Effect of Nerve Regeneration on Fracture Healing of Rat Tibia. Histological and Radiological Study

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Abstract: Injuries from road traffic crashes form a major public health problem. The resulting bone fractures are usually associated with nerve injuries in the form of nerve degeneration followed by regeneration. This regeneration process is expected to affect the amount and quality of the formed calluses. This study aims to investigate the effect of sciatic nerve crushing as a model for nerve regeneration on bone healing in rat tibial fracture. Forty five rats were used in this study. They were divided into three groups (Fifteen each) after induction of tibial fracture and internal fixation. Sciatic nerve was kept intact in rats of group I, cut in group II and crushed in group III. The tibial fractures were examined histologically and radiologically. The amount of the bridging calluses was more evident in groups II and III after 2 weeks. Remodeling of the calluses was better in group III at 4 weeks. Our results suggests that deinnervation induced a large but irregular fracture callus and that regeneration showed faster and more regular healing. These results may be of significance to clinicians and researchers who are seeking to improve fracture-healing in patients with associated nerve injury as spinal cord trauma.

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Key words: sciatic nerve crushing, tibial fracture, nerve regeneration.

1. Introduction

Clinical observations on enhanced healing of fractures in extremities of patients with spinal cord or head trauma had been previously reported (Garland *et al.,* 1980, Newman *et al.,* 1987, Bidner *et al.,* 1990 and Citta-Pietrolungo *et al.,* 1992). Various clinical observations suggested that the nervous system interacts with and promotes fracture-healing (Peter *et al.,* 2009). Increased incidence of heterotopic ossification been reported, abundant callus formation and rapid progress to union were observed (Smith, 1987 and Spencer, 1987). Although there is increasing understanding of both the molecular basis and metabolic regulation of bone healing, the knowledge of what controls the rate of fracture healing is incomplete (Pape *et al.,* 2001).

It was proven that nerves have extensive and decisive effects on the development, formation and metabolism of bone tissues (Shang *et al.,* 2001). Accordingly denervation, caused irregularities in the radiopacity and increased the length of fracture callus (Aro, *et al.,* 1981). Moreover, spinal lesion was reported to increases the rate of callus ossification and the variance of callus size in tibial fractures of rats (Aro *et al.,* 1985). These results suggest that neural regulation plays a role in the type of fracture healing and in the amount and quality of the callus (Nordsletten *et al.,* 1994).

There is evidence of high level of fractures in urbanized communities (Khoja *et al.,* 2007). Injuries from road traffic crashes have become a major public health and socio-economic problems. Traffic crashes usually induce traumatic nerve injury (Lundborg, 1988; Zochodne and Ho, 1990). This condition in turn induces demyelinization, remyelinization, axonal degeneration, axonal regeneration, focal, multifocal or diffuse nerve fiber loss and endoneural edema (Zochodne and Ho, 1990 and Bagdatoglu *et al.,* 2002).

Wallerian degeneration takes place during the first few days postinjury. The axons degenerate, their myelin sheath detaches and degrades, and the degradation products, together with macrophage secretion, stimulate the Schwann cells within the distal stump to proliferate within their basal lamina tubes. This proliferation continues for approximately 2 weeks (Salzer & Bunge, 1980; Salzer *et al.,* 1980; De Vries, 1993).

Sciatic nerve crush is widely accepted as a valid model for peripheral nerve regeneration. In this model, nerve damage results in rapid disruption of nerve function as evidenced by electromyography measurement. Recovery of nerve function occurs within 2 weeks and by week 4 post-lesioning significant remyelination of the regenerated axons is observed. The recovery of sensory functions after sciatic nerve crushing occurs more sooner than the recovery of motor functions of sciatic nerve. The motor function of the sciatic nerve shows full recovery within 21 days (Fournier *et al.,* 1993).

Interactions between the peripheral nervous system and the healing skeleton are poorly understood (Peter *et al.,* 2009). To our knowledge the literatures dealing with the effect of nerve regeneration on healing of the bone fracture are scarce.

This study aims to investigate the effect of sciatic nerve crushing as a model for peripheral nerve regeneration on bone healing in rat tibial shaft fracture.

2.Materials and Methodology

Animals:

Forty five adult male albino rats (weighing 200 – 250 gm and 4 – 5 months old) were housed at room temperature and supplied with standard pellet food with tap water *ad libitum*.

Surgical procedures:

All animals were anaesthetized with an intraperitoneal injection of ketamine and xylazine (30 mg and 5 mg/kg body weight respectively). The Right hind limb was shaved, scrubbed with Betadine Solution, and draped with sterile sheets. A medial incision was made at the knee, the patella was deflected laterally and 21-G sterile needles (0.9 mm in diameter, 2.5 cm in length) were inserted into the intercondylar area of the tibia. The incision was extended down to the mid leg to expose the tibia. Open tibia fracture was inflicted manually on the middle of the bone using bone cutting forceps.

Fifteen rats were selected randomly and subjected to sciatic nerve paralysis (group II). In this group the sciatic nerve was exposed by upward extension of the incision to the mid-thigh. The muscles were separated in order to expose the nerve. One cm of the nerve was resected (Madsen *et al.,* 1998).

Another fifteen rats were selected randomly and subjected to sciatic nerve crush (group III). The nerve was crushed with a 1 mm wide non-serrated hemostatic clamp at a standardized force at mid-thight level for 30 seconds. The remaining fifteen rats were considered as the control group (group I).

The incisions in all rats were closed with absorbable sutures and local antibiotic is applied. Good longitudinal alignment of the intramedullary needle, the type of the fracture and the position of the intramedullary needle were determined radiographically at the end of the experiments (Aro *et al.,* 1981).

Four animals showed unsuitable position of intramedullary needle and six showed comminuted factures. These ten animals were excluded and immediately replaced by others.

After recovery from anesthesia, completeness of the crush was established by examining the loss of sensory and motor function in the operated limb. Digits in the operated limb were pinched using a blunt forceps. Absence of foot withdrawal and vocalization were noted as loss of sensory and motor function.

Radiology:

Animals were anesthetized, and fixed in supine position at their fore limbs and hind limbs on a surgical board with the two hind limbs completely abducted. One week, two weeks and four weeks postsurgury, radiographs were taken and the fracture healing of each specimen was graded for callus maturity by an independent radiologist who were blinded to the identity of the specimens using the Lane-Sandhu Scoring System (Lane and Sandhu 1987) as follows: 0 - no callus ; 1 minimal callus formation; 2 – callus evident but healing incomplete; 3 – callus evident and stability expected (cilinically healed); 4- complete healing with complete bone remodeling. The mean radiographic score were calculated for each treatment group.

Histology:

Five animals from each group were killed 1, 2 & 4 weeks after operation. The specimens, which include the fracture line, were excised as en bloc. The internal fixation needles were removed. They were fixed in 10% neutral buffered formaline for 24 hrs and then decalcified in 10% formic acid solution at room temperature for 10 days. The decalcification solutions were changed every 3 days. Longitudinal sections of the tibia including the fracture area with intact external callus were histologically examined by Malloy Trichrome stain. Mallory staining was chosen because the staining of collagen in the demineralized sections provided an accurate representation of the fracture callus bony tissues.

Morphometric assessment:

Radiographs of the fractured tibias were assessed morphometrically. Five measurements proximal and distal to the fracture line were sampled along the longitudinal axis of the bone at 0.25, 0.5, 0.75, 1.0 and 1.25 mm from the fracture itself. The measurements were taken from the lateral side of the intramedullary needle to the outer bridging shell. Morphometric measurements were taken using an Image Analysis system (Leica Q Win standard, digital camera CH-9435 DFC 290, Germany)

Data were analyzed using Statistical Analysis System package (SAS; version 12). The mean callus lateral thickness in the studied three groups by time was compared using one way ANOVA test. *P* value was considered significant at < 0.05 level.

3.Results

There were no bacterial deep wound infection in any of the rats that underwent the procedure. No rat died after the end of the experiment.

Histology

Examination of paraffin sections in the fractured tibia of the control rats revealed the presence of collagenous fibers and irregularly deposited bone matrix 1 week after facture induction

(Fig.1). Degenerated chondrocytes and newly synthesized fibers of cancellous bone were detected at 2 weeks. (Fig.2). Thereafter, regular bone matrix with regular chondrocytes and few osteocytes were noticed 4 weeks after induction of the fracture. (Fig.3).

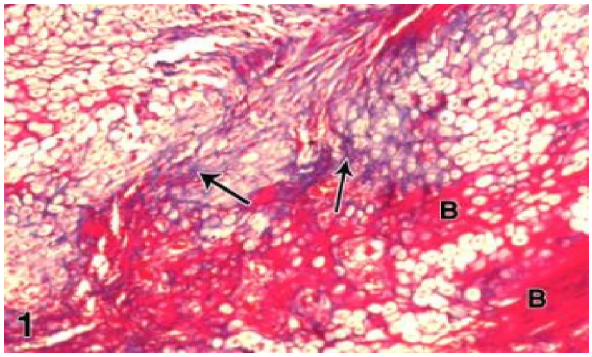

Fig. (1):A photomicrograph of a paraffin section of the control group, group I, showing the fracture site one week after induction, showing collagenous fibers (arrow) and irregularly deposited bone matrix (B). (Mallory trichrome X 250)

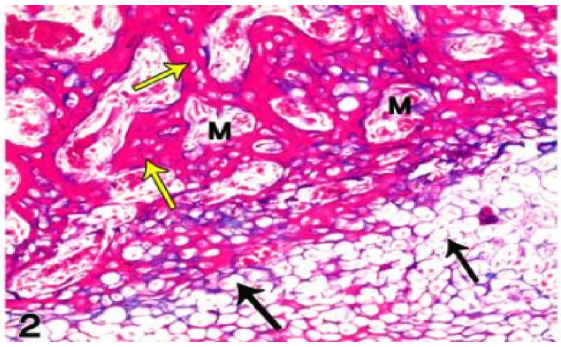

Fig. (2):A paraffin section of the control (group I), 2 weeks after induction of the tibial fracture showing degenerated chondrocytes (black arrow) and newly synthesized bars of cancellous bone (yellow arrow) separated by marrow spaces (M). (Mallory trichrome X 250)

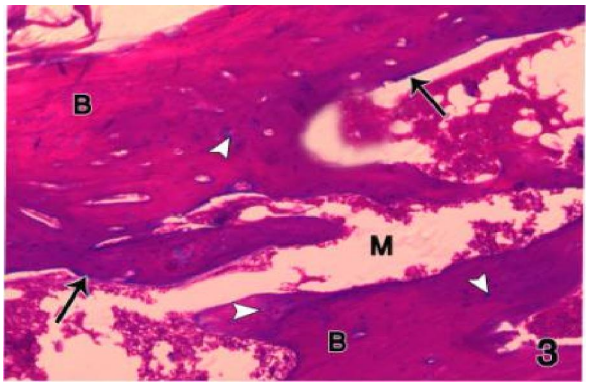

Fig. (3):A paraffin section of the control (group I), 4 weeks after induction of the tibial fracture demonstrating regularly deposited bone matrix (B) with few osteocytes (white arrow heads), regular endosteum (arrows) and medullary cavity (M) are seen. (Mallory trichrome X 400)

Tibial fracture in animals subjected to sciatic nerve cutting (Group II) exhibited markedly irregular bone bars with relatively numerous osteocytes one week after fracture induction. (Fig. 4). One week after, markedly irregular bone bars with numerous osteocytes and heavily collagenous deposition were observed. (Fig. 5). The compact bone formed after 4 weeks was irregular and thin with numerous osteocytes. (Fig.6).

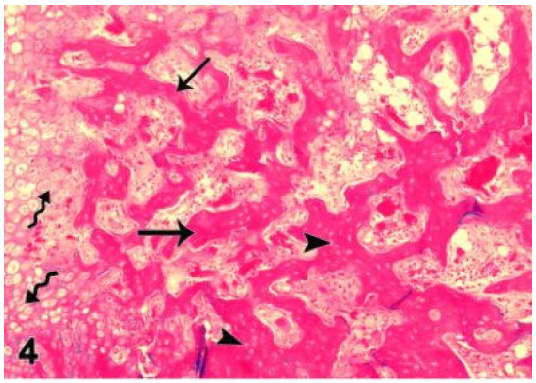

Fig. (4):A paraffin section in the site of the tibial fracture of group (II) one week after sciatic nerve cutting exhibiting markedly irregular bone bars (arrow) with relatively numerous osteocytes (arrow head). The curved arrows point to degenerated chondrocytes. Compare with fig.1. (Mallory trichrome X 250)

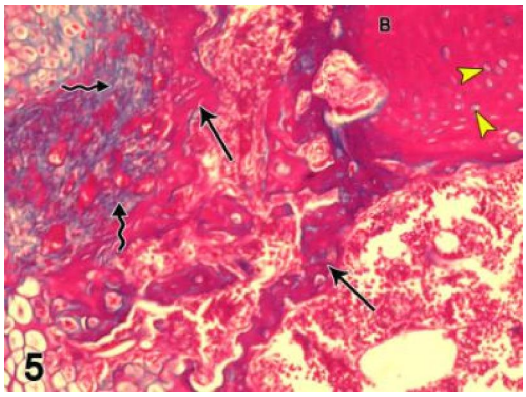

Fig. (5):The site of tibial fracture in group (II) rat, with cut sciatic nerve, two weeks after induction of fracture exhibiting markedly irregular bone bars (arrow) and a sheet of compact bone (B) with numerous osteocytes (yellow arrow heads). The curved arrows point to the heavy collagen deposition. Compare with fig. 2. (Mallory trichrome X 400)

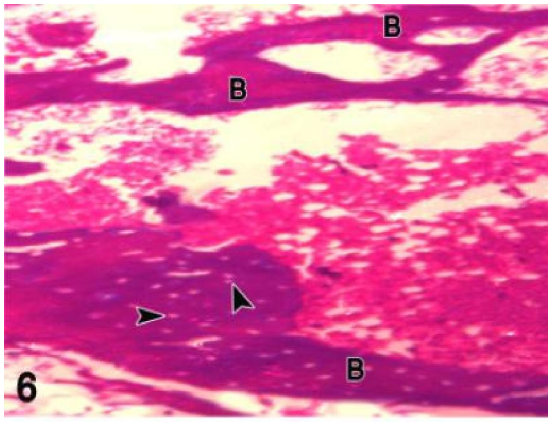

Fig. (6):The healed tibial fracture in group (II) rat, with cut of the sciatic nerve four weeks after induction of facture showing thin and irregular compact bone (B) with numerous osteocytes (arrow head), compare with fig. (3). (Mallory trichrome X 250)

Induced tibial fracture associated with crushing of the sciatic nerve (Group III) resulted in heavy collagen deposition and irregularly synthesized bone bars after 1 week (Fig. 7). One week after, irregular bone with numerous osteocytes and irregular collagen deposition were manifested. (Fig. 8). Thereafter, the final compact bone was regular, with typical Haversian canals and few osteocytes, 4 weeks after induction of the fractures. (Fig.9).

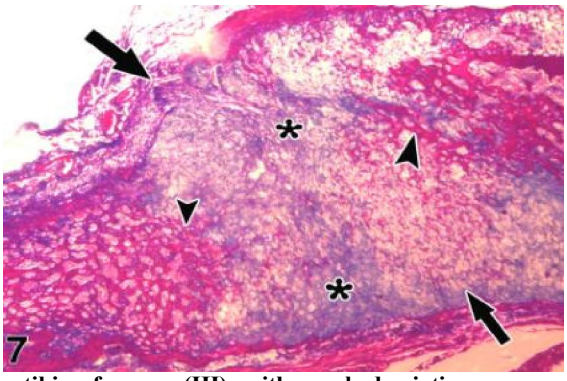

Fig. (7):A paraffin section in the tibia of group (III), with crushed sciatic nerve, one week after fracture induction, demonstrating the site of induced fracture (thick arrows). Heavy collagen deposition (asterisk) and irregularly synthesized bars of cancellous bone (arrow heads) are shown, compared with figs 1 and 4. (Mallory trichrome X 100)

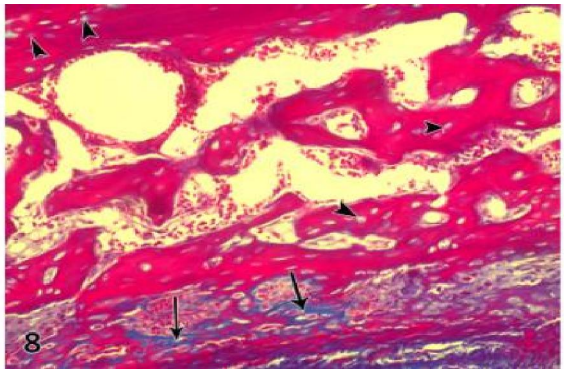

Fig. (8):The site of tibial fracture in group (III), 2 weeks after induction of fracture showing irregular bone with numerous osteocytes (arrow head) and irregular collagen deposition (arrows). (Mallory trichrome X 400)

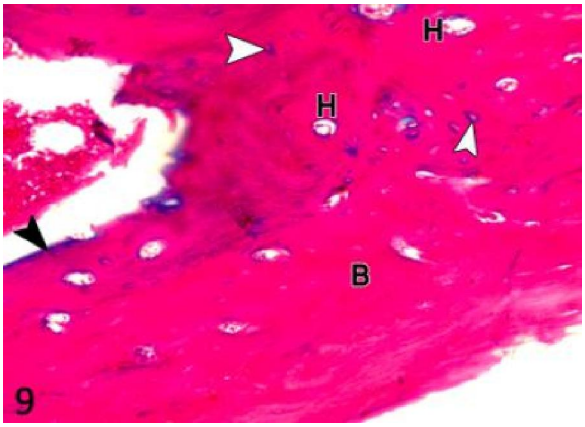

Fig. (9):Four weeks after induction of fracture in group (III) rats demonstrating a regularly deposited bone matrix (B) with Haversian canals (H) and few osteocytes (white arrow heads). The black arrow head refers to the regular endosteum. Compare with figs. 3 and 4. (Mallory trichrome X 400)

Radiology

One week after operation, radiological examination of the three groups showed clear fracture line and soft tissue swellings.

Two weeks after fracture induction, group I showed minimal amount of bridging callus and evidence of ossification. These changes were more evident in groups II and III.

After the 4th week, remodeling of the factures varies between the examined groups and was noted to be better in group III. No fracture gabs were observed in the three groups and complete union of the bones was observed, the amount of bridging callus appeared more in group II. In group II and III the fracture line disappeared completely at the majority of the examined animals. (Fig. 10).

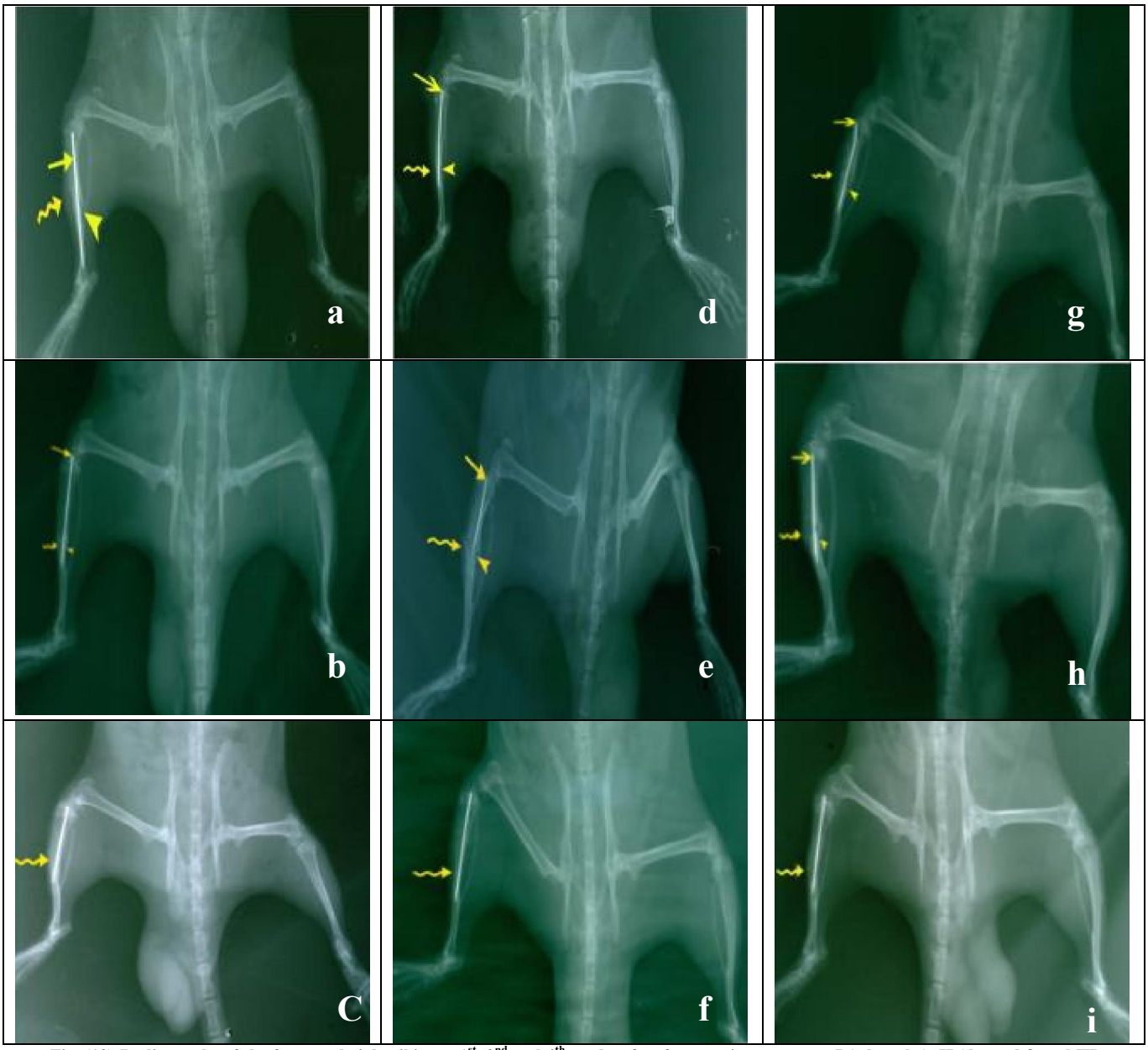

Fig. (10):Radiographs of the fractured right tibias at 1st, 2nd and 4th weeks after fracture in rat groups I (a,b and c), II (d,e and f) and III (g,h and i) showing the site of fracture (arrow head), the intermedullary needle(arrow) and the calluses (curved arrow).

Radiological scoring of callus maturity is shown in table 1. According to Lane-Sandhu Scoring System, the differences inside the same groups were significant at different times. At the second and fourth weeks the fracture healing score was low in group I, high in group II and highest in group III. There was a statistically significant deference between the groups (p= 0.008 and 0.01 respectively).

Table 1: Results of radiological scores were cited as median (min-max) value. A *p* value <0.05 was regarded as statistically significant.

Radiological score	1 week Median (Min-max)	2 week Median (Min-max)	4 week Median (Min-max)	*P*
Group I	1 (1,1)	1 (1,2)	3 (3,4)	<0.0001
Group II	1 (1,1)	2 (1,2)	3.5 (3,4)	<0.0001
Group III	1 (1,1)	2 (1,2)	4 (3,4)	<0.0001
P	1.0	0.008	0.01	

Morphometric assessment:

Mean callus lateral thickness in the studied groups by time is shown in table 2. In the first week, there is no statistically significant difference (p= 0.06) among all the studied groups. In the 2nd week, there is a statistically significant difference between 2nd group and both 1st and 3rd groups with the highest callus lateral thickness in the 2nd group (252.2 ± 16.5). In the 4th week, the callus lateral thickness is highest in group II (194.2 ± 23.7) compared with group I and group III with statistically significant difference ($p < .0001$).

Table 2: shows the comparison of mean callus lateral thickness in the studied three groups in the first, second and third weeks in micrometer..

	Week 1	Week 2	Week 4	*P* value
Group I	13.72 ± 2.5	128.8 ± 6.9	54.8 ± 7.1	<.0001*
Group II	10.4 ±0.58	252.2 ± 16.5	194.2 ± 23.7	<.0001*
Group III	11.4 ± 0.37	156.2 ± 21.6	51.2 ± 8.9	<.0001*
P value	0.06	<.0001*	<.0001*	

*Significant difference.

4.Discussion

When a bone is fractured, bone matrix is destroyed and bone cells adjoining the fracture die. The damaged blood vessels produce a localized hemorrhage and form a blood clot. During repair, the blood clot, cells and damaged bone matrix are removed by macrophages. The periosteum and endosteum around the fracture respond with intense proliferation producing a tissue that surrounds the fracture and penetrates between the extremities of the fractured bone (Einhorn, 1998 and Junqueira and Carneiro, 2005). Primary bone is then formed by endochondoral and intramembranous ossification, both processes contributing simultaneously to the healing of fracture.

Repair progresses in such a way that irregularly formed trabeculae of primary bone temporarily unite the extremities of the fracture bone, forming a bone callus (Einhorn *et al.,* 1995 and Junqueira and Carneiro, 2005).

Stresses imposed on the bone during repair serve to remodel the bone callus. If these stresses are identical to those that occurred during growth of the bone, and therefore influence its structure, the primary bone tissue in the callus is gradually resorbed and replaced by secondary tissue, remodeling the bone and restoring its original structure. Unlike other types of connective tissue, bone tissue heals without forming scar (Gerstenfeld *et al.,* 2003 and Junqueira and Carneiro, 2005).

It has been reported that intact innervation is essential for normal fracture healing because nerve injury induces a large, but mechanically insufficient, fracture callus (Madsen *et al.,* 1998).

It was also proven that nerves have extensive and decisive effects on the development, formation and metabolism of bone tissues (Shang *et al.,* 2001).

In the current study, both cutting and crushing of the sciatic nerve resulted early in the formation of irregular bone bars associated with numerous osteocytes and heavy collagen deposition. Thereafter, variations in the histological picture was noted between the two groups; the compact formed in group III after 4 weeks was regular with typical Haversian system and few osteocytes. However, cutting the nerve in group II led to the formation of thin and irregular compact bone with numerous osteocytes. These findings came in accordance with the results of previous papers of Aro *et al.,* 1981 and Madsen *et al.,* 1989. They reported that nerve resection produced a remarkable increase in the length of callus in experimentally-induced tibial fractures.

Radiologically, remodeling of the fractures varied between the examined groups. The amount of the bridging callus appeared much more evident in group II, 4 weeks after induction of the fracture. Meanwhile, remodeling and radiological scoring of callus maturity were highest in the animals of group III suggested to crushing the nerve as compared with the other two groups. Similar data was previously demonstrated by Aro *et al.* (1981). They mentioned that osseous union of the tibial fractures after nerve resection was radiologically evident or at least the same time with the control.

Certainly, we can't decide that calluses of the regenerated rats are more mechanically strong, as we didn't examine the mechanical strength of the formed callus in the three groups. It was previously noticed that mechanical strength of the formed callus after nerve resection was insufficient (Madsen *et al.,* 1998 and Lyrits and Boscainos, 2001). In addiction they concluded that the calluses formed after nerve resection were larger in size but mechanically weaker, indicating a defection either tissue composition or organization.

However, caution must be exercised while applying these data on human beings as the biological response differs from human to rat. These results suggest that intact innervations is essential for normal fracture healing because nerve cutting induced a large but irregular fracture callus and that deinnervation followed by reinnervation showed faster and more regular healing than the deinnervated group.

The question now is how to find an applicable technique resembling nerve regeneration

in patients with bone fracture to have rapid and regular callus. These results may be of significance to clinicians and researchers who are seeking to improve fracture-healing in patients with associated nerve injury as spinal cord trauma.

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