



Pharmacokinetics and toxicology studies of new neuronal growth factor

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ABSTRACT

The objective of this study was to examine the pharmacokinetics and toxicology of major organs after intramuscular injection of new neuronal growth factor (NNGF) in rats and sheep and to gather evidence for future clinical trials so that new treatment modalities of successful treatment can be available for peripheral nerve injuries. For pharmacokinetics, we injected 70 skeletally mature Sprague Dawley rats intramuscularly with 5 mg/kg of body weight of NNGF, and we collected blood samples at 0, 30, 60, 120, 240, 360, and 480 minutes. We quantified the concentration of peptide present in the rat plasma samples collected at multiple time intervals using high-performance liquid chromatography. For toxicology, we included 20 rats, 10 in the control group and 10 in the study group, and 20 adult sheep, 10 in the control group and 10 in the study group. We injected 100 mg/kg of body weight of the NNGF intramuscularly for 7 days for rats and sheep. We observed all animals clinically daily to assess behavioral and dietary activities. After 2 weeks, we euthanized the animals and observed and weighed each one's heart, kidney, liver, and brain. We harvested the organs and sent them out for histopathological studies. We found no significant difference in body weight or the food residue observed between the control and the study groups. All animals withstood the study well, and no deaths occurred in any group until we finished the experiment. We detected NNGF content in the plasma by liquid chromatography. Immediately after injection, the NNGF level was $0.108 \pm 0.034 \mu\text{M}$. It rose to $0.166 \pm 0.024 \mu\text{M}$ at 30 minutes and peaked at 60 minutes at $0.362 \pm 0.409 \mu\text{M}$. At 120 minutes, the level started decreasing ($0.202 \pm 0.209 \mu\text{M}$), and after 240 minutes, it reached $0.086 \pm 0.044 \mu\text{M}$. At 320 minutes, we detected no NNGF in the plasma and no abnormality in any of the animals' organs compared to the control group in the sheep or rats. This study showed that with an intramuscular injection of 5 mg/kg of body weight of NNGF, the serum levels peaked at 60 minutes, showing rapid absorption and excretion. Moreover, the histopathology showed no toxic effects on the organs tested.

INTRODUCTION

Neurotrauma is one of the major causes of morbidity and mortality globally, more so in developing countries [1]. Taylor *et al.* [2] found that roughly 2%–3% of all patients admitted to a Level I trauma center have peripheral nerve injury (PNI). Recently, the PNI rate increased by over 15%, and less than 50%

of such individuals undergo nerve repair surgery. Of those who do, only 40%–50% recover normal function [3,4]. PNIs usually occur in the young and entail significant socioeconomic hardships [5]. Despite clear progress in the understanding of the healing process of injured nervous tissue and the recent advancements in surgical nerve repair techniques, complete success still seems far off [6,7]. Recently, growth factors have come into vogue in the healing of nervous tissue, and the other recently identified members of the neurotrophin family have been used to heal the peripheral nerves [8–13]. The new neuronal growth (NNGF), a placenta-derived polypeptide now sequenced and laboratory synthesized, could heal iatrogenic divided sciatic nerves in small

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and large experimental animals [14,15]. Therefore, we conducted this study to examine pharmacokinetics and toxicology in rats and sheep to gather experimental evidence for the clinical application of an investigational new drug.

MATERIALS AND METHODS

The study was conducted in accordance with the basic & clinical pharmacology & toxicology policy for experimental and clinical studies [16] and as per the ARRIVE guidelines. The study was approved by the Institutional Review Board of Imam Abdulrahman Bin Faisal University, Dammam, after the approval of the Animal Welfare and Ethics Committee vide #2021-01-465 dated 12/12/2021. We acquired 90 Sprague Dawley adult male rats and 20 adult male sheep and acclimated them to the study environment for 15 days before dose administration. We housed the animals in individually ventilated cages and kept them on a 12/12 hours light/dark cycle except when interrupting it for study procedures. We maintained the average room temperature at 25°C, average relative humidity at 30%–70% and an average daily airflow >10 fresh air changes/hour. We fed the rats Lab Diet Certified Rodent Diet 5002 Meal *ad libitum* and gave them access to water *ad libitum*. As a standard practice, we kept the sheep separated in groups, not mixing them with other breeds to avoid stress for individual animals, which could have altered their physiology and stereotypic behaviors. We provided well-designed feed troughs with sufficient space for all animals to feed simultaneously with plenty of water.

The formula of NNGF is C₄₇H₇₇N₁₁O₁₁ (H-Pro-Lys-Val-Phe-Leu-Gln-Gln-Leu-OH_{is}-OH) with a molecular weight of 1,223.49 g/mol. For pharmacokinetics, we used 70 skeletally mature Sprague Dawley rats and injected 5 mg/kg of body weight of NNGF intramuscularly [17] and we collected blood samples at 0, 30, 60, 120, 240, 360, and 480 minutes. We quantified the concentration of peptide present in the rat plasma samples using liquid chromatography and the standard curve method.

Pharmacokinetic studies

Chromatographic equipment and conditions

We used an high-performance liquid chromatography (HPLC) system from waters to quantify the NNGF (Waters®, USA). The instrument comprised an e2695 pump, an autosampler, and a UV/visible detector 2,489. We set the detector at a wavelength of 215 nm. The mobile phase (consisting of acetonitrile and water at a ratio of 76:24) was circulated through a waters symmetry RP-C18 analytical column (WAT045905) with dimensions of 5 µm, 4.6 × 150 mm, maintained at an ambient temperature, with a flow rate of 1 ml/minute. We used the isocratic mobile phase because selectivity does not change according to the column dimensions; it maintains a constant concentration in the mobile phase throughout the process. After each run, we rinsed the column for 15 minutes with water and methanol (90:10) to remove debris. The total run time was 4 minutes, and the retention time was 1.7 minutes. We controlled the HPLC system using Empower software.

Preparation of standard and samples

We prepared the master stock solution of 25 µM by dissolving 25 mg of lyophilized standard compound in 1 ml of deionized water and stored the stock at –20°C. We serially

diluted the master stock to obtain the standard working stock solutions with concentrations of 12.5, 4.17, 1.39, 0.46, 0.15, 0.05, 0.017, and 0.00 µM.

We positioned a total of 12.5 µM of the standard samples at the beginning, followed by two levels of quality controls, 1.5 and 0.01 µM, and the rest of the standard samples throughout the analytical run. We based the optimization on the shorter retention time (1.7 minutes) and lower detection (STD 0.05 µM). We froze the collected blood samples at –20°C until analysis. We thawed the samples and deproteinized them using acetonitrile. We vortexed the sample vigorously for 30 seconds and then centrifuged it at 15,000 rpm for 15 minutes. After centrifugation, we injected 20 µl of supernatant directly into the HPLC system. We calculated the NNGF level and presented it as mean and standard deviation.

Specificity

Specificity is the ability of the analytical method to distinguish between the analyte(s) and the other components in the sample matrix. In the case of our HPLC method, it is assured by the complete separation of peak(s) of analyte(s) from other peaks originating from the sample matrix. Specificity is the ability to assess the exact component in a mixture, and retention time is an accurate way to specify components.

Recovery

The recovery is the ratio of the concentration of analyte found to that stated to be present. The acceptable range of recovery is between 70% and 120%.

Toxicology studies

For toxicology studies, we injected 100 mg/kg of body weight per day of NNGF intramuscularly for 7 consecutive days into 10 rats and 10 adult sheep. We used 10 rats and 10 sheep as control subjects. All animals underwent clinical observation daily and as necessary. After 2 weeks, we euthanized the animals and observed and weighed each animal's organs, including the heart, kidney, liver, spleen, and brain. We stained the organs with hematoxylin and hematoxylin and then qualitatively analyzed them.

RESULTS

Weight and food residues in animals

We found no significant change in body weight in the control or study group or any change in the food residue.

Effects of NNGF injections

All animals withstood the study well, and no deaths occurred in any of the groups during the experiment. This result confirms that the animals could withstand the tests and adapted well to their environment.

Effects of NNGF injection on pharmacokinetics in rats

We detected NNGF in the plasma through liquid chromatography. Immediately after injection, the level was 0.108 ± 0.034 µM. It rose to 0.166 ± 0.024 µM at 30 minutes and peaked at 60 minutes at 0.362 ± 0.409 µM. At 120 minutes, the level started to decrease (0.202 ± 0.209 µM). After 240

minutes, it reached $0.086 \pm 0.044 \mu\text{M}$, and at 320 minutes, we detected no NNGF in the plasma (Fig. 1).

Effects of NNGF injection on the morphology of animal tissues

There was no difference between the weights of the animals and the organs tested (Table 1).

No abnormality was detected in the organs of all animals in comparison with the control group in sheep and rats (Figs. 2–6).

DISCUSSION

In this study on NNGF's pharmacokinetics and toxicology, we used the standard dose of 5 mg/kg of body weight, which was used in two prior studies on rats and rabbits, and we found the drug efficacious. The regular clinical assessment and results of animal weight and lack of deaths in the animals demonstrated that the NNGF was well tolerated. The NNGF, an 8-amino-acid-chain peptide, as expected, had a shorter half-life in widespread distribution and reached its peak level in 60 minutes. In the histological studies on both animals, the toxic doses did not cause any damage or adverse effects on the organs tested.

The literature contains sparse data on the biodistribution of externally derived and delivered nerve growth factor (NGF). The available data came from studies on rats and cynomolgus monkeys. The administration route was intravenous and subcutaneous in rats, with a single injection of 35 mg/kg, a single dose or a continuous infusion with an osmotic mini-pump at a rate of 50–450 mg/pump. In both studies, maximum plasma concentrations (T_{max}) occurred in 2–3 hours [18,19]. In our study, a single intramuscular injection of 5 mg/kg of body weight T_{max} peaked in 60 minutes.

The use of NGF has immense potential in clinical situations in PNIs and general metabolic diseases. Several studies

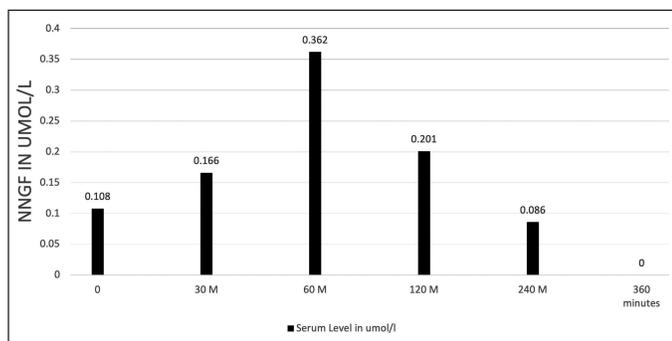


Figure 1. Serum level of NNGF in Umol/l.

Table 1. Body and organ weights (grams) of toxicology study in Sprague Dawley rats.

	Control group	Study group	<i>p</i> -value
Body weight	332.9 ± 29.7	337.19 ± 17.2	0.5
Kidney	1.28 ± 0.27	1.26 ± 0.24	0.9
Liver	3.1 ± 0.18	3.2 ± 0.18	0.5
Heart	2.9 ± 0.18	2.8 ± 0.13	0.7
Brain	1.87 ± 0.01	1.90 ± 0.08	0.6

have suggested that the use of NGFs has potential beneficial effects. Further animal studies have shown that exogenous administration of NGFs can promote peripheral nerve growth and re-establish the functional activity of peripheral nerve fibers and damaged neurons [20–23]. NNGF was studied earlier in the iatrogenic division of sciatic nerves in rats and rabbits and showed promising results [14,15]. Successful animal studies have made some human trials possible regarding using NGF in diabetic neuropathy, diabetic ulcers, and vascular ulcers secondary to rheumatoid arthritis [24–28]. But in most of the studies, only topical application was used, leading to a successful outcome. The clinical trials of rhNGF were discontinued due to the reported

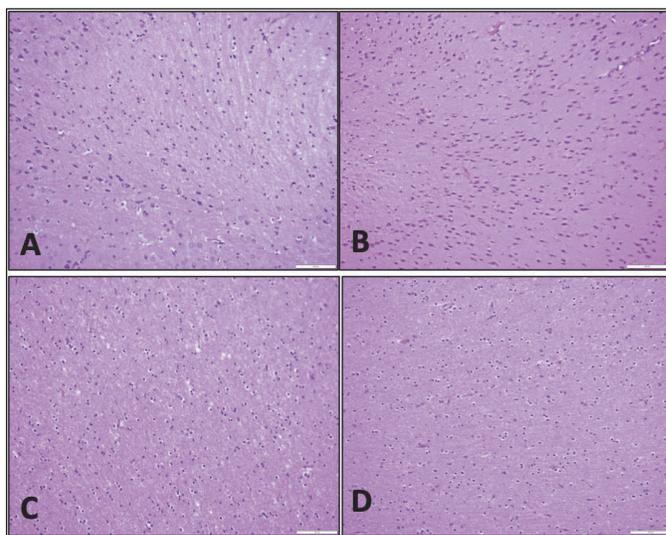


Figure 2. Brain tissue. Specimens taken from the animals are comparable histologically to the controls; there is no gliosis, necrosis, inflammation, hemorrhage, or congestion. (A) Rat control, (B) rat study, (C) sheep control, and (D) sheep study (H&E; 20 \times).

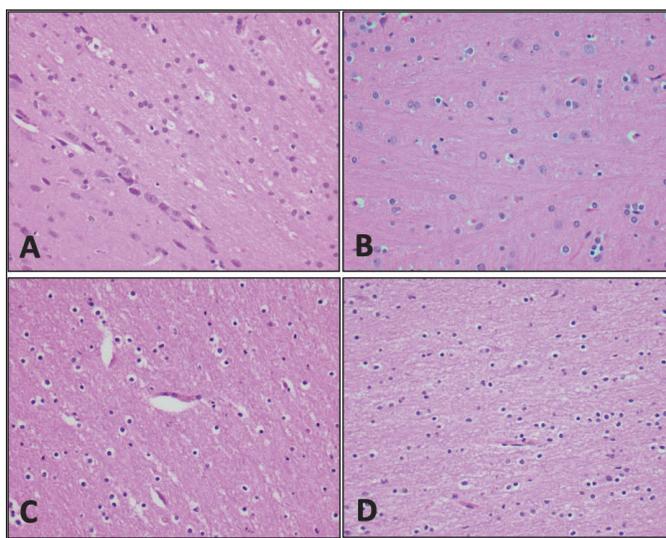


Figure 3. Brain tissue. Specimens taken from the animals are comparable histologically to the controls; there is no gliosis, necrosis, inflammation, hemorrhage, or congestion. (A) Rat control, (B) rat subject, (C) sheep control, and (D) sheep subject (H&E; 40 \times).

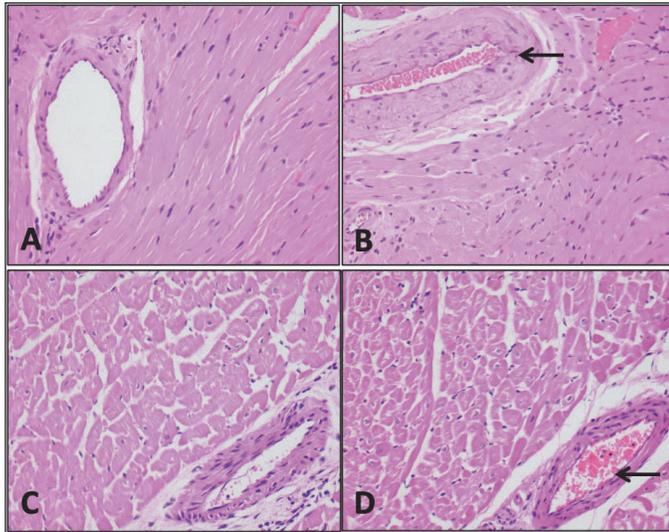


Figure 4. Heart tissue. Specimens taken from the animals are comparable histologically to the controls; there is no fatty infiltration, necrosis, inflammation, hemorrhage, or congestion. The blood vessels (arrows) also appear normal without calcification in the wall or any element of vasculitis. (A) Rat control, (B) rat study, (C) sheep control, and (D) sheep study (H&E; 40 \times).

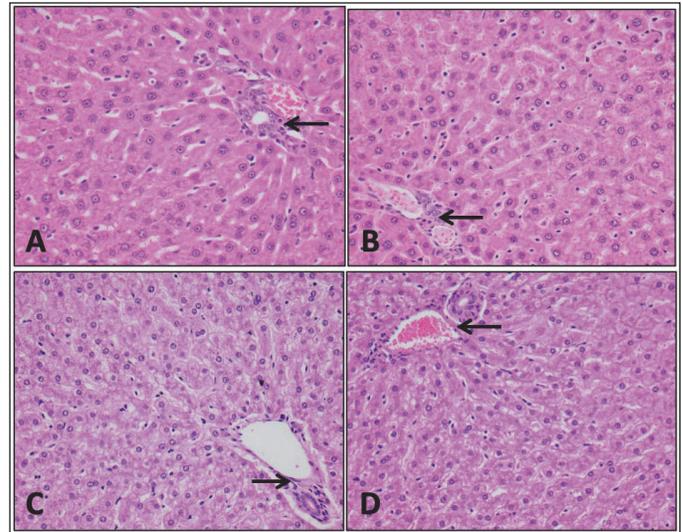


Figure 6. Liver tissue. Specimens taken from the animals are comparable histologically to the controls; there is no apoptosis, necrosis, hemorrhage, or congestion. The portal tracts (arrows) also appear normal without inflammation. The hepatocytes do not show fatty change or ballooning degeneration. (A) Rat control, (B) rat study, (C) sheep control, and (D) sheep study (H&E; 40 \times).

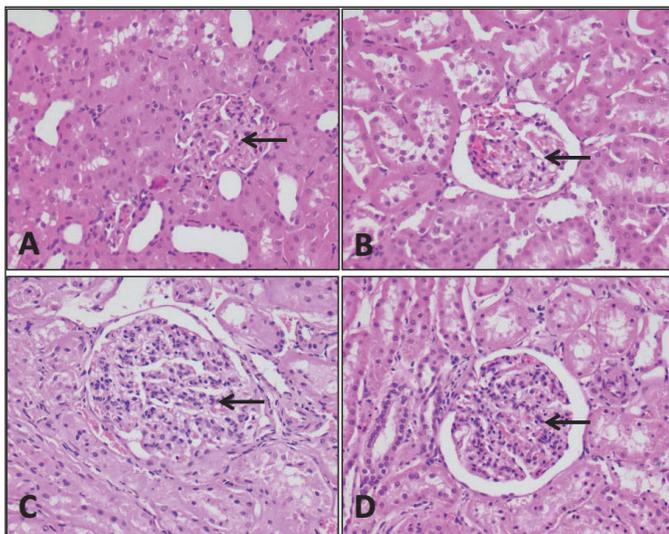


Figure 5. Kidney tissue. Specimens taken from the animals are comparable histologically to the controls; there is no necrosis, inflammation, hemorrhage, or congestion. The glomeruli (arrows) also appear normal and the surrounding tubules do not show an element of acute tubular injury. (A) Rat control, (B) rat study, (C) sheep control, and (D) sheep control (H&E; 40 \times).

side effects in clinical trials after intramuscular, intravenous, and subcutaneous injections, even though they proved to ameliorate symptoms effectively [29]. Studies are ongoing to overcome and limit the side effects of injectable NGF to accepted levels [30,31].

Our study has some limitations. First, we used small animals to assess serum levels after injecting a single dose, and we completed only one aspect of the pharmacokinetics study.

Second, we observed rats and sheep for 15 days after the toxic dose. A longer period of NNGF has to be given to check the toxic effect if any comes out. We need to study

pharmacokinetics in large animals like sheep and look for chronic toxicity. Toxicity studies are also needed in different animal species and to monitor physiological effects and assess histological abnormalities. This study's strength was that it was a single-dose study, which is generally more sensitive than multiple-dose studies in determining the release of a drug into the systemic circulation. Second, we examined toxicology in two animals. In conclusion, our pharmacokinetics and toxicology study of NNGF has shown that 5 mg/kg of body weight of intramuscular injection of NNGF and there are no acute toxic effects and the peptide is cleared out of the plasma after a peak of 60 minutes, which was expected. The results further give us credence for future research in human trials to confirm the efficacy and suitability to use in PNIs.

AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

The study was approved by the Institutional Review Board of Imam Abdulrahman Bin Faisal University, Dammam, after the approval of the Animal Welfare and Ethics Committee vide #2021-01-465 dated 12/12/2021.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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