mice receiving 14 days of *H. erinaceus* treatment at a dose of 300 mg/kg prior to 4-hr MCA occlusion. Pre-treatment significantly increased the levels of NGF in both the cortex and striatum of mice subjected to 4-hr MCA occlusion. Therefore, *H. erinaceus* provides neuroprotection against focal cerebral ischemia by increasing NGF levels and may be clinically useful for preventing cerebral infarction.

A double-blind trial was performed on 50- to 80-year-old Japanese men and women diagnosed with mild cognitive impairment in order to examine the efficacy of oral administration of *H. erinaceus* in improving cognitive functioning by using a cognitive function scale based on the Revised Hasegawa Dementia Scale (HDS-R).[87] The subjects of the *H. erinaceus* group took four 250-mg tablets containing 96% of dry powder three times a day for 16 weeks. Cognitive function scale scores increased with the duration of intake. Laboratory tests showed no adverse effect of *H. erinaceus* and it was effective in improving mild cognitive impairment.[87]

Nagano et al.[88] investigated the clinical effects of *H. erinaceus* on menopause, depression, sleep quality, and indefinite complaints, by means of a questionnaire investigation. They detected a difference between groups using the Kupperman Menopausal Index (KMI), the Center for Epidemiologic Studies Depression Scale (CES-D), the Pittsburgh Sleep Quality Index (PSQI), and the Indefinite Complaints Index (ICI). Their results showed that *H. erinaceus* intake may reduce depression and anxiety. It may also be relevant to frustration and palpitation because *H. erinaceus* intake lowers scores for the terms *frustrating* and *palpitation*. For this reason, they suggested a mechanism that is different from the NGF-enhancing action of *H. erinaceus*. Mori et al.[89] examined the effects of *H. erinaceus* on amyloid β(25-35) peptide–induced learning and memory deficits in mice. *H. erinaceus* prevented impairments of spatial short-term and visual recognition memory induced by intracerebroventricular administration of amyloid β(25-35) peptide and may be useful in the prevention of cognitive dysfunction.

Neurotrophic effects of *H. erinaceus* dried fruit bodies on the neurons of hippocampal slices in rats (nerve cell spike activity) have been studied.[61,62] They exert neurotrophic action or excitation of neu-

rons at concentrations that did not affect the growth of nerve cells *in vitro*, and also did not evoke a toxic effect or nerve cell damage. Neurons of the hippocampus as part of the limbic system are closely related to the regulation of motivation-emotional responses, memory, and other mental functions.[62] A characteristic feature of this structure is an extreme sensitivity to slight changes in the intercellular liquid composition, which is considerably greater than that of neocortex and cerebellum neurons.[90] The extract also promoted normal development of cultivated cerebellar cells and demonstrated a regulatory effect on the process of myelin genesis *in vitro* after myelin damage.[91] The myelin sheath is an important component of neurons that is involved in the transmission of nerve messages. Injury of myelin's compact structure leads to an impairment and severe illness of the nerve system.

VI. A MODEL FOR THE STUDY OF PERIPHERAL NERVE REGENERATION FOLLOWING CRUSH INJURY

We investigated the effects of aqueous extract of *H. erinaceus* fresh fruit bodies on the recovery of peroneal nerve of the right hind limb following crush injury in adult female Sprague-Dawley rats.[37,38] The study included: (1) functional recovery of the peroneal nerve as assessed by walking track analysis and toe-spreading reflex, (2) axonal reinnervation pattern of the extensor digitorum longus (EDL) muscle in unoperated and operated limbs, (3) expression of the protein kinase B (Akt) and mitogen-activated protein kinase (MAPK) pathways, and expression of c-Jun and c-Fos in the ipsilateral DRG from injured nerve, and (4) axonal regeneration and protein synthesis in the injured nerves.

A. Preparation of Aqueous Extract

Mushrooms have always been prepared for medicinal use by hot-water extraction, as in brewing of teas or decoctions in traditional Chinese medicine for prevention of oxidative stress-related diseases.[92] In our study, *H. erinaceus* fresh fruit bodies were obtained from Vita Agrotech in Tanjung Sepat, Selangor, Malaysia. Ten grams of fresh fruit bodies were boiled with 10 mL of distilled water [ratio of 1:1 (w/v)] for 30 min, cooled, and filtered.[8] In an earlier study of stimulation of neurite outgrowth activity, aqueous extract of fresh fruit bodies has been shown to be a potent enhancer of neurite outgrowth activity *in vitro*.[8] Neurotrophic components of *H. erinaceus* dissolved readily in water and retained their effectiveness even after one month of storage at 4°C.[8] The aqueous extract was used within a week after preparation.

B. Induction of Peripheral Nerve Injury

The peroneal nerve is a branch of sciatic nerve, which controls movement and sensation of the lower leg, foot, and toes. The sciatic nerve contains a larger number of fibers compared to the peroneal nerve. Injury to the peroneal nerve normally has a greater potential for regeneration and recovers faster than injury to the sciatic nerve.[93] Therefore, we used peroneal nerve to achieve complete functional recovery following crush injury.

Eighteen adult female Sprague-Dawley rats weighing 200 ± 20 g were randomly assigned to three groups of six rats each. A negative control group received daily oral administration of distilled water (10 mL/kg body weight/day); the experimental group received aqueous extract of fresh fruit bodies (10 mL/kg body weight/day); and the positive control group received mecobalamin (130 μg/kg body weight/day). All treatments were administered with an 18-gauge stainless steel feeding needle for 14 days as pre-treatment before surgery.

