Guinea pigs as an animal model for sciatic nerve injury

Malik Abu Rafee1, Amarpal1,*, Prakash Kinjavdekar1, Hari Prasad Aithal1, Sajad Ahmad Wani2, Irfan Ahmad Bhat1
1 Division of Surgery, Indian Veterinary Research Institute, Izatnagar, Bareilly, UP, India
2 Division of Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar, Bareilly, UP, India


Abstract
The overwhelming use of rat models in nerve regeneration studies is likely to induce skewness in treatment outcomes. To address the problem, this study was conducted in 8 adult guinea pigs of either sex to investigate the suitability of guinea pig as an alternative model for nerve regeneration studies. A crush injury was inflicted to the sciatic nerve of the left limb, which led to significant decrease in the pain perception and neurorecovery up to the 4th week. Lengthening of foot print and shortening of toe spread were observed in the paw after nerve injury. A 3.49 ± 0.35 fold increase in expression of neuropilin 1 (NRP1) gene and 2.09 ± 0.51 fold increase in neuropilin 2 (NRP2) gene were recorded 1 week after nerve injury as compared to the normal nerve. Ratios of gastrocnemius muscle weight and volume of the experimental limb to control limb showed more than 50% decrease on the 30th day. Histopathologically, vacuolated appearance of the nerve was observed with presence of degenerated myelin debris in digestion chambers. Gastrocnemius muscle also showed degenerative changes. Scanning electron microscopy revealed loose and rough arrangement of connective tissue fibrils and presence of large spherical globules in crushed sciatic nerve. The findings suggest that guinea pigs could be used as an alternative animal model for nerve regeneration studies and might be preferred over rats due to their cooperative nature while recording different parameters.

Key Words: nerve regeneration; Guinea pigs; animal model; sciatic nerve injury; foot print length; neuropilin; histopathology; neural regeneration

Introduction
Sciatic nerve injury is the most frequently encountered peripheral nerve injury. Different animal models have been established to study the regenerative capacity of the peripheral nerve using various therapeutic agents (Ranjan et al., 2015). In rats, sciatic nerve crush injury model has been established to study various aspects of nerve regeneration (Algora et al., 1996; Daglioglu et al., 2010; Park et al., 2011; Raducan et al., 2013; Tamaddonfard et al., 2013; Tan et al., 2013). Induced sciatic nerve injury is manifested in limb weakness, altered gait and lengthening of foot prints (Khan et al., 2014). Foot prints are measured one day before and then at weekly interval after induction of the nerve injury for calculation of sciatic functional index (Santiago et al., 2009; Daglioglu et al., 2010; Raducan et al., 2013; Tamaddonfard et al., 2013). Extent of gastrocnemius muscle atrophy, measured as reduction in the ratio of muscle weight and/or volume of the gastrocnemius muscle of the experimental leg versus the control leg, has been used as an indirect indicator of the nerve injury (Santiago et al., 2009). Histopathological changes of sciatic nerve by light microscopy (Algora et al., 1996; Daglioglu et al., 2010; Park et al., 2011; Tamaddonfard et al., 2013; Tan et al., 2013; Bansode et al., 2014) and changes under scanning electron microscopic (SEM) examination (Tan et al., 2013) are the other important parameters studied to describe the changes following the injury and regeneration of sciatic nerve.

Rat and mice are the most common animal species used for models of nerve regeneration studies. Hilton et al. (2015) found a total of 284 in vivo studies on nerve regeneration, 222 were in rats and mice (78 %), 34 in rabbits (12 %), 11 in dogs, 6 in monkeys, 4 in sheep, 4 in cats and 3 in pigs. They considered that rats and mice are not the best models for the evolving complexities faced in designing nerve repair strategies. The preponderance of nerve regeneration data in a single species due to the overwhelming use of rat models in nerve regeneration is likely to skew treatment outcomes and leads to inappropriate evaluation of the risks and benefits.

