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The Effect of *Curcuma longa* Alcoholic Extract on Cell Regeneration (Neurons and Neuroglias) after Sciatic Nerve Injury in Diabetic Rats

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Abstract: Background: Wallerian degeneration in the CNS and PNS consists of degradation and phagocytosis of axons and their myelin sheath distal to the site of injury that progress in diabetic condition. Context and purpose of the study: The present study was undertaken to evaluate the effect of Curcuma Longa alcoholic extract on cell regeneration (neurons and neuroglias) after sciatic nerve injury in diabetic rats. Forty rats were divided to five groups (control, compression and three experimental groups). In compression and experimental groups right sciatic nerve were highly compressed for 30 s, assigned to experimental groups (Diabetic+Compression+Curcumin extract injections (0, 25, 50 mg kg^{-1}, i.p., 3 time) (N = 8). After 4 weeks post-operative the lumbar segments of spinal cord were sampled, processed, sectioned, stained and the number of cells were counted by stereological study. **Results:** Statistical analyses showed remarkable increase in the neuronal and neuroglias density in the all experimental groups. **Conclusions:** This effect of *Curcuma longa* extract is for antioxidative effect. This effect has the potential role to protect the neurons in neurodegenerative process in diabetic condition.

Key words: *Curcuma longa*, neuroprotective, compression, serological study, chromatolysis

INTRODUCTION

The cell body of neuron is the place to synthesize cytoplasm members and cell membrane of all cells. When nervous fiber is cut or crushed degeneration wallerian is occurred (Rosenfield and Paksima, 2001). Then myelin is divided to geom like pieces. Finally, macrophages and Schwann cells raid in to neurollema (shown) cover and swallow axonal myelin pieces (McPhail *et al*., 2004).

The course of defeated axons destruction is different from wallerian process, because the destruction phagocytosis of these pieces in CNS is carried out at very lower speeds (Siniscalco *et al*., 2007).The defeated pieces of myelinated axons can be seen even after months. Phagocyte cell containing a parted pieces romaine in the place even after years and show the place of destroyed fibers (Siniscalco *et al*., 2007). Chromatolysis is a procedure following axonal defeat in neurons cellular body so sometimes this phenomenon is called axonal reaction (Baker and Hagg, 2005).

Diabetes mellitus can lead to functional and structural deficits in both the peripheral and central nervous system. Underlying diabetic complications such as nephropathy, neuropathy, retinopathy, cardiovascular disease and peripheral vascular disease, diabetes can be present for many years before an actual diagnosis is made (Sima and Li, 2005).

The pathogenesis of these deficits is multifactor and may involve microvascular dysfunction and oxidative stress (Patil *et al*., 2006). Cognitive deficits are also reported to occur in animal models of diabetes (Stroptozotocin induced)which can be prevented, but not fully reversed by insulin treatment (Kuhad *et al*., 2008).

Many deleterious events contribute to oxidative damage to neurons in diabetes: because of high levels of polyunsaturated lipids in the brain, direct lipperoxidation frequently occurs causing lipid membrane disruption and consequent neurodegeneration (Ristow, 2004). Regeneration of diabetic axons has delays in onset, rate and maturation. It is possible that microangiopathy of vase nervorum, the vascular supply of the peripheral nerve, may render an unfavorable local environment for nerve regeneration. Diabetic patients are susceptible to peripheral nerve injury from entrapment and other causes. Regeneration in peripheral nerves can be complicated by relative ischemia, as may occur in cases of nerve infarction (Bansal *et al*., 2006). The intact peripheral nerve endoneurial vascular nutritive compartment is well supplied by extrinsic "feeding" vessels arising from its epineuria plexus. Ischemia after nerve injury may be "normally" averted by increasing endoneurial nerve blood flow to address the increased nutrient and oxygen consumption of regenerating axons and cellular elements during repair (Zochodne *et al*., 1996). Oxidative stress

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results from a cell or tissue failing to detoxify the free radicals that are produced during metabolic activity. Diabetes is characterized by chronic hyperglycemia that produces deregulation of cellular metabolism. Accumulated data support that oxidative stress induced by chronic hyperglycemia plays a key role in both central and peripheral nervous system complications of diabetes (Kennedy and Zochodne, 2002).

