# Effects of glycine on electrical and histological properties of a rat peripheral nerve injury model

## Glisinin sıçan periferik sinir yaralanması modelinde elektriksel ve histolojik özellikler üzerine etkisi

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***BACKGROUND***

Treatment of peripheral nerve injuries focuses on lesion type, from expectant to interfascicular repair. Many experiments have been undertaken using different factors to facilitate better or faster nerve stump growth: nerve growth factor (NGF), plaque growth factor (PGF), hyaluronic acid, leukemic inhibiting factor, and GABA, etc. Glycine is an inhibitory neurotransmitter in the brain stem and spinal cord, and it also plays a critical role as a modulator of NMDA receptors. We studied the potential regenerative effect of glycine administered for different periods of time and compared results with a control group.

***METHODS***

The sciatic nerve of Wistar rats was exposed and the electrophysiology procedure was performed: the nerve was cut transversally and stitched back in place with four isolated cardinal 9/0 nylon stitches on each end. Study group rats were administered glycine 40 mM/kg daily for 15, 30, and 60 days, while control group rats were medicated with isotonic saline solution 0.9% for the same time periods. At the end of each study time period, the electrophysiological study was repeated. Animals were sacrificed on the 15th, 30th and 60th postoperative day and the sciatic nerve was exposed and prepared for histological studies.

***RESULTS***

According to our results, glycine was effective in the morphologic regeneration and functional recovery of the sciatic nerve post-injury in Wistar rats with one month administration.

***CONCLUSION***

We observed that nerve histology with glycine administration was more similar to that of normal nerves.

***Key Words:*** Animal model; glycine; nerve injury; nerve stump growth; NMDA receptor; peripheral nerve.

***AMAÇ***

Periferik sinir yaralanmalarında tedavi, interfasiküler onarım beklentisiyle lezyon tipine odaklanır. Sinir kesiğinin daha iyi veya daha hızlı büyümesine yardım etmek üzere, bazıları da aşağıda belirtilen değişik faktörle birlikte birçok deney gerçekleştirilmiştir: Sinir büyüme faktörü (NGF), plak büyüme faktörü (PGF) hiyalüronik asit, lösemi inhibitör faktör, GABA, vb. Glisin; beyin sapında ve spinal kordda görev yapan bir inhibitör nörotransmitter olup, aynı zamanda bir NMDA reseptör modülatörü olarak da önemli bir rol oynamaktadır. Glisinin olası rejeneratif etkisi, farklı uygulama zamanlarında araştırıldı ve sonuçları kontrol grubuyla karşılaştırıldı.

***GEREÇ VE YÖNTEM***

Wistar cinsi sıçanların siyatik sinirleri ortaya konuldu ve elektrofizyolojik işlem gerçekleştirildi: Sinir enine olarak kesildi ve kesik kısımlar dört adet izole temel 9/0 naylon dikiş ile yeniden yerlerine dikildi. Çalışma gruplarına 15, 30 ve 60 gün süreyle, günlük 40 mM/kg glisin uygulanırken, kontrol gruplarına aynı zaman zarfında serum fizyolojik verildi. Her çalışma döneminin sonunda elektrofizyolojik çalışma tekrarlandı. Denekler cerrrahi sonrası 15., 30. ve 60. günlerde öldürülerek siyatik sinirleri çıkarıldı ve histolojik çalışmalar için hazırlandı.

***BULGULAR***

Glisin, Wistar sıçanlarındaki deneysel yaralanma sonrasında gerçekleştirilen bir ay süreli uygulama boyunca siyatik sinirin morfolojik rejenerasyonu ve fonksiyonel düzelmesi üzerine etkili oldu.

***SONUÇ***

Glisin uygulaması ile birlikte oluşan sinir histolojisinin normal sinirlerdekine çok benzer olduğunu gözlemledik.

***Anahtar Sözcükler:*** Hayvan modeli; glisin; sinir hasarı; sinir kesiği büyümesi; NMDA reseptörü; periferik sinir.

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In the United States, peripheral nerve injuries are common, occurring in 2.8% of traumatic injuries in general, and 65% occur in the upper limbs, especially in radial nerves; peripheral nerve injuries result in some nervous compromise.[1] When a peripheral nerve is heavily damaged, sensorial fibers produce several impulses that last from seconds to several minutes, which are known as injury discharge.[2]