After pre-treatment, the right sciatic nerve and its two major branches were exposed through a gluteal muscle-splitting incision. A crush injury was created using a fine watchmaker forceps (no. 4) for 10 s on the peroneal nerve at 10 mm from the EDL muscle, and complete crush was confirmed by the presence of a translucent band across the nerve (Fig. 2). After surgery, distilled water, aqueous extracts, or mecobalamin was continuously fed for another 20 days or until the complete return of hind limb function.

Pre-treatment was used to examine the prevention effect of the aqueous extract. There are no hard rules regarding the period of pre-treatment because medicinal mushrooms will not cause any side effects. Pre-treatment was used to examine the prevention effect of the aqueous extract. There are no hard rules regarding the period of pre-treatment because medicinal mushrooms will not cause any side effects. However, pre-treatment must be done at least 7 days before injury because oral drugs take time to be absorbed and get into the general blood circulation.

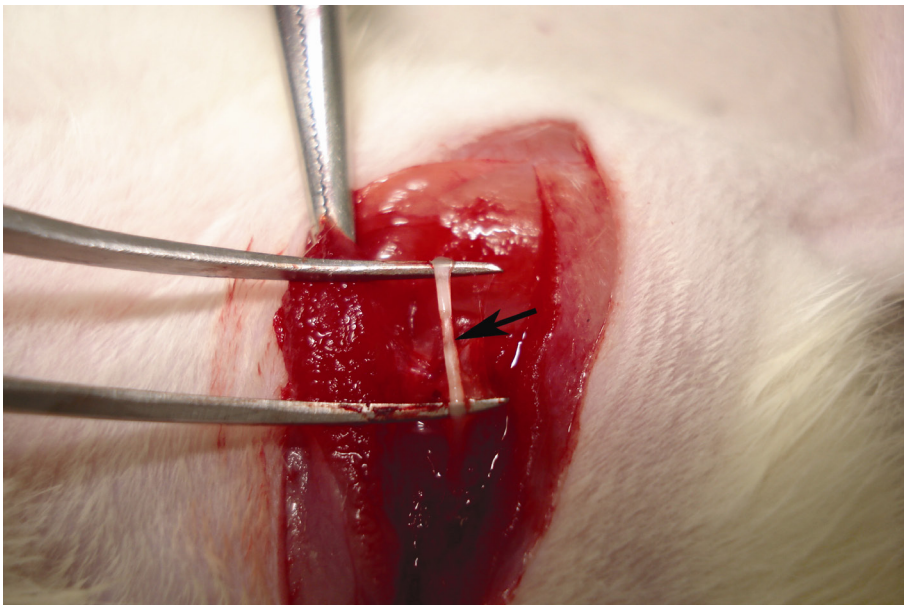

FIGURE 2. Complete crush of peroneal nerve is confirmed by presence of a translucent band (as indicated by an arrow) across the nerve.

C. Assessment of Peripheral Nerve Regeneration

The effects of aqueous extract of *H. erinaceus* on the rate of hind limb recovery were assessed by walking track analysis[93] and toe-spreading reflex.[94] Rats were allowed conditioning trials in a walking track (8.2 × 42 cm) darkened at one end. The rat's hind limbs were dipped in Chinese ink, and the rat was permitted to walk down the track, leaving its hind footprints on the paper (Fig. 3). The peroneal functional index (PFI) was determined based on multiple linear regression analysis of factors derived from measurements of walking tracks in rats with peroneal nerve injury. The factors that contributed to PFI were print length factor (PLF) and toe-spread factor (TSF). As for toe-spreading reflex, activities were classified according to the affected right hind limb: no spreading, minimal spreading, average spreading, and normal spreading.

As for microscopic investigation, frozen sections (50 μm thick) of EDL muscle were cut longitudinally in a cryostat microtome at –20°C and stained for neuromuscular junction by a combined silver-cholinesterase method.[95] Expression of Akt and MAPK signaling pathways as well as c-Jun and c-Fos genes were also studied in DRG; axonal regeneration and axonal protein synthesis[96] were studied in peroneal nerve by an immunohistochemical method.

VII. THE POTENTIAL OF *H. ERINACEUS* IN PERIPHERAL NERVE REGENERATION

Accumulating lines of evidence show that free radicals generated after injury play the predominant role in retarding functional recovery. Studies on crush injury models in peripheral nerves have shown better functional recovery when therapies were directed against ischemia-reperfusion injury, using antioxidants, lipid peroxidation inhibitors, and anti-inflammatory agents.[97,98] With this in mind, medicinal mushrooms may be potential alternatives to neurotrophic factors for peripheral nerve repair. Although less effective than neurotrophins, aqueous extract of *H. erinaceus* may be used to assist in the application of neurotrophins to enhance axonal regeneration in the nervous system and cut down the dosage of neurotrophins to reduce the toxic effects in humans. Our findings showed that daily oral administration of aqueous extract of *H. erinaceus* fresh fruit bodies enhanced recovery of damaged peripheral nerve in rats.[37,38]

A. The Effects of Aqueous Extract of *Hericium erinaceus* on Functional Recovery Following Crush Injury

It was observed that functional recovery in the mecobalamin and aqueous extract groups was on day 4, while the crushed limb in the negative con-

FIGURE 3. Walking track apparatus. Rat in an 8.2 x 42 cm walking track apparatus lined with white office paper. After the hind limbs of the rat are dipped in Chinese ink, the rat walks towards the darkened end of the corridor.

trol group remained dysfunctional (Fig. 4). Rats in the negative control group showed clumping of toes and dragging of the injured foot. On the other hand, the mecobalamin and aqueous extract groups demonstrated toe spreading (Fig. 5) and clear footprints on the walking tracks. The peroneal functional index in the aqueous extract group returned to pre-surgery values 4 to 7 days earlier than for rats in the negative control group as measured by walking track analysis, and normal toe spreading was achieved 5 to 10 days earlier in the aqueous extract group than in the negative control group.