Curcumin, a yellow pigment extracted from the rhizome of the plant *Curcuma longa* (Zingiberaceae) has been shown to have antioxidative properties due to the presence of chain breaking or H donating phonologic groups in it's molecular structure (Bala *et al*., 2006). Curcumin is widely used as a food additive. This pigment used throughout Asia as herbal medicine (Bala *et al*., 2006). Curcumin use as a therapy for malignant and inflammatory diseases and it's potential use in the treatment of degenerative neurologic diseases (Cheng *et al*., 2001), cystic fibrosis, cardiovascular diseases (Strimpakos and Sharma, 2008) and cancer (Lin and Lin-Shiau, 2001).

The neuroprotective effect of this substance was discovered during a screening of its potential to protect against the harmful component. Since them, studies have indicated potential benefits for Alzheimer's disease and parkinson's disease based on laboratory models (Ringman *et al*., 2005).

Turmeric is used as a tonic and as a blood purifier. Its role in the treatment of skin diseases and its ability to soften rough skin resulted in the prolific use of turmeric in topical creams and bath soaps In India. Tumeric is also used in home remedies in the treatment of cuts, wounds, bruises and sprains. It is use as an anti-inflammatory and antimicrobial agent. This substance has been recognized for more than a century (Joe *et al*., 2004).The importance of turmeric in medicine took a new twist when it was discovered that the rhizome of *curcuma longa* is very rich in phenolics, whose structures have been identified as curcuminoids. Phenolics are known to possess antioxidant properties (Joe *et al*., 2004).

The aim of this study is to evaluate the effect of curcumin on cell regeneration (neurons and neurogelias) after sciatic nerve injury in diabetic rats.

MATERIALS AND METHODS

All experiment was conducted in faculty of science, Islamic Azad University of Mashhad, Iran (2010).

Animal subjects: Forty male, Wistar rats weighting between 300-350 g served as Subjects for these experiments. All animals were housed individually and maintained on a 12/12 light/dark cycle, with lights on at 6.00 h. Ambient temperature in the animal facility was kept at 22±2°C. Food and water was given *ad libitum* to animals.

Extraction: Curcuma Longa was collected from a reign around mashhad and was coded with Islamic Azad University of Mashhad, Iran herbarium. Its extract was performed with Soxhlet apparatus (Cicchetti and Chaintreau, 2009). After obtaining extract, it was situated in oven with temperature (45±2°C) for 48 h to remove solvent.

Surgery: Animals were anesthetized under intraperitoneal injection of 60 mg kg^{-1} ketamine i.p. and 6 mg kg^{-1} xylazine i.p. Right sciatic nerve was exposed and crushed for 30 seconds period between prongs of #5 clamp forceps (Behnam *et al*., 2000).

Inducing diabetes: Diabetes was induced by a single injection (i.p.,) of streptozotocin (55 mg kg^{-1}) dissolved in sterile phosphate buffered saline (Maryam *et al*., 2008). Control group received only buffer. Then animals were housed under standard condition and received food and water. Induction of diabetes was confirmed by blood glucose level (glycemia>400).

Groups:

- Controls (N = 8) For baseline measurement in this group on the right side an operation was performed which exposed the sciatic nerve but did not disturb it (Just for induced stress effect of operation)
- Compression or Sham-operated controls groups (N = 8)
- In this group after operation the right sciatic nerve was crushed
- Compression+induce diabetic (N = 8)
- In this diabetic animal sciatic nerve was crushed
- Diabetic+Compression+Curcumin extract injections (25 mg kg^{-1}, i.p., 3 time) (N = 8)
- Diabetic+Compression+Curcumin extract injections (50 mg kg^{-1}, i.p., 3 time) (N = 8)

In these diabetic animal Coordinated with sciatic nerve crush curcumin extract were given three times. (Every 7 day one injection)

Sampling: At the selected post-operative time (4 weeks), rats were anesthetized and intracardially perfused with

formaldehyde. Immediately following perfusion a block of the spinal cord segments L4 to L6 (approximately 8 mM length) was removed while sciatic nerve roots of both sides were still attached it. The spinal blocks were placed in the same fixative for post sampling fixation overnight and then processed and embedded in paraffin. The blocks were sectioned serially at 7 micrometer. A uniform random sampling scheme was employed so that about 10 sections from each block was sampled. Sections were stained with toluidine blue staining method with special buffer of acetic acid, sodium acetate and distilled water (pH = 4.65). After permanent mounting the number of motor neurons and neurogelias in right sides of ventro-lateral regions of the spinal cord ventral horns (L4 to L6) were determined, using stereological counting technique; physical dissector (Tehranipour and Kabiri, 2009).