In nerve reconstruction, treatment focuses on lesion type, from the expectant to the interfascicular repair. Many experiments have been undertaken using different factors to facilitate better or faster nerve stump growth: NGF (nerve growth factor), PGF (plaque growth factor), hyaluronic acid, leukemic inhibiting factor, GABA, and more.[3,4] Growth velocity in the regeneration unit depends on the species -- in rats it is from 2-3.5 mm per day, while in humans, the fastest is between 1 to 2 mm per day with a progressive deceleration when the axon is more distal.[4]

In glycine, peptides $\alpha$ subunit (ligation and adhesion) and $\beta$ subunit (structural) were considered necessary building blocks in order to form the glycine receptor in comparison with the large and peripheral polypeptide known as a structural protein, the gephyrin, which joins receptors to synapses, joining them to the adjacent microtubules.[5,6]

Significant glycine overflow opens the NMDA (N-methyl-D-aspartate) receptor ionic channels that are highly permeable to $Ca^+$, which regulates the extracellular magnesium under voltage influence. In order to be activated, these receptors depend on two agonists: the glutamate and the glycine.[6,7]

The NMDA ionic channel is located in the dorsal horn post-synaptic neurons; therefore, a nervous injury causes important increase in spinal glutamate that opens the NMDA channels, causing an internal $Ca^+$ and $Na^+$ influx, and an increase in post-synaptic cell response.[8,9]

In an injury, when the generation of free radicals is more important than the antioxidant mechanisms, an increase in high cell permeability is induced as well as a decrease in the membrane potential. The effect of glycine over antioxidant enzymes could be due to the block of this amino acid in the activation of Kupffer cells, which are oxygen, nitrogen and cytokine free radical producers, the concentrations of which increase in ischemic/reperfusion injury. This blockade stops the activation of these factors over antioxidant enzymes, with the eventual restoration of normal values in activity as well as in enzymes RNAm.[10]

In animal models of peripheral nerve injury, intrathecal infusion of glycine has been proven to diminish terminal hyperalgesia, which suggests that glycine can reduce neuropathic painful symptoms.[11-13] Other studies report that glycine administration in spinal cord injury rats results in better and faster recovery than in animals without this amino acid intake.[14,15]

## MATERIALS AND METHODS

### Animals

Thirty-five adult male Wistar rats weighing 200-225 g were used: 15 were allocated as study subjects and 15 as controls, and 5 served as the sham group. Rats were housed five per cage in standard laboratory conditions under 12-h light-dark cycle. The rats were given free access to food and water. Animals were treated according to international standards on animal handling (NOM-062-Z00-1999).[16] The procedure was approved by the Ethics and Investigation Committees of the National Institute of Rehabilitation of Mexico City.

Animals were divided into six groups according to the drug administration days:

Group 0: sham operation (n=5);

Groups 1, 2 and 3: peripheral nerve injury without drugs over 15, 30 and 60 days (n=15);

Group 4: peripheral nerve injury and glycine administration for 15 days (n=5);

Group 5: peripheral nerve injury and glycine administration for 30 days (n=5);

Group 6: peripheral nerve injury and glycine administration for 60 days (n=5).

All animals were operated under general anesthesia with a single injection of a mixture of ketamine and xylazine (80 mg/kg and 8 mg/kg body weight) i.p., which was sufficient for the entire procedure. The medial surface of the right and left thigh and hips was routinely prepared by shaving and antisepsis with a 20% iodine alcohol solution, and the operative field was protected with sterile towels. The sciatic nerve was exposed by an anteromedial longitudinal straight incision going from the greater trochanter down to the lateral condyle of the femur,

followed by blunt dissection between the anterior adductor muscles. The entire length of the nerve was made visible but the nerve was only detached from the surrounding soft tissues at the level of its middle third. A surgical microscope set for 40x magnification was used to handle the sciatic nerve during every surgical step. In all groups, the sciatic nerve was left exposed and the electrophysiology procedure was performed; then, the left nerve was cut transversally and stitched back in place with four isolated cardinal 9/0 nylon stitches on either end. The electrophysiology test was repeated to ensure there were no collateral fibers, and then the wound was stitched.

After the anesthetic effect, rats were put back in the case and were fed *ad libitum* until the end of the experiment.

**Drugs**

Study groups (4, 5, 6) (n=5 per group) were administrated glycine (SIGMA) i.p., in a concentration of 40 mM/kg of isotonic solution. It was administered daily between 11:00 am and 12:00 pm according to the drug programmed time for 15, 30, and 60 days, respectively.

Control groups (1, 2, 3) were divided similarly until the end of the programmed time; isotonic saline solution 0.9% i.p. was administered in the same quantity and on the same days as with glycine administration.

**Electrophysiology**

The electrophysiology study was done in rats under the same anesthetic conditions mentioned previously. The register was done before and after the injury to verify there was no conduction through adjacent nerve fibers. The sciatic nerve was exposed completely until it was visible; electrical stimuli were then applied with a single 0.2 ms supra maximal (8 V) pulse through a bipolar electrode. Potentials were recorded from the gastrocnemius muscle with a needle electrode and displayed on a digital storage (model Cadwell 3000).

At the end of the programmed time for each group, rats were anesthetized again and a new electrophysiological register, proximal to the nerve repair, was taken. Only the nerve conduction existence was verified; wave latencies and amplitudes were measured to evaluate if there were significant differences between the control and study groups.

**Pathological evaluation**

Before animals were killed on the 15th, 30th and 60th postoperative day with an intraperitoneal injection of sodium pentobarbital (Anestesal Pfizer®, 120 mg/kg body weight), the sciatic nerve was entirely removed through a posterolateral approach to the thigh with the site of end-to-end repair in the middle portion in nerve specimens that were prepared for histological studies.

Nerve fragments were then dehydrated in an ethyl alcohol aqueous solution of growing concentrations, and embedded in epoxy resin (Poly Bed-812®, Polysciences Inc.). Serial sections were cut with an ultra-microtome (MT 6000-XL, RMC Inc.) from the nerve segments, beginning from the repair site, downwards. The sections were stained with 1% toluidine blue, and examined with a light microscope (Zeiss Axiophoto) equipped with a video camera linked to a microcomputer loaded with the KS 400 Measure Interactive (version 2.0) software.

**Data analysis**

All data are expressed as the means ± SD. The Wilcoxon parametric comparison test was used for statistical analysis of latencies and amplitudes of the same group before and after the injury at the level of significance ($p \leq 0.05$). The Mann-Whitney non-parametric test was preferred for statistical analysis to compare latency and amplitude means of the control and study groups 60 days after surgery using SPSS 10.0 (SPSS Inc., Chicago, IL). The level of significance was set at $*p \leq 0.05$.

## RESULTS

Both anesthetic and operative procedures were well tolerated by all animals, and there were no signs of infection at any time. In Group 0 (sham operation), the right hind paw toes were totally spread, around the second postoperative day, and weight was completely normal. In the other groups (end-to-end repair), the initial flexion contracture of the paw and adduction of toes gradually recovered, as well as the capacity to stand and walk on the paw; however, completely normal weight was never regained, even during the four postoperative weeks.

**Electrophysiological evaluation**

The Wilcoxon test was done to compare amplitude and latency means in groups before and after the peripheral nerve injury, and p values were reported. The test showed that there were no significant

**Table 1.** Wilcoxon test significance

| Group | Amplitude initial (Mean) | Amplitude last (Mean) | p | Latency initial (Mean) | Latency last (Mean) | p |
|---|---|---|---|---|---|---|
| Control 2 months | 4.4 | 6.50 | p≤0.144 | 1.94 | 1.46 | p≤0.285 |
| Control 3 months | 8.75 | 5.50 | p≤0.345 | 2.77 | 1.73 | p≤0.715 |
| Control 5 months | 12.2 | 10.1 | p≤0.053 | 1.25 | 1.33 | p≤1.000 |
| Control 6 months | 15.1 | 12.5 | p≤0.500 | .75 | 1.6 | p≤0.068 |

p≤0.05.

differences between groups. The 15-day group does not appear in the Table because these rats did not present electrophysiological register in this period (Table 1).

The Mann-Whitney test was done to compare the results of amplitudes and latencies between the control and study groups two months after the peripheral nerve injury with and without glycine treatment, and p values of 0.690 for amplitudes and 0.841 for latencies were determined.

**Pathological evaluation**

Fig. 1 shows nerve injury photomicrographs at 15, 30 and 60 days after surgery, with injury and recovery with and without glycine treatment.

Internal nerve morphology was entirely normal in Group 0 (Fig. 1 A, B, C), with typical myelinated fibers of several diameters distributed into a single fascicle.

In Groups 1, 2 and 3 (without glycine), the appearance shows a typical Wallerian degeneration, with advanced axonal loss, myelin remainder, vacuoles and macrophages phagocytizing degenerated myelin and mast cells. At 15 days (Fig. 1 D), there were axonal rests and zones with normal appearance, with macrophages and mast cells. At one month (Fig. 1 E), macrophages had increased, and a zone with great vacuoles and axons with normal appearance was noted. At two months (Fig. 1 F), connective tissue predominated, with an undifferentiated cells nucleus; in some samples, small axons groups could be seen that would probably regenerate.

