A Review Analyzing In Vivo and In Vitro Testing Models on Nerve Conduits of the Peripheral Nervous System

Ali A. Al-Hamdi *MBChB, MSc*, Zain Bukamal *MBChB, MSc*, Billy C. Leung *MBBS, MSc, MRCS*

Dept. of Surgery and Interventional Science, University College of London, Royal Free Hospital, Pond, St, London NW3 2QG

Abstract

The gold standard method for nerve reconstruction involve the use of autologous graft, however, major drawbacks included limited availability, donor-site morbidities and requirement of multiple surgeries. Researchers worldwide had aimed to produce alternative tissue-engineered synthetic nerve conduits, but development had been slow, with only four FDA (US Food and Drug Administration) approved conduits for human subjects in the past 50 years of research. This slow progress may potentially be related to the lack of standardized guideline for nerve conduit testing. This review aims to summarize the methodologies used in the testing of nerve conduits in vivo and in vitro. The review demonstrated a lack of consensus and consistency in the study methodologies, including various measures of functional assessment, over 8 different types of animal species, 17 peripheral nerves and varied gap lengths ranging between 1 mm and 90 mm. In vitro models demonstrate more consistencies in testing models, but have been discarded in recent years for functional nerve testing, and had been employed for preliminary testing in nerve toxicity and compatibility instead. This study emphasizes the urgent need for a more standardized approach for in vivo testing, and the need to re-utilize in vitro studies for functional testing purposes.

Key words: nerve conduits; tissue engineering; peripheral nerves; testing methodology

Introduction

Tissue engineering has advanced as an integrative field, which incorporates cells, growth factors, biomaterials and engineering to produce an artificial section or system capable of replacing damaged human tissue or to improve its functional effectiveness [1]. The nervous system is one of the numerous areas in which tissue engineering is focusing on; nerve conduits being a crucial element of that advancement [2,3].

A nerve conduit is a guide tube manufactured from either natural or synthetic materials. It aims to restore sensitivity to nerve gaps caused by trauma, degenerative disease or tissue loss due to tumor resection [4]. Autologous nerve conduits are the current gold-standard tool [5] for repair of injured or diseased nerves, the sural nerve being the most commonly used for nerve grafting in humans [6]. However, complications such as sensory loss, neuroma and scar formation [4] may arise following peripheral nerve harvesting. Due to the resulting donor site morbidity and graft mismatch, an alternative is currently needed. Thus, the development of artificial nerve conduits began to progress toward replicating a nerve that may match the former's functional capabilities. In that context, nerve conduits' function is being tested throughout research for the last 50 years by varying techniques and methods, yet, a standard testing method does not exist [7,8].

Nerve tubulation (conduits) was first introduced in the 19th century by Gluck; he has proposed the use of nerve conduits in 1880 whereby he employed the use of a bone as a tube for nerve repair [9]. Gluck has adapted his idea from

Neuber who had used a bone tube in 1879 to serve as a resorbable wound drain [9]. In current practice, the US Food and Drug Administration (FDA) and the conformity European (CE) approved the clinical use of four artificial nerve conduits; two are type 1 collagen nerve conduits and the other two conduits are synthetic polyester-based [10,11].

This review aims to summarize the existing testing methodologies of artificial nerve conduits in the setting of both in-vitro and in-vivo models and to analyze the outcome of these methods in order to attain a standardized method of research for future nerve conduit studies in the peripheral nervous system.

Review

In-Vivo models

The use of animal models for nerve conduit testing flourished in the past decade producing abundant volumes of published studies. A systematic review conducted by Angius *et al* in 2012 analyzed the methodologies of more than 416 published in-vivo nerve conduit studies and concluded there was genuine lack of consensus and consistency in researchers' choice of methods [12]. The variability of methods included the choice of animal model, tested nerve, gap length, and assessment tool.

Choice of animal

The most popular choice of animal was rats, which accounted for up to 70% of all in-vivo studies [12]. The advantages of using rats included low maintenance cost, resilience to surgical intervention and infections, availability, and production of consistent assessment outcomes [13-15]. However, the drawbacks included the relatively small gap length compared to common human nerve lesions, the difference in neurophysiology to humans i.e., nerve axotomy produces full recovery in rats but not in human nerves and nerve regeneration is slower in humans [12,16,17]. Furthermore, there are different species of rats which have unknown variations in their physiological response to foreign materials for nerve regeneration [12].