The dissector principle was used to determine the numbers of motoneurons in each section (Gundersen *et al*., 1988). Those cell nuclei selected by the frame on the reference plane but not appearing on the adjacent look-up frame section were deemed to have their tops in the volume described by the product of the area of the counting frame and the distance between sections. These nuclei were counted (Q) to provide the Numerical density of cells (NV) in the ventral horns of spinal cord according to the equation:

$$NV = \frac{\sum a}{\sum frame \times V_{disector}}$$

$$Vdisector = A\ frame \times H$$

where, Σa the sum of counted neurons, H is the depth of the dissector equal to the section thickness (7 micron) and a (frame) is the scaled area of the dissector frame.

Statistical analyze: The ratio of numerical density of neurons and neuroglia in samples of spinal cord was used as an index. All quantitative data were analyzed using ANOVA and t-test. Statistical significance was chosen as $p<0.05$. All results are reported as Mean±SEM.

RESULTS

Diabetes was assessed in this study by monitoring the blood glucose levels in both PBS and STZ injected rats. There was a significant increase ($p<0.001$) in blood glucose levels from 100±5 mg dL^{-1} in control to 470±18 mg mL^{-1} in diabetic rats (Fig. 1).

The control group revealed healthy neuronal cells amounted by (1766±70) intact neurons. Sciatic nerve

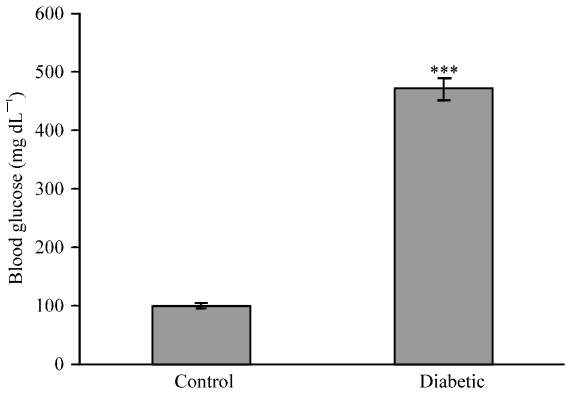

Fig. 1: Comparison between blood glucose levels in control and diabetic groups. Results are expressed as Mean±SD (n = 8). ***p<0.001

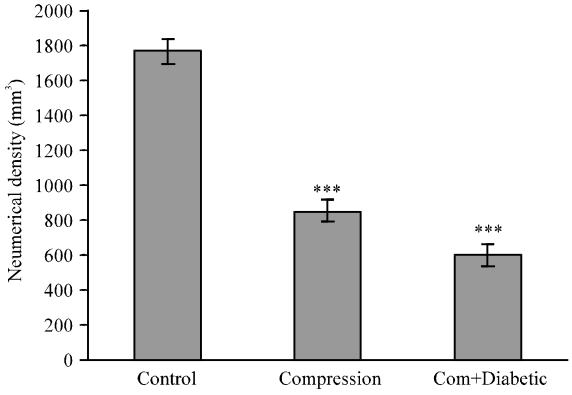

Fig. 2: Comparison between control, compression and compression+diabetic groups. Results are expressed as Mean±SD (n = 8). ***p<0.001

crush resulted in massive neuronal damage manifested as a significant ($p<0.05$) 50% decrease in the number of normal appearing neurons. This decrease is very obviously in diabetic group (Fig. 2).

Animal treated with curcumin extract immediately after compression (Sciatic nerve injury) and continued for 4 week resulted in a significant ($p<0.05$) increase in the number of intact neurons respectively as compared to compression+diabetic group (Fig. 3).

The effects of curcumin extract treatment on the numbers of neuroglia in the right ventral horn of spinal cord region at 28 days after sciatic nerve compression in rats are shown in (Fig. 4).

However, when control group was compared to treatment groups still a significant decrease in the number of intact neurons has remained.