In the study group (Fig. 1 G, H, I) (with glycine treatment), nerve regeneration was present, and the samples presented a greater quantity of connective tissue and fat tissue. There were more fibers with myelin, with a smaller diameter than in controls and irregular disposition groups. The minor axon density and the presence of fibers with smaller diameter demonstrate that there were sprouts in damaged axons, with different growth velocities, as shown in the pictures with different diameters.

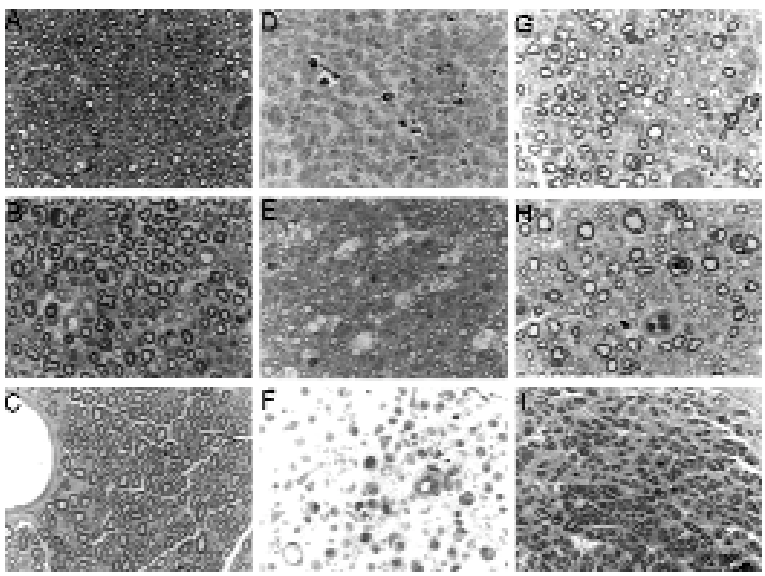


**Fig 1.** Representative photomicrographs of the sciatic nerve.
*(A, B, C) Sciatic nerve in control without injury. (D) Control without treatment 15 days after injury. (E) Control without treatment 1 month after injury. (F) Control without treatment 2 months after injury. (G) A study group rat with glycine treatment 15 days after injury. (H) A study group rat with glycine treatment 1 month after injury. (I) A study group rat with glycine treatment 2 months after injury.*

## DISCUSSION

Electrophysiological results showed that at 15 days after injury and repair, there was no nerve conduction; however, at one and two months after injury and repair in the groups both with and without glycine treatment, conduction was present.

Every peripheral nerve injury is followed by an axon membrane collapse, axoplasm outflow and intense ion

exchange; on the other hand, extracellular ions ($Na^+$, $Ca^+$) enter into the fiber while intracellular ions ($K^+$) leak out; therefore, the axoplasm becomes more positive. Intracellular components such as mitochondrion migrate towards the injury site; those in the proximal segment move distally and those in the distal segment move proximally, that is, from a less positive environment toward a more positive environment.[17,18]

According to our results, quite a morphologic regeneration had occurred one month after an end-to-side nerve repair in the groups without glycine, and this was accompanied by progressive functional recovery, even though this recovery was not yet as complete as that which appeared at two months after injury; however, according to histology, there were great differences between the nerves of rats administered glycine compared to those without glycine treatment. Nerve histology with glycine administration was observed to be more similar to that of the normal nerve.

The findings can be explained by the action of glycine over Kupffer and other white cells, inducing intracellular $Cl^-$ influx and producing a cellular membrane hyperpolarization, preventing the intracellular $Ca^{++}$ increment,[19-21] which is produced for many stimuli such as nerve injury and endotoxin, among others. However, glycine blocks intracellular stimuli and cytokine production, inhibiting the systemic inflammatory process and the Wallerian degeneration, and also keeping out the cumulus of intra- and extra-axonal nerve cicatrization products.[22-24]

Obviously, the nerve was too deteriorated as time passed but it is important to note that the presence of small groups of axons at two months indicated reinnervation, and the glycine treatment made the difference with respect to the control group. In these groups, axons were destroyed and, at two months, some samples were observed to have some regeneration fibers.

Finally, it is necessary to expand this study to the next investigation level with glycine to be proposed as a co-adjuvant treatment in cases of peripheral nerve injury in humans, mainly in thoracic injuries, since it has been demonstrated that glycine has no undesirable effects in animals or humans. Glycine diet allows glycine blood concentration to increase at more than 1 mM from 0.1 to 0.2 mM concentrations and protects from shocks caused by endotoxins.[25-27]

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