After peripheral nerve lesions, a simple, precise, and inherently meaningful measure is the return of toe spreading.[99] Toe spreading requires reinnervation of the most distal muscles in the sciatic distribution, the intrinsic muscles of the foot including the interossei. Its absence provided visible evidence of interruption of the nervous pathway

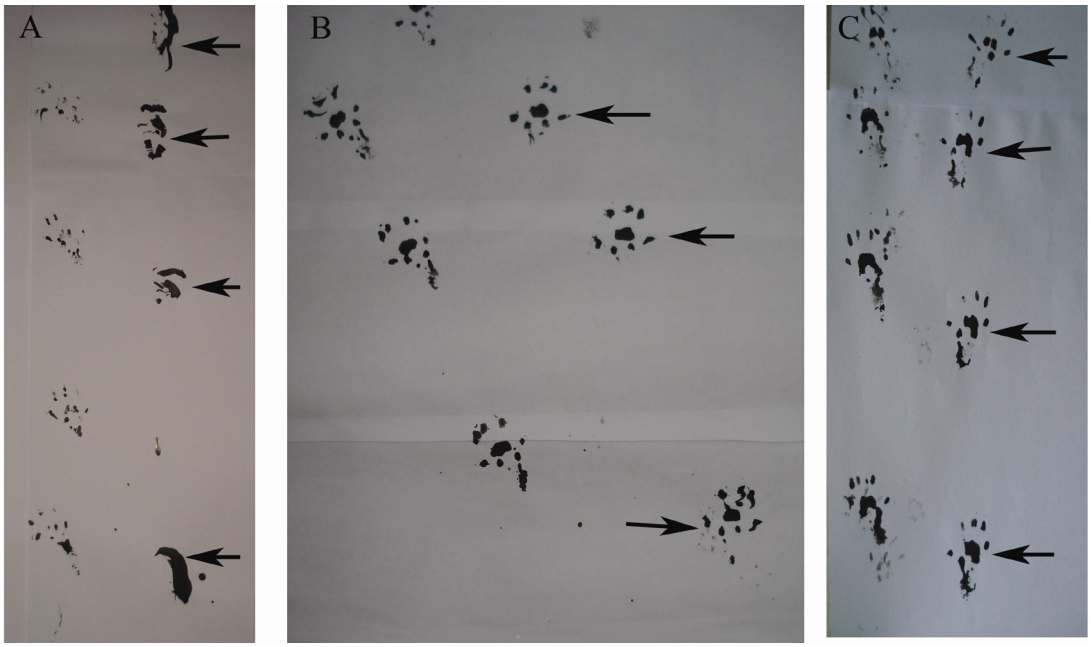

FIGURE 4. Walking tracks of footprints after 4 days of peroneal nerve crush injury. Arrows indicate footprints of the operated limb. (A) Footprints in negative control group—distilled water (10 mL/kg body weight/day). The palsy after interruption of the peroneal nerve is characterized by flexion contracture of the paws ("footdrop"), absence of toe-spreading reflex, and some dragging of the operated limb. (B) Footprints in positive control group—mecobalamin (130 μg/kg body weight/day). Clear footprints of the operated limb can be seen. (C) Footprints in aqueous extract group—*H. erinaceus* fresh fruit bodies (10 mL/kg body weight/day). Aqueous extract group demonstrated toe spreading and clear footprints of the operated limbs on the walking tracks.

and its reappearance indicated the regeneration of the nerve and the reestablishment of the nervous circuit.

Functional data show that the toe-spreading reflex appeared 2 to 3 days earlier than recovery of motor function as observed in walking track analysis. Toe spreading precedes recovery of locomotion following peroneal nerve crush injury because proper walking requires coordinated function involving sensory input, motor response, and cortical integration. Locomotion includes the return of ankle dorsiflexion and toe spreading during walking.

B. The Effects of Aqueous Extract of *H. erinaceus* on Axonal Reinnervation of Motor Endplates in EDL Muscle Following Crush Injury

Adult mammalian muscles recover virtually completely from temporary denervation provided that the reinnervation is allowed to proceed unhindered.[100,101] Axonal reinnervation of the EDL muscle was more enhanced in the aqueous extract group than in the negative control group after 14 days of crush injury as demonstrated by combined silver-cholinesterase staining (Fig. 6).

An important difference between the muscles of the groups occurs with respect to the regenerating axons. Extensor digitorum longus muscles of the negative control group contained a mixture of degenerating and regenerating axons, and migration of macrophages to remove degenerated myelin and axon fragments, a process called Wallerian degeneration.[2] The functional connection between motor neuron and EDL muscle fibers has not been reestablished at this stage. In rats treated with mecobalamin and aqueous extract, loose axon bundles indicate that the regeneration process is ongoing. Axon bundles are more compact and the regeneration process is more advanced after 14 days of crush injury compared to 7 days after injury. In treated groups, a high density of regenerating axons reinnervating motor endplates can be observed. This indicates reestablishment of connection between motor neuron and EDL muscle fibers, leading to functional recovery.[38]

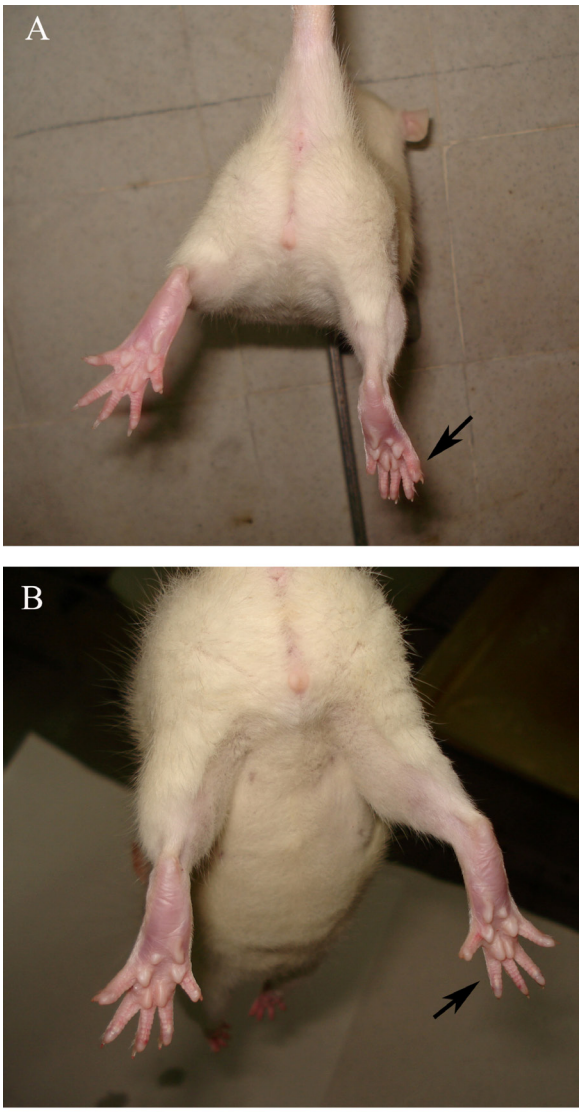

FIGURE 5. Recovery of toe-spreading reflex after 7 days of peroneal nerve crush injury. Arrows indicate the operated limb. (A) Minimal toe spreading on right limb in negative control group—distilled water (10 mL/kg body weight/day). (B) Normal toe spreading on right limb in treated groups—mecobalamin (130 μg/kg body weight/day) and aqueous extract of *H. erinaceus* fresh fruit bodies (10 mL /kg body weight/day).

C. The Effects of Aqueous Extract of *H. erinaceus* on Expression of Akt and MAPK Signaling Pathways as well as c-Jun and c-Fos Genes in the DRG Following Crush Injury

Expression of Akt and MAPK pathways as well as expression of c-Jun and c-Fos in the ipsilateral DRG from injured nerve was more enhanced in the aqueous extract group than in the negative control group after 7 days of crush injury as demonstrated by an immunohistochemical method. There was bright immunofluorescence for Akt, MAPK, c-Jun, and c-Fos in small neurons of ipsilateral DRG from injured nerve in the aqueous extract group (Fig. 7). The population of large neurons is defined by the expression of NF-200 as green fluorescence. Akt, MAPK, c-Jun, and c-Fos staining as orange fluorescence was targeted to small neurons. Up-regulation of Akt, MAPK, c-Jun, and c-Fos is crucial in facilitating axonal regeneration and subsequent reinnervation of target muscle, which brings a return of hind limb function.

Injury to neurons results in complex sequences of molecular responses that play an important role in the successful regenerative response and the eventual recovery of function. In the injured neurons, the rapid arrival of injury-induced signals is followed by the induction of transcription factors, adhesion molecules, growth-associated proteins, and structural components needed for axonal regrowth.[102] Injury to adult peripheral neurons, but not to CNS neurons, reactivates the intrinsic growth capacity and allows regeneration to occur. Primary sensory neurons with cell bodies in the DRG provide a useful model system to study the mechanisms that regulate regeneration.

Peripheral nerve injury induced peripheral sensitization, causing activation of Akt, MAPK, c-Jun, and c-Fos in small DRG neurons. These contributed to pain hypersensitivity found at the site of tissue damage and inflammation. In general, DRG neurons can be divided into large (>1200 μm^2), medium (600–1200 μm^2), and small (<600 μm^2) neurons. Small neurons respond to thermal, mechanical, and chemical nociceptive stimulations, whereas large neurons transmit touch and proprioceptive sensations.[103] Akt, MAPK, c-Jun, or c-Fos did not co-localize with NF-200 in large DRG neurons. It is possible that aqueous extract of *H. erinaceus* could trigger the expression of protein kinases and early genes that regulate nociceptive function and inflammation associated with nerve recovery.

Activation of Akt, MAPK, c-Jun, and c-Fos persisted for 1 week after injury until axonal regeneration occured. The findings are in accordance with a study by Naidu et al.[104] In their study, immunoreactivity for phospho-Akt was detected in ipsilateral DRG after 2, 4, and 7 days of

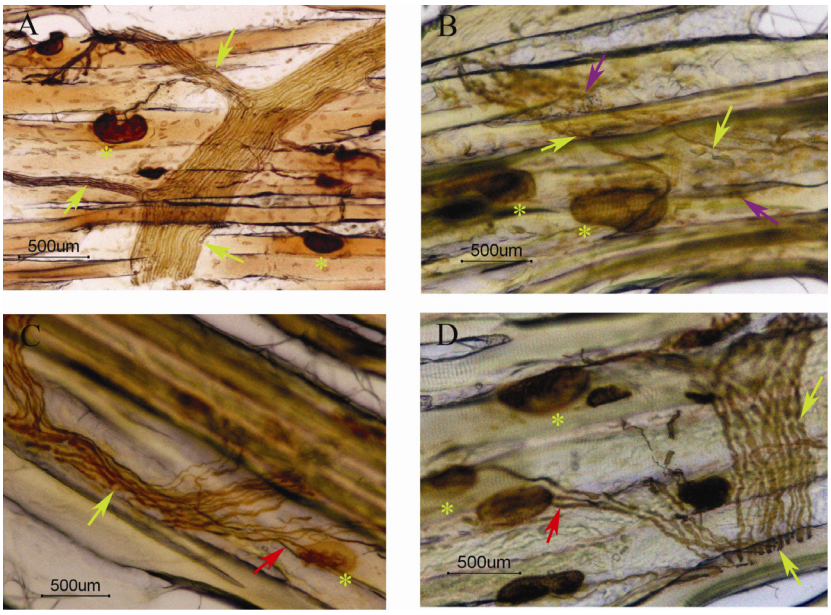

FIGURE 6. The morphology of silver-cholinesterase-stained longitudinal section of EDL muscle in normal unoperated limb and operated limb after 14 days of peroneal nerve crush injury. *Yellow arrows* indicate the axons. *Violet arrows* indicate the degenerating axons. *Red arrows* indicate polyneuronal innervation. *Asterisks* indicate the motor endplates. Scale bar = 500 μm. (A) Normal unoperated limb. Axon bundles are clear and compact. (B) Operated limb in negative control group—distilled water (10 mL/kg body weight/day). Wallerian degeneration can be detected. Degenerated axons are being phagocytosed by the cooperative action of denervated Schwann cells and infiltrating macrophages. (C) Operated limb in positive control group—mecobalamin (130 μg/kg body weight/day). Loose axon bundles indicate regeneration process is ongoing. (D) Operated limb in aqueous extract group—*H. erinaceus* fresh fruit bodies (10 mL/kg body weight/day). Axon bundles are more compact and regeneration process is more advanced compared to positive control group.

sciatic nerve crush by using western blot analysis, whereas clear up-regulation of both phospho p44 MAPK and phospho p42 MAPK was detected after 7 days of injury. In a study using *in situ* hybridization, Ito et al.[105] reported enhanced gene expression for PI3K in the hypoglossal motor neurons following axonal crush in the first week after injury. Phospho-Akt was clearly expressed in the normal DRG and nerve, and was up-regulated during nerve regeneration in the DRG neurons. The expression of Akt in the normal DRG and nerve may be required to maintain day-to-day cell survival, and an up-regulation could be essential to prevent mass cell death as a result of nerve injury.

MAPK pathway activation in neurons has been demonstrated to be important for neurite outgrowth, regeneration, synaptic plasticity, and memory functions in mature neurons. Naidu et al.[104] showed that phospho-MAPK was expressed in normal DRG but confirmed its presence in the rat sciatic nerves even after injury. This was probably needed for axonal regeneration. Further, Cheung et al.[56] utilized a model system, PC12 cells, to demonstrate the presence of neuroactive compounds in *G. lucidum*. Incubation with the mushroom extract resulted in a reduction of cell proliferation and induction of neuronal differentiation of PC12 cells. The ability of the extract to induce phosphorylation of ERK1/2 in PC12 cells suggests that it may be mediated via the ras/ERK signaling pathway.

Downstream events influenced by crush injury–activated kinases include up-regulation or activation of several transcription factors. Activated Akt and MAPK induce up-regulation and phosphorylation of the transcription factors c-Jun and c-Fos into the nucleus, leading to formation of AP-1 complexes that activate many downstream genes. In this study, up-regulation of c-Jun and c-Fos occurred in ipsilateral DRG neurons after 7 days of crush injury. Similar to the study on signaling pathways, activation of c-Jun and c-Fos also persisted for 1 week after injury until axonal

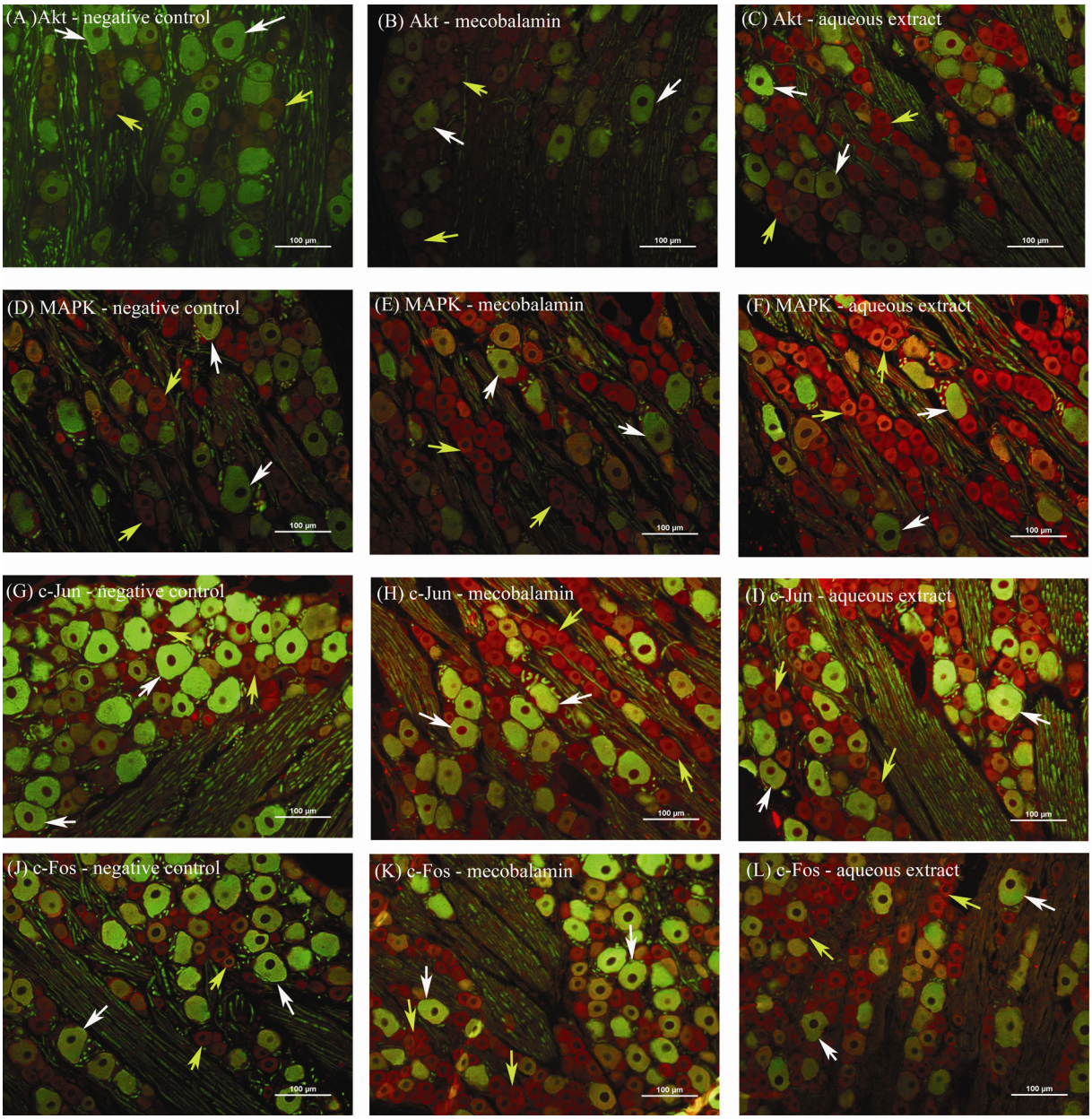

FIGURE 7. Expression of Akt, MAPK, c-Jun, and c-Fos in the DRG after crush injury. Double immunofluorescence staining between expression of signaling pathways or genes (orange) and NF-200 (green) in ipsilateral DRG from injured nerve. The expressions did not co-localize with large neurons. Although all small DRG neurons are stained brightly with Akt, MAPK, c-Jun, or c-Fos, immunoreactivity was higher in the ipsilateral DRG from injured nerve in rats treated with aqueous extract or mecobalamin than in negative control. *White arrows* indicate large neurons whereas *yellow arrows* indicate small neurons. Scale bar = 100 µm. (A-C): Akt activation. (D-F): MAPK activation. (G-I): c-Jun activation. (J-L) : c-Fos activation.

regeneration took place.

c-Jun is a protein that may be important for successful axonal regeneration. Studies by Broude et al.[106] on adult rat DRG showed that c-Jun was substantially up-regulated in DRG neurons following a peripheral axotomy, but after a central axotomy, only 18% of the neurons expressed c-Jun. In a study by Chi et al.,[107] c-Fos expression in the lumbar spinal cord increased in the superficial dorsal horn and deep dorsal horn within hours, and lasted for at least 4 weeks following sciatic nerve transection in rats.

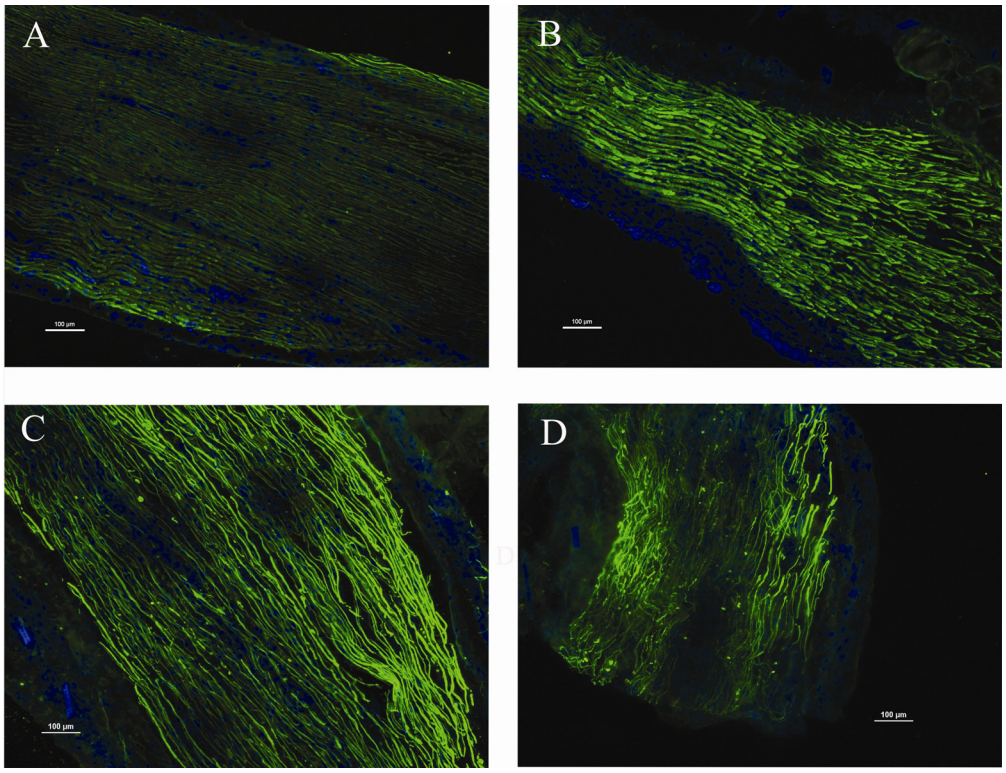

FIGURE 8. Representative photomicrographs of longitudinally sectioned peroneal nerves distal to the injury site after 7 days of peroneal nerve crush injury and the pathologic scale used for depicting these extents of injury or axonal loss. The green fluorescent strands represent individual axon fibers stained with anti-neurofilament 200 and blue fluorescent patches indicate DNA. Scale bar = 100 µm. (A): 0 = normal nerve of unoperated limb. (B): 1 = mild axonal damage of operated limb. (C): 2 = moderate axonal damage of operated limb. (D): 3 = severe axonal damage of operated limb.

D. The Effects of Aqueous Extract of *H. erinaceus* on Axonal Regeneration and Axonal Protein Synthesis Following Crush Injury

Crushing of peripheral mammalian axons initiates the process of axonal regeneration. During this process, fine-caliber axonal sprouts emerge from the parent stump of the axon, which remains attached to the neuronal cell body, and the new sprouts elongate distally. Provided that environmental conditions are supportive, regeneration continues until the new axons elongate sufficiently to reconnect with appropriate target tissues. The final stage of the regeneration process involves the radial growth of the new axons. The cytoskeletal polymers, microfilaments, microtubules, and neurofilaments are well known to have a central role in the regeneration process since they provide the basis for structure, motility, and final stability of the new axons.[108]

On injury to peripheral nerve, the neuronal cell bodies contributing to the damaged nerve respond to the insult in various ways. One of the most noted changes is a decrease in the production and anterograde transportation of neurofilament protein.[109] Consequently, axonal continuity and transport are disrupted by crush, and the portion distal to the site of injury is no longer supplied with neurofilament protein. Neurofilaments function chiefly as architectural elements that operate by displacing volume within the neuron and thereby increasing the volume of the neuron.[110] Specifically, neurofilament proteins are assembled into neurofilaments in the nerve cell body, and are then transported into the axon, where they contribute to the radial dimensions of the axon.

Promotion of axonal regeneration and protein synthesis in the injured peroneal nerves was more accelerated in the aqueous extract group than in the negative control group after 7 days of crush injury. Anti-neurofilament immunohistochemistry was used to compare the peroneal nerve regeneration.

Microscopic evaluation of the axon was performed in a double-blind fashion by three individuals. Each peroneal nerve was graded for damage on a qualitative 4-point scale.[111] Normal nerve received a score of 0. Mild, moderate, and severe axonal damage received a score of 1, 2, and 3, respectively (Fig. 8). Axons from each group were evaluated to determine the proportion of nerves with an injury greater than or equal to the moderate (≥2) level. In a normal peroneal nerve section from an unoperated limb, axons appeared to have normal morphology, were arranged more densely, and had uniform neurofilament immunostaining. In crushed peroneal nerve sections, axonal regeneration distal to the site of injury can be observed. Regenerating axons sprouted aberrantly and formed tangled masses or neuromas. Nerve fibers are nonparallel. Five rats in the negative control group and two rats in the mecobalamin or aqueous extract groups demonstrated moderate or severe axonal damage after 7 days of crush injury.[38]

Protein synthesis has been shown to be involved in axonal regeneration. There was bright immunofluorescence for nuclear ribonucleoprotein along the regenerating axons distal to the injury site after 7 days of crush injury, coincident with the synthesis of neurofilament proteins. Local protein synthesis occurred in regenerating axons and was up-regulated by aqueous extract of *H. erinaceus* after injury (Fig. 9). The original dogma was that necessary components for axonal growth and regeneration are usually synthesized by the cell body and sent along the axons by fast or slow axonal transport to their respective targets, which are usually hundreds of micrometers away from the cell body. These processes are crucial in facilitating axonal regeneration and subsequently reinnervation of target muscle.

The pool of newly synthesised cytoskeletal proteins is likely to act as a source of structural proteins for growth cone reformation and axonal growth,[112] as well as to regulate cytoskeletal dynamics.[113] Axonal regeneration has been thought to recapitulate development in many aspects. The injured axon must transition to a growth state, and this transition distinguishes protein synthesis in adult axons from that in developing axons. The old arguments that adult axons do not synthesize proteins were based largely on the absence of polysomes in axons compared with those in dendrites.[114] This scarcity of protein synthetic machinery suggests that the mature axon must recruit (and activate) ribosomes and mRNAs to the injury site. It is not clear what triggers this recruitment of protein synthetic machinery after injury, but an increase in axoplasmic [Ca^{2+}] is a likely candidate. Adult vertebrate axons have the capacity to synthesize many different proteins.[115]

VIII. CONCLUSIONS

This is the first study revealing the relationship between activation of Akt and MAPK signaling pathways, c-Jun and c-Fos genes, axonal regeneration, protein synthesis and degradation, and peripheral nerve regeneration following crush injury in an *in vivo* experiment using *H. erinaceus*, a medicinal mushroom known for its neurological effects. Up-regulation of Akt, MAPK, c-Jun, and c-Fos in ipsilateral DRG neurons, as well as axonal protein synthesis and degradation after 7 days of crush injury, are consistent with the beginning of functional recovery of injured right hind limb.

Aqueous extract of *H. erinaceus* enhanced nerve regeneration and accelerated motor functional recovery after crush injury. Patients who receive *H. erinaceus* may experience a more expeditious improvement in the quality of life after injury. The functional evaluation combined with morphological examination of regenerated nerves, ipsilateral DRG, and target EDL muscle revealed that the aqueous extract promoted peripheral nerve regeneration with significant functional recovery. It was also noted that the neurotherapeutic effects of the aqueous extract were comparable to those elicited by mecobalamin, a CNS drug used in peripheral nerve disorders. However, taking mecobalamin for the treatment of nerve injury gives rise to side effects such as gastrointestinal and dermatological problems.[116]

It will be of interest to examine the potential of different signaling pathways that underlie peripheral nerve regeneration. There is a possibility that *H. erinaceus* may mediate neuronal functions by modulating the activities of different signaling pathways such as activation of cAMP response element-binding (CREB). The importance of CREB signaling on learning and memory, as well as hyperphosphorylation of CREB, plays an important role in the long-term potentiation of the hippocampus.

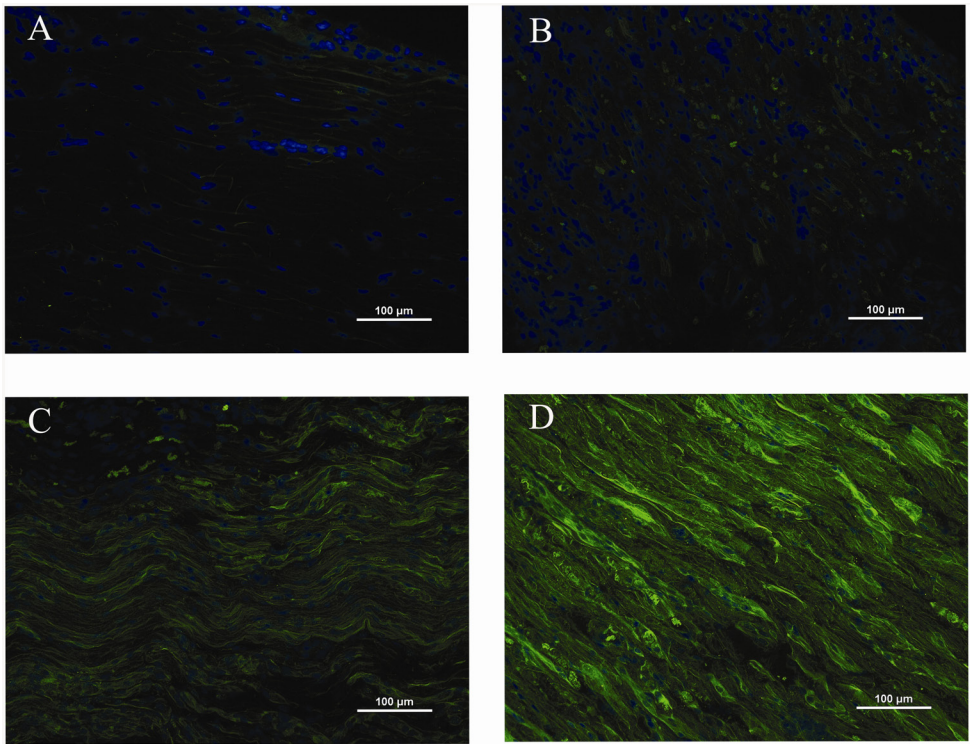

FIGURE 9. Distribution of protein synthetic machinery in peroneal nerve axons after 7 days of crush injury. Fluorescence imaging demonstrating staining for nuclear ribonucleoprotein in peroneal nerve axons as green fluorescent patches and DNA as blue fluorescent patches. Scale bar = 100 µm. (A): Normal nerve of unoperated limb. Patches of nuclear ribonucleoprotein was not detected in the uninjured nerve. (B): Negative control group—distilled water (10 mL/kg body weight/day). Immunoreactivity for nuclear ribonucleoprotein in regenerating axon was less than in treated groups. (C): Positive control group—mecobalamin (130 µg/kg body weight/day). Bright immunofluorescence for nuclear ribonucleoprotein along the regenerating axons can be noticed. (D): Aqueous extract group—*H. erinaceus* fresh fruit bodies (10 mL/kg body weight/day). Nuclear ribonucleoproteins are brightly stained by immunofluorescence.

At the present time, many of the medicinal properties attributed to mushroom products are based on data obtained from *in vitro* and animal-based experiments. The protective effects of *H. erinaceus* in humans require further research, as the *in vivo* trial in rats has limited scope. Much more advanced science is required to demonstrate that claims of enhanced function and reduced disease risks are also applicable in the human context.[117] Long-term *in vivo* trials may need to be assessed as there may be a cumulative effect.

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