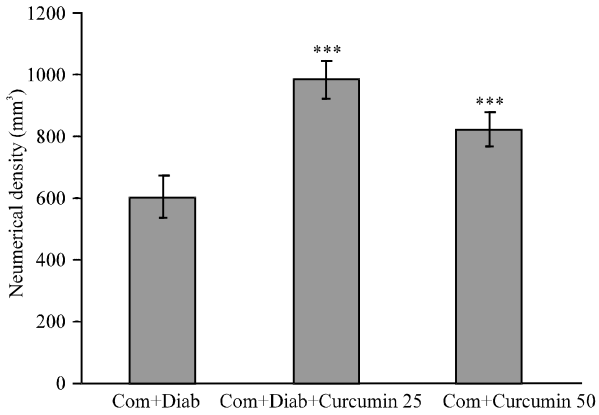

Fig. 3: Effects of compression and Curcumin extract on the number of intact neurons of right ventral horn of spinal cord in diabetic rats. Results are expressed as Mean±SD of 8 rats and data were analyzed by one-way ANOVA followed by Tukey-kramer multiple comparisons test.***p<0.001

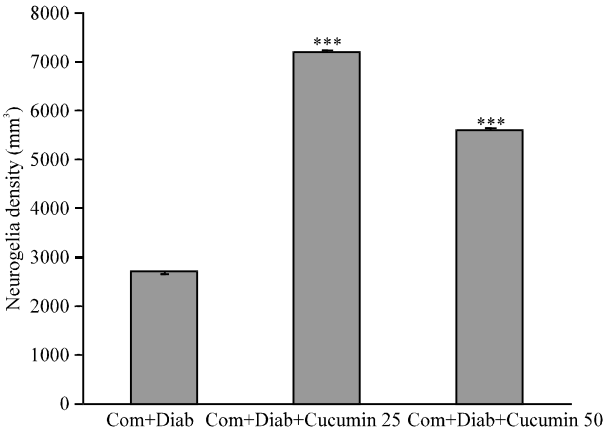

Fig. 4: Effects of compression and Curcumin extract on the number of neuroglia of right ventral horn of spinal cord in diabetic rats. Results are expressed as Mean±SD of 8 rats and data were analyzed by one-way ANOVA followed by Tukey-kramer multiple comparisons test

Some changes were shown in different groups At Photomicrographs (Fig. 5). The shape and place of nucleus is changed in compression, diabetic and treatment.

DISCUSSION

Our findings demonstrate that Curcumin plays an important role in the maintenance and repair of the nervous system after injury or disease that is correlated with Bala *et al.* (2006). There is a remarkable change in the number of Alpha motor neurons and neurogelias in different groups. Animals treated with Curcumin immediately after compression of sciatic nerve (for 4 weeks) resulted in a significant ($p<0.05$) increase in the number of intact neurons and neurogelias, respectively as compared to compression and compression+Diabetic groups (Fig. 1-3). However, still there is a significant ($p<0.05$) decrease in the number of intact neurons when compared to the control group. We have demonstrated that the Curcumin has therapeutic and repairing effects in the spinal cord, protecting motor neurons from atrophy after the death of neighboring motor neurons. These degenerative effects are very strong in diabetic condition.

One of the very important phenomena in diabetic condition is oxidative stress. Oxidative stress increase tissue levels of highly reactive and toxic substances and effects signal transduction pathways involved in neuronal and endothelial cell function. As we see in diabetic group the rate of injury increased. Primary diabetic encephalopathy is recognized as a late complication of both type 1 and type 2 diabetes (Kinney *et al.*, 2003). Impairments in learning, memory, problem salving and mental and motor speed are more common in type 1 diabetic patients than in the general population (Deregnier *et al.*, 2000).

Several recent studies have implicated abnormal function of the insulin/IGF axis in the early pathogenesis of Alzheimer's disease. Insulin and IGF-1 are believed to regulate ß-amyloid levels and tau phosphorylation (Gasparini and Xu, 2003). Curcumin's neuroprotective role has been recently demonstrated in a few studies (Ghoneim *et al.*, 2002). For example, Manikandan *et al.* (2004) have evaluated the free radical-scavenging and neuropotective potential of the manganese complexes of curcumin Manikandan *et al.* (2004). Other has demonstrated the neuroprotective effects of curcumin against the effects of middle cerebral artery occlusion (Thiyagarajan and Sharma, 2004). Free radical mediated damage to biological systems is recognized as the initiating agent for many diseases, such as cardiovascular diseases, cancer and arthritis. Tumeric and its constituents show beneficial effects on these diseases and on other illnesses (Al-Omar *et al.*, 2006).

It has been well known that the survival and functional maintenance of neurons is clearly dependent upon retrogradly transport of neurotrophins. Peripheral nerve transaction or crush, blockade axonal transport and therefore might produce chromatolysis and cell death (Perrin *et al.*, 2005).

It is known that clinical motor function deteriorates in a delayed manner after sciatic nerve compression. We

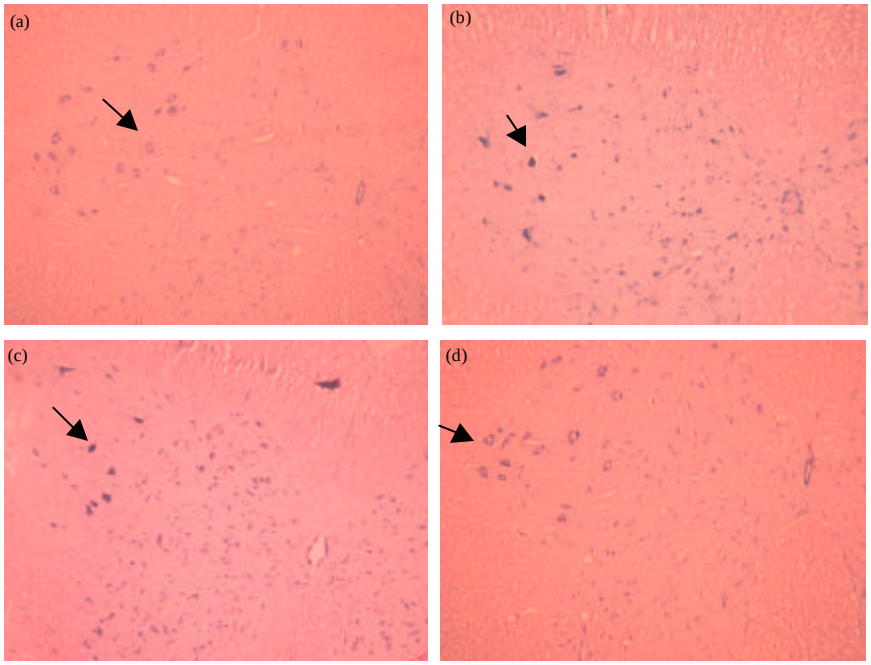

Fig. 5(a-d): Photomicrographs illustrate neurons of the anterior horn of spinal cord stained with toluidine blue and eosin at magnification of (20x) 28 days after injury. Alpha motonurons were shown with spikes (a) Control, (b) Compression, (c) Compression+Diabetic and (d) Compression+Diabetic+Curcumin injection

show that 50% of motor neurons were selectively lost after compression (Fig. 2) and the motor neuronal loss could be the reason for the delayed neurological dysfunction.

Progressive dysfunction and death of neurons characterize neurodegenerative diseases (Baker and Hagg, 2005). There are some evidences supporting the hypothesis that curcumin may act protectively in some neurodegenerative disorders (Sicotte *et al*., 2007). The most important factors contributing to neuronal cell death are: genetic factors, glutamate mediated excitotoxicity leading to disturbances in intracellular calcium and sodium metabolism, mitochondrial dysfunction, oxidative stress, growth factor with drawl, cytokines and toxins (Singh *et al*., 2008).

In addition after compression, inflammatory process was started some inflammatory mediators, such as bradykinine, prostaglandin and serotonin enhance the excitability of normal and injured neurons.

Researchers suggest that inflammation is a critical factor for progressing degeneration process. Tissues inflammation factors were surround surface of injured sciatic nerve after chronic compression. In this situation existence of Anti inflammation factors could suppress degeneration. Curcumin extract has Anti inflammation role (Wang *et al*., 2005) and could reduce the rate of degeneration as happen in this study. In all treatment groups, there is a remarkable changes in number of alpha motoneurons in compare to compression group ($p<0.05$) (Fig. 2).

Although the number of intact alpha motoneuron in all treatment groups was increased but in treatment group with does (25 mg kg^{-1}, i.p., 3 time) the best result was seen. In this group the mean of alpha motoneuron after compression is very near to control group (Fig. 2). Result shows that curcumin could have protection effect even in diabetic condition. Although in diabetes the rate of degeneration was increased but this extract could act as survival factor.

Finally, histological and stereological assessment showed that Curcumin with a dose of 25 mg kg^{-1} attenuated neuronal damage after sciatic nerve compression in diabetic rats. Curcumin is relatively safe in human. If curcumin extract provides neuroprotection against sciatic nerve injury in humans, as seen in rats, curcumin treatment would act to save a number of patients from CNS damage.

The Diabetes Control and Complications Trial (DCCT) established the importance of hyperglycemia and other consequences of insulin deficiency in the

pathogenesis of diabetic neuropathy, but the precise mechanisms by which metabolic alterations produce peripheral nerve fiber damage and loss remain unclear. Plants have been used for medical purposes since many centuries. Different cultures have tried different kinds of plants for traditional treatment. Due to a least side effect, trend to use of plants medications have been increased recently (Deputy, 2002).

CONCLUSION

In total result had shown that curcuma longa extract has protective effect on neurons and neurogelias after sciatic nerve injury even in diabetic condition.

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REFERENCES

Al-Omar, F.A., M.N. Nagi, M.M. Abdulgadir, K.S. Al-Joni and A.A. Al-Majed, 2006. Immediate and delayed treatments with curcumin prevents forebrain Ischemia induced neuronal damage and oxidative insult in the rat hippocampus. Neurochem. Res., 31: 611-618.

Baker, K.A. and T. Hagg, 2005. An adult rat spinal cord contusion model of sensory axon degeneration: The estrus cycle or a preconditioning lesion do not affect outcome. J. Neurotrauma, 22: 415-428.

Bala, K., B. Tripathy and D. Sharma, 2006. Neuroprotective and anti-ageing effects of curcumin in aged rat brain regions. Biogerontology, 7: 81-89.

Bansal, V., J. Kalita and U.K. Misra, 2006. Diabetic neuropathy. Post Grad. Med. J., 82: 95-100.

Behnam-Rasouli, M., M. Nikravesh, M. Mahdavi and M. Tehranipour, 2000. Post-operative time effects after sciatic nerve crush on the number of alpha motoneurons, using a stereological counting method (Disector). Iran. Biomed. J., 4: 45-49.

Cheng, A.L., C.H. Hsu, J.K. Lin, M.M. Hsu and Y.F. Ho *et al*., 2001. Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. Anticancer Res., 21: 2895-2900..

Cicchetti, E. and A. Chaintreau, 2009. Comparison of extraction techniques and modeling of accelerated solvent extraction for the authentication of natural vanilla flavors. J. Sep. Sci., 32: 1957-1964.

Deputy, 2002. The Iranian plant's pharmacopoeia. Iranian Ministry of Health and Medical Education, Deputy of Food and Medication (Report), pp: 1-3.

Deregnier, R.A., C.A. Nelson, K.M. Thomas, S. Wewerka and M.K. Georgieff, 2000. Neurophysiologic evaluation of auditory recognition memory in healthy newborn infants and infants of diabetic mothers. J. Pediatr., 137: 777-784.

Gasparini, L. and H. Xu, 2003. Potential roles of insulin and IGF-1 in Alzheimer's disease. Trends Neurosci., 26: 404-406.

Ghoneim, A.I., A.B. Abdel-Naim, A.E. Khalifa and E.S. El-Denshary, 2002. Protective effects of curcumin against ischaemia/reperfusion insult in rat forebrain. Phamacol. Res., 46: 273-279.

Gundersen, H.J.G., T.F. Bendtsen, L. Korbo, N. Marcussen and A. Moller *et al*., 1988. Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS., 96: 379-394.

Joe, B., M. Vijaykumar and B.R. Lokesh, 2004. Biological properties of curcumin-cellular and molecular mechanisms of action. Crit. Rev. Food Sci. Nutr., 44: 97-111.

Kennedy, J.M. and D.W. Zochodne, 2002. Influence of experimental diabetes on the microcirculation of injured peripheral nerve: Functional and morphological aspects. Diabetes, 51: 2233-2240.

Kinney, B.A., M.B. Rabe, R.A. Jensen and R.W. Steger, 2003. Maternal hyperglycemia leads to gender-dependent deficits in learning and memory in offspring. Exp. Biol. Med., 228: 152-159.