Guinea pig is a popular laboratory animal and can be used as an alternative animal model in studies on sciatic nerve injury. Guinea pig being more docile in nature and easy to handle as compared to rats and mice can allow easy gait analysis and functional evaluation as well as recording of clinical parameters. In this study, we attempted to determine whether guinea pig can be an alternative to rat and mouse models for nerve degeneration/regeneration studies based on clinical parameters like pain perception and neurological recovery, histopathological and ultrastructural changes in nerve and associated muscle and changes in expression of certain genes.
Materials and Methods

Animals
Eight healthy guinea pigs (cavia porcellus) of either sex were used in this study. All the animals were provided with the standard diet, ad libitum water and allowed to acclimatize for approaching, handling and animal house conditions for a period of 10 days before initiation of the study. The study was approved by Institute Animal Ethics Committee of the Indian Veterinary Research Institute Vide order No.F.26-1/2015-16/J.D(R), dated 18th January, 2016.

Before induction of the injury, each animal was subjected to superficial and deep pain in order to assess the integrity of pain pathways and their response was graded using a 1–3 score scale (Tarlov and Klinger, 1954; Table 1). Similarly, neurological recovery score was recorded using ordinal scale of Tarlov and Klinger (1954) with slight modifications (Table 1). The total neurological recovery score was obtained by summing up pain perception and neurological recovery scores. The animals showing total neurological recovery score of 7 were assumed to have normal neurological function of the sciatic nerve and were included in the study.

Surgical intervention/procedure
The animals were anesthetized by intramuscular injections of xylazine (6 mg/kg) and ketamine (60 mg/kg) in the thigh muscles as per standard protocol. Left thigh region was prepared for aseptic surgery and the animal was secured in right lateral recumbency. A linear skin incision of 3–4 cm length was made at the caudo-lateral surface of the animal’s left thigh and blunt dissection was performed to separate the biceps femoris and semitendinous muscle to expose the sciatic nerve. Few drops of local anaesthetic (Lignocaine 2%) were instilled over the nerve to provide local analgesia. Five minutes later, the nerve was subjected to crush injury with the help of a curved hemostatic forceps (jaw width 3 mm). The strength used for compression was standardized at the second locking position of the hemostatic forceps (MICROSD INDIA) for 60 seconds. The site of the crush injury was the intermediate region of the sciatic nerve in its course down the thigh region before bifurcation into the tibial and peroneal nerves. The muscle and skin were sutured in standard manner. Postoperatively, all the animals were housed in individual boxes and administered with broad spectrum antibiotic enrofloxacin for 5 consecutive days. The animals were then euthanized and crushed nerve and the gastrocnemius muscle of experimental (left) and control limbs (right) of guinea pigs. Of xylazine (6 mg/kg) and ketamine (60 mg/kg) in the thigh muscles as per standard protocol. Left thigh region was prepared for aseptic surgery and the animal was secured in right lateral recumbency. A linear skin incision of 3–4 cm length was made at the caudo-lateral surface of the animal’s left thigh and blunt dissection was performed to separate the biceps femoris and semitendinous muscle to expose the sciatic nerve. Few drops of local anaesthetic (Lignocaine 2%) were instilled over the nerve to provide local analgesia. Five minutes later, the nerve was subjected to crush injury with the help of a curved hemostatic forceps (jaw width 3 mm). The strength used for compression was standardized at the second locking position of the hemostatic forceps (MICROSD INDIA) for 60 seconds. The site of the crush injury was the intermediate region of the sciatic nerve in its course down the thigh region before bifurcation into the tibial and peroneal nerves. The muscle and skin were sutured in standard manner. Postoperatively, all the animals were housed in individual boxes and administered with broad spectrum antibiotic enrofloxacin for 5 consecutive days. The animals were then euthanized and crushed nerve and the gastrocnemius muscle of experimental (left) and control limbs (right) of guinea pigs.
Histopathology of nerve and muscle samples
The samples collected were stored in 10% formalin to evaluate the microstructural changes. The nerve sample from the fourth animal was stored in 2.5% glutaraldehyde to observe ultrastructural changes using scanning electron microscopy. Two of the formalin fixed muscle and nerve samples were processed by paraffin embedding and cut into 4–5 μm thick sections and stained with hematoxylin and eosin. The third formalin fixed nerve sample was processed for Marchi’s technique (Marchi, 1886) for assessment of myelin degeneration in one sample. For this purpose, the formalin fixed specimen was post-chromed for 5 days in 3% potassium dichromate and then transferred to Marchi’s fluid for 15 days. Thereafter, the specimen was processed by paraffin embedding and cut into 4–5 μm thick slices. One slide was subjected to hematoxylin-eosin staining and the other observed as such for histopathological changes.