The remaining 30% of animal models were accounted for by mouse, rabbits, dogs, cats and monkeys, with a few scattered studies on sheep and guinea pigs [12] (Fig. 1).
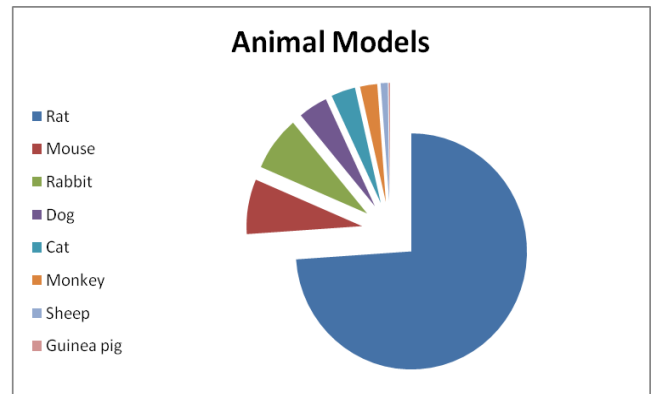

Fig. 1. Pie chart illustrating the types of animal model used in 'in vivo' studies

The mouse model was used in 7.5% of all studies and shared similar advantages and disadvantages to the rat model. One unique advantage in the mouse model was the ability to genetically modify mouse to allow imaging of nerve regeneration of fluorescent-induced axons [18,19]. The major disadvantage of mouse model was its limited gap length of less than 13mm [12].

The rabbit model had been one of the more frequently used models amongst the larger animals (up to 7.5% of studies). The rabbit model facilitated testing of larger nerve gap lengths and produced reliable results from neuromorphometric and electrophysiological testing methods [12]. However, its disadvantages included cost, difficulty of care, limited molecular probes for mechanistic analysis and most importantly, the difference in anatomy e.g., hind limb muscle in rabbits functions to hop, this may reduce its strength for human clinical trials [12]. Nerve studies on dogs and cats also allowed large testing nerve gap, and commonly produced reliable neuromorpho-metric analysis [12]. One major advantage in the use of dogs was the ability to train the animal for functional motor and sensory analysis, however, major drawbacks, together with cats,

included maintenance cost, ethical concerns in their role as domestic animals, and the lack of molecular probes present for mechanistic analysis [12].

There use of larger animals such as monkeys, sheep and guinea pigs in nerve conduit testing were less common (approximately 10, 4 and 1 reported cases, respectively). These animals allowed larger nerve gap length up to 60mm to be tested. However, these studies were restricted due to high cost and limited range of assessment tools available, including difficulties in training these species for functional testing compared to dogs [12]. Although the study of nonhuman primates i.e. monkey, would provide presumably the most reliable outcomes for a step toward human trials, recent reports from the Institute of Medicine had pledged their disagreement to nonhuman primates testing [20,21].

Overall, the selection of the animal type for clinical trials was essential, and researchers must consider the cost, availability, ethical issues and importantly, the physiology of the species e.g. lifespan, inter-variation of the species, susceptibly of infection and ability to withstand surgical interventions [22,23].

Furthermore, compatibility of the neuro-physiology of the animal species to the human being must also be considered i.e., neuromicrostructure, inflammatory response, degeneration process (Wallerian), and regeneration capacity [24,25]. It is important to make aware that the testing model used will depend on the experimental question, thus most authors would agree that no single testing model will fit all, nevertheless, the call for a more standardized methodology and guidelines will aid research forward [13,23,26]. In addition, strict adherence to national regulation in animal-testing policies is vital [27].