Kuhad, A., R. Sethi and K. Chopra, 2008. Lycopene attenuates diabetes-associated cognitive decline in rats. Life Sci., 83: 128-134.

Lin, J.K. and S.Y. Lin-Shiau, 2001. Mechanisms of cancer chemoprevention by curcumin. Proc Natl. Sci. Counc. Repub. China B., 25: 59-66.

Manikandan, P., M. Sumitra, S. Aishwarya, B.M. Manohar, B.Lokanadam and. R.Puvanakrishnan, 2004. Curcumin modulates free radical quenching in myocardial ischaemia in rats. Int. J. Biochem. Cell Biol., 36: 1967-1980.

Maryam, T., B.R. Morteza and R. Amin, 2008. Maternal hyperglycemia proliferate choroids plexus and enlarge the lateral ventricle in brain of newborn rats. J. Boil. Sci., 8: 799-803.

McPhail, L.T., D.P. Stirling, W. Tetzlaff, J.M. Kwiecien and M.S. Ramer, 2004. The contribution of activated phagocytes and myelin degeneration to axonal retraction-dieback following spinal cord injury. Eur. J. Neurosci., 20: 1984-1994.

Patil, C.S., V.P. Singh and S.K. Kulkarni, 2006. Modulatory effect of sildenafil in diabetes and electroconvulsive shock-induced cognitive dysfunction in rats. Pharmacol. Rep., 58: 373-380.

Perrin, F.P., S. Lacroix, M. Aviles-Trigueros and S. David, 2005. Involvement of monocyte chemoattractant protein-1, macrophage inflammatory protein-1α and interlukin-1β in wallerain degeneration. Brain, 131: 2620-2631.

Ringman, J.M., S.A. Frautschy, G.M. Cole, D.L. Masterman and J.L. Cummings, 2005. A potential role of the curry spice curcumin I alzheimers disease. Curr. Alzheimer Res., 2: 131-136.

Ristow, M., 2004. Neurodegenerative disorders associated with diabetes mellitus. J. Mol. Med., 82: 510-529.

Rosenfield, J. and N. Paksima, 2001. Peripheral nerve injuries and repair in the upper extremity. Bull. Hospital Joint Dis., 60: 155-161.

Sicotte, N.L., B.S. Giesser, V. Tandon, R . Klutch and B. Steiner *et al*., 2007. Testosterone treatment in multiple sclerosis: A pilot study. Arch. Neurol., 64: 683-688.

Sima, A.A. and Z.G. Li, 2005. The effect of C-peptide on cognitive dysfunction and hippocampal apoptosis in type 1 diabetic rats. Diabetes, 54: 1497-1505.

Singh, M., N. Sumien, C. Kyser and J.W. Simpkins, 2008. Estrogens and progesterone as neuroprotectants: What animal models teach us? Front Biosci., 13: 1083-1089.

Siniscalco, D., C. Fuccio, C. Giordano, F. Ferraraccio and E. Palazzo *et al*., 2007. Role of reactive oxygen species and spinal cord apoptotic genes in the development of neuropathic pain. Pharmacol. Res., 55: 158-166.

Strimpakos, A.S. and R.A. Sharma, 2008. Curcumin: Preventive and therapeutic properties in laboratory studies and clinical trials. Antioxid. Redox Signal., 10: 511-545.

Tehranipour, M. and M. Kabiri, 2009. The effect of exogenous testosterone administration on peripheral nerves regeneration after sciatic nerve compression in rat. J. Biol. Sci., 9: 692-696.

Thiyagarajan, M. and S.S. Sharma, 2004. Neuroprotective effect of curcumin in middle cerebral artery occlusion induced focal cerebral ischemia in rats. Life Sci., 74: 969-985.

Wang, Q., A.Y. Sun, A. Simonyi, M.D. Jensen and P.B. Shelat *et al*., 2005. Neuroprotective mechanisms of curcumin against cerebral ischema-induced neuronal apoptosis and behavioral deficits. J. Neurosci. Res., 82: 138-148.

Zochodne, DW., C. Cheng and H. Sun, 1996. Diabetes increases sciatic nerve susceptibility to endothelin-induced ischemia. Diabetes, 45: 627-632.