Scanning electron microscopy
The nerve sample preserved in 2.5% glutaraldehyde was post-

Table 1 Pain and neurorecovery scores

<table>
<thead>
<tr>
<th>Score</th>
<th>Pain perception scores</th>
<th>Neurorecovery scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>No reaction to forceful compression (absence of deep pain)</td>
<td>Complete paralysis: complete loss of sensation below the stifle joint except medial aspect</td>
</tr>
<tr>
<td>2</td>
<td>Mild reaction to forceful compression of the toe (absence of superficial pain but presence of deep pain)</td>
<td>Lifted animal shows extension of the hock joint, absence of web paw. While walking/sitting, there is lateral deviation/abduction of the limb, knuckling of the paw</td>
</tr>
<tr>
<td>3</td>
<td>Strong reaction to light compression (superficial pain intact)</td>
<td>Lifted animal shows angulations (flexion) of the hock joint, web paw. While walking/sitting, there is slight lateral deviation/abduction, slight knuckling of the paw</td>
</tr>
<tr>
<td>4</td>
<td>Normal positioning of the limb while walking/sitting</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Primers used for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence (5’–3’)</th>
<th>Accession number</th>
<th>Amplicon size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NRP-1</td>
<td>Forward</td>
<td>GAA TCT ACC CTG AGC GAG CC</td>
<td>XM_005003202.2</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAT TGG GAG TCG GTC CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRP-2</td>
<td>Forward</td>
<td>GGA GGT CGC CTG AAT TCC AA</td>
<td>XM_005005763.2</td>
<td>167</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AAC CTT AAG TCC GCT GGA GG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-actin</td>
<td>Forward</td>
<td>ACT CCT CCA CAG AGG GC</td>
<td>NM_001172909.1</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGT TGG GGG ACA AAA AGG GG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3 Scores of pain perception, neurorecovery, and total neurological recovery

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 1</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
<th>Day 28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain</td>
<td>3.00±.00</td>
<td>1.00±.38</td>
<td>1.00±.54</td>
<td>2.00±.54</td>
<td>2.00±.00</td>
<td>2.00±.00</td>
</tr>
<tr>
<td>Neurorecovery</td>
<td>4.00±.00</td>
<td>1.00±.38</td>
<td>1.00±.38</td>
<td>1.00±.38</td>
<td>2.00±.49</td>
<td>2.00±.49</td>
</tr>
<tr>
<td>Total neurological recovery</td>
<td>7.00±.00</td>
<td>2.00±.49</td>
<td>3.00±.76</td>
<td>3.00±.76</td>
<td>4.00±.49</td>
<td>4.00±.49</td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD. *P < 0.05, vs. day 0.

Table 4 NRPI1 and NRPI2 gene expression, print length, toe spread, and ratios of gastrocnemius muscle weight and volume of experimental (injured) limb to control (normal) limb

<table>
<thead>
<tr>
<th></th>
<th>Normal side</th>
<th>Injured side</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sciatic nerve</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRPI gene expression</td>
<td>–0.99±.33</td>
<td>–2.79±1.02</td>
</tr>
<tr>
<td>NRPI2 gene expression</td>
<td>–2.34±.30</td>
<td>–3.40±1.60</td>
</tr>
<tr>
<td>Limb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Print length (cm)</td>
<td>3.195±0.115</td>
<td>3.653±0.306</td>
</tr>
<tr>
<td>Toe spread (cm)</td>
<td>1.718±0.164</td>
<td>0.921±0.187</td>
</tr>
<tr>
<td>Ratio of gastrocnemius muscle weight of experimental limb to control limb</td>
<td>0.369±0.027</td>
<td></td>
</tr>
<tr>
<td>Ratio of gastrocnemius muscle volume of experimental limb to control limb</td>
<td>0.384±0.105</td>
<td></td>
</tr>
</tbody>
</table>

Data are expressed as the mean ± SD (n = 4). *P < 0.05, **P = 0.01, vs. normal side. NRPI: Neuropilin.
fixed in 2% OsO₄, washed with distilled water and then dehydrated through a graded series of ethanol from 30%, 50%, 70%, 90% to 100%. The dehydrated specimen was critical point dried and mounted on aluminium stub in longitudinal orientation, using adhesive silicon tape, sputter coated with Au-Pd (80:20) and then visualized using FEI Quanta 250 scanning electron microscope (Houston, TX, USA).

**Statistical analysis**
All data were statistically analyzed using SPSS 16.0 software (SPSS, Chicago, IL, USA) with one-way analysis of variance and expressed as the mean ± SD. P < 0.05 was considered statistically significant.

**Results**

**Clinical observations**
Before the injury, pain pathways were fully intact for both superficial and deep pain. 24 hours after the injury, the score of pain decreased significantly from the normal and it remained significantly decreased until the 4th week, though there was some improvement in the score from the 2nd week onwards (Table 3). The similar pattern was observed in neurological recovery and total neurological recovery score. The animals were cooperative while recording the parameters.

**Quantitative real-time PCR (changes in gene expression)**
Cycle threshold (Ct) values of target genes were normalized to internal reference β-actin. Normalized gene expression data from biological replicates were averaged and calibrated against the normal nerve; \( \Delta \Delta Ct = [(Ct_{target} - Ct_{internal control})_{sample} - (Ct_{target} - Ct_{internal control})_{calibrator}] \). Expression of NRP1 was significantly increased in the tested nerve (\( \Delta \Delta Ct = -2.79 \pm 1.02 \)) as compared to its expression in the normal nerve (\( \Delta \Delta Ct = -0.99 \pm 0.33 \)). The expression of NRP2 gene was appreciable but statistically non-significantly higher in the tested nerve (\( \Delta \Delta Ct = -3.40 \pm 1.6 \)) as compared to its expression in the normal nerve (\( \Delta \Delta Ct = -2.34 \pm 0.30 \) (Table 4). There was 3.49 ± 0.35 fold increase in NRP1 gene and 2.09 ± 0.51 fold increase in NRP2 gene 1 week after the nerve injury as compared to the normal nerve.

**Changes in weight and volume of gastrocnemius muscle**
Grossly the mass of the normal gastrocnemius muscle appeared greater than the muscle of the test limb on the 30th day (Figure 1). Ratios of gastrocnemius muscle weight and volume of experimental limb to control limb 30 days after sciatic nerve injury are shown in Table 4.

**PL and TS**
Lengthening of foot print and shortening of toe spread were observed in guinea pigs after injury to the sciatic nerve. Data of PL of the paw in right (normal) and left (experimental) limbs are shown in Table 4. The difference was significant (\( P < 0.05 \)). TS in the right (normal) and left (experimental) limb are shown in Table 4. The difference was highly significant (\( P = 0.01 \)).

**Histopathological changes in nerve and muscle**
Vacuolated appearance of the nerve was observed. Different types of vacuoles ranging from vacuolated foci-containing eosinophilic material and associated with a distorted cell nucleus to larger, multilocular, linear array of compartmentalized myelin debris (digestion chambers) (Figure 2B) were observed. Degenerated myelin stained black after Marchi’s staining, whereas normal myelin stained light brown. The degenerated myelin was found as aggregates/deposits (Figure 2C). Combining Marchi’s staining and hematoxylin and eosin staining procedures revealed the presence of degenerated myelin deposits in digestion chambers (Figure 2D). Hematoxylin and eosin stained sections of gastrocnemius muscle samples showed absence of striations, multinucleated myofiber and degenerative changes (Figure 3B). In longitudinal sections, the muscle fibers were shirked, broken and wiggly. Areas of myofibril fragmentation and rupture (called retraction caps) were observed. Eosinophilic homogenous, hypercoagulated myofibers (hyalinization) indicating degeneration was also observed. Spaces between the muscle bundles were widened. In crosssections, angular (triangular) and shirked bundles were observed. Hyalinization of the muscle bundles was also evident.

**Ultra-structural changes in the nerve**
Scanning electron microscopy revealed loose and rough arrangement of connective tissue fibrils and presence of large spherical globules in the degenerating fibers oriented along the major axis of the nerve on day 30 (Figure 4).

**Discussion**
In an earlier study, Park et al. (2011) showed that the expression of neuronal markers was increased 1 week after nerve injury. The same was observed in this study with regard to the expression of NRP1 and NRP2 genes. NRP2 and NRP1 are members of a well-documented class of receptors that respond to ligands, termed semaphorins, of which there are a number of subclasses (Rore and Piischel, 2003). NRP1 and NRP2 each responds to selective members of subclass-3 semaphorins, secreted proteins that guide migration of axonal growth cones to their appropriate target tissue during development (Kitsukawa et al., 1997; Kolodkin et al., 1997; Bagnard et al., 2000). A marked induction, at the messenger ribonucleic acid (mRNA) level, of NRP2 in Schwann cells within the crush site and distal stumps of crushed rodent nerves has been reported earlier (Scariato et al., 2003; Ara et al., 2004). Locomotion is a well co-ordinated event that requires precise timing (speed) of impulse conduction to get meaningful movements. Demyelination by means of crush injury may alter this normal coordination as action potential could be delayed and movements become uncoordinated (Zachary, 2007). Impaired coordinated movements and decreased pain perception and decreased recovery scores noticed in the present study might be attributed to demyelination and delayed action potential caused by crush injury.

The ratios of gastrocnemius muscle weight and volume of experimental limb to control limb indicate more than 50% weight and volume loss 30 days after nerve injury. Previous
studies also reported significant reduction in the muscle weight in rats after nerve injury (Higashino et al., 2013). Khan et al. (2014) reported lengthening of foot prints in rats after nerve injury. Bain et al. (1989) reported increased PL and decreased TS in rats after complete sciatic nerve lesion. Similar findings were noticed in guinea pigs after sciatic nerve injury in the present study. Previous studies have observed that TS represents the major alteration after sciatic nerve injury and may be used alone as an indicative of the sciatic nerve injury stage (Walker et al., 1994; Bervar, 2000; Baptista et al., 2007).

Histology is a traditional method to evaluate degeneration/regeneration of the nerves. The response of the peripheral nervous system following injury varies with the cause and extent of the injury, and different pathological changes depend on the degree of injury. After sciatic nerve injury, Wallerian degeneration occurs in the distal stump consisting of a series of processes, including axonal degeneration, myelin degeneration and disintegration, Schwann cell proliferation, infiltration of macrophages and mast cells, and axonal and myelin debris clearance (Dubovy, 2011). In the present study, histological examination of regenerated nerves was done by conventional hematoxylin-eosin staining, Marchi’s staining and combined Marchi’s and hematoxylin-eosin staining protocols. Hematoxylin-eosin staining of nerve revealed the presence of vacuoles, gitter (foamy) cells and digestion chambers indicating phagocytosis and lysosomal degradation of myelin by Schwann cells and/or haematogenous macrophages. These different types of vacuoles gave the tissue a vacuolated appearance. The degenerated myelin was found as black aggregates/deposits after Marchi’s staining. These black aggregates were found in digestion chambers and vacuoles on pre-treated samples with Marchi’s procedure followed by hematoxylin-eosin staining procedure. Similar changes have been observed in rat nerve injury models (Holtzman and Novikoff, 1965; Amniattalab et al., 2010; Khan et al., 2014; Gomez-Sanchez et al., 2015). The gastrocnemius muscle is supplied by the sciatic nerve and crush injury would lead to quick atrophy of the muscle. Atrophic changes in gastrocnemius muscle could be correlated with the degeneration in sciatic nerve. Similar histopathological changes have been observed in rats after nerve injury (Higashino et al., 2013).

Electron microscopy enables analysis of the ultra-structural events associated with Wallerian degeneration and peripheral nerve regeneration (Ohmi, 1961). The normal myelinated axons are seen as relatively smooth cylindrical structures which interweave with strands of collagen and elastic fibers that course over their length. The axons vary in diameter and are closely packed within the nerve bundles. After degen-
eration, the myelin sheaths of nerve fibers are distorted and swollen with numerous cytoplasmic bulges occurring along the length of the degenerating axons. The amount of the connective tissue covering the damaged fibers was increased due to which the axons have lost their smooth appearance. Many degenerating fibers began to fragment into large spherical globules which were oriented in a linear fashion along the major axis of the nerve. Similar changes have been observed in rat nerve injury model (Gershenbaum and Roisen, 1978).

The functional, macroscopic, microscopic and ultrastructural changes observed in sciatic nerve and gastrocnemius muscle changes in guinea pigs mimic the alterations observed in rat and other species following sciatic nerve injury. Further, the assessment of functional parameters like pain perception, neurological recovery and TS and PL was facilitated by cooperative nature and the larger size of the guinea pigs as compared to rats and mice. Taken together, guinea pigs can prove to be an alternative model for sciatic nerve regeneration studies, which can address to the problem of overwhelming use of rat models in nerve regeneration and thereby reduce the skew in treatment outcomes.

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Peer review: This paper was double-blinded and stringently reviewed by international expert reviewers.

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