Type of peripheral nerve and length of nerve gap tested

The most commonly tested nerve was the sciatic nerve, accounted for over 70% of all studies [12]. The popular use of the sciatic nerve was likely to be due to its relatively anatomical accessibility and size compared to other peripheral nerves. The peroneal, tibial and facial nerve accounted for approximately 5-7% of the studies. A total of 17 different types of peripheral nerves that had been used for nerve conduits studies [12] (Fig. 2).
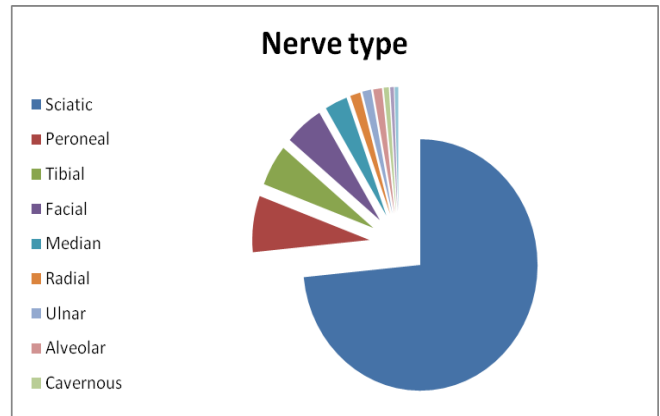

Fig. 2. An illustration showing the types of peripheral nerves used in 'in vivo' studies

Small volumes of individual studies used the median, radial, ulnar, alveolar, cavernous, saphenous, hypogastric, sural, optic, phrenic, recurrent laryngeal lingual and femoral nerves [12]. Overall, the selection of nerves was likely to be governed by resources, animal variability, and most importantly, intended purpose of the clinical trial.

As previously described above, the length of nerve gap examined were influenced greatly by the selection of the tested animal: rats 1-50 mm, mouse 2-13 mm, rabbits 2-50 mm, dogs 10-90 mm, cats 1-50 mm, monkeys 1-50 mm, pigs 8mm and no nerve gap was examined in the sheep study (Table 1).

Table 1: Table illustrating gap lengths (mm) used in 'in vivo' studies

Nerve gap length	
1-5 mm	29.5%
6-10mm	54%
11-15mm	14%
16-20mm	7%
24-30mm	3.4%
40-90mm	3.6%

The ideal nerve gap length studied would mimic distances commonly encountered in human nerve injuries, which vary tremendously. In most studies, the selection for gap lengths were >2 mm, which were decided upon the concept of critical length i.e., gap distance which regeneration would not occur unless nerve grafting or bridging occurs. Studies that conducted testing gap lengths < 2mm were not clear in the reason behind their selection. The range of gap lengths tested was from 1mm to 90mm. The most frequently used gap lengths were small distances of 1-5 mm and 6-10 mm, which accounted for 80% of all studies, followed by intermediate lengths of 11-15 mm, 16-20 mm and 24-30mm (25% of cases). Larger gap lengths of 40-90mm were less commonly tested.

Assessment tool for testing nerve conduit

There were a vast number of available testing tools used to assess nerve recovery and function (Table 2).

Table 2. Illustrates the common types of assessment methodologies in nerve conduit studies (in vivo)

Testing Methodologies	
Histological Analysis	80%
Neuromorphometric Analysis	50%
Electrophysiological	40%
Functional Analysis	27%
Immunohistological Analysis	25%

Most studies used more than one testing method [28], but there were great inconsistencies in their selection, with little consensus on the definitive testing tool. Furthermore, studies rarely explained or rationalized their choice of testing tool. This inconsistency could not simply be explained by the differences in experimental outcome or the influence of the type of animal used i.e., dog models were ideal for observational functional outcomes [29-31].

The most common assessment tool was qualitative histological analysis, which was present in around 80% of studies, followed by neuromorphometric analysis and electro-physiological analysis, which was present in 40-50%. A quarter of the studies utilized functional analysis and immunohistological analysis as their assessment tool. Other less common methods included gene analysis (RNA, DNA expression), stain analysis (retrograde labeling, BrdU staining), observational analysis (fast axonal transport assay, fiberscopic), muscle analysis (weight, contraction test, morphometric analysis) and imaging analysis (radiological, ultrasound) [32-34].

Histological and neuromorphometric analysis consistently reported myelinated-fiber count, nerve-fiber count, axon diameter, myelin thickness and g-ratio as endpoints, but failed to comment on which portion of the nerve was examined i.e. distal, central or proximal part. Furthermore, method of tissue processing were not often discussed i.e. tissue collection and sampling procedures. Electrophysiological analysis commonly measured the amplitude and latency of compound muscle action potentials (CMAP) or sensory nerve action potentials (SNAP), and occasionally, centrally recorded somatosensory evoked potentials. However, there were no consistencies amongst studies in their stimulation and recording parameters, as well as the location of nerve stimulated.

The majority of studies that tested functional analysis measured motor function, which included gait studies (static or dynamic), strength measurement (grip strength), and task assessments (object transfer). A common standardized motor test was the 'sciatic function index' that measured functional gait of rat (sciatic nerve) by standardized walking tracks [35]. There are over 20 different types of cellular markers for immunohistological analysis. Other less common used assessment tools included gene expression, muscle integrity, and imaging i.e., one study used ultrasound imaging.

This variability in testing methods potentially

highlights the poor communication amongst researchers, suboptimal available testing methods, and complexity and heterogeneity of nerve testing. However, we believe the utilization of a combination of testing methods within studies appeared constructive and logical, as it provided broaden ranges of analytical data. The use of the combination of histological, neuromorphometric or electro-physiological analysis provided valuable information in the different aspects of neurophysiology in nerve regeneration [33].

Functional analysis provided gross scale nerve recovery and was particularly testing models using dogs. Advancing techniques with cellular markers in immunohistological analysis offered targeted analysis of nerve regeneration. Therefore, the ideal testing methodology should in theory target a range of parameters; nevertheless, a standardized guideline, potentially containing various ranges of assessment tool, is essential to formulate a more structured approach to in vivo nerve conduit testing [36,37].

Nerve conduit composition and reconstruction

The type of nerve conduits used would be the tested variable in studies and would influence the assessment tool used, and to a degree influence the selection of animal model, nerve type or gap length. This further highlighted the complexity of selection in nerve conduit testing. There were more than 70 different synthetic nerve conduit materials being tested, broadly categorized into synthetic biodegradable, synthetic non-biodegradable and semi-synthetic materials derived from biological source, e.g., collagen, chitosan and silk.

Furthermore, methods of material extraction, processing, and scaffold integration for biological materials e.g., collagen, in the construction of semi-synthetic conduits, greatly varied between studies [12].

This demonstrated another inconsistency in testing methodologies. It was important to note that the differences in nerve conduit composition and reconstruction techniques used would be variable-tested, therefore would be independent from the selected testing method [38].

In-Vitro model

The testing of nerve conduit in recent years had favored in vivo animal studies to in vitro models, with the majority of in vivo studies functioning to assess biological safety and biocompatibility rather than functional outcome. At present, in vitro studies fail to mimic in vitro nerve environment, thus fail to assess immune response or tissue reaction secondary to vascularisation, oxygen supply and waste elimination present in in-vitro studies [12]. Nevertheless, in vivo nerve conduit testing plays an essential part in clinical approval.

All biomaterial have to pass in vitro and in vivo tests to get FDA and CE approval, there are only four type of nerve conduits were approved (2 collagen- and 2 synthetic- polyester based conduits). The International Standard Organization in ISO 10993-11 put the criteria for biological safety but not for the functional outcome.

In our literature search, there were relatively less published studies on in vitro models compared to in vivo. In vitro models can be categorized into the properties the study is testing for: physicochemical and biological (Table 3).

Table 3. Illustrates the types of in vitro testing models

Physicochemical properties	Biological properties
1. Mechanical (stress , strain, maximal load)	1. Cytotoxicity
2. Flexibility	2. Genotoxicity
3. Topography (spatial structure)	3. Enzymatic degradation
4. Degree of polymerization	4. Cell proliferation
5. Surface chemistry	5. Cell adhesion
	6. Length of the neurite growth
	7. Cell density

Main vitro methods were testing

1. The physicochemical properties of the conduit include:
   i. Maximum load and breaking load using Instron Series IX Automated Materials Testing System.
   ii. Flexibility of the conduit was assessed by texture evaluation methods.
   iii. Spatial structure of the nerve guide conduit analyzed by scanning electron microscope (SEM).
   iv. The distribution of the microspheres analyzed by was light microscope (LMS).
   v. Degree of polymerization by Gel Electrophoresis.
   vi. Tensile strength and tensile strain: analyzed by scanning electron microscope (SEM) [3].

2. Biological properties of nerve conduit include:
   i. Cytotoxicity assessed by cell culturing and then electron microscopy coupled with immunocytochemistry [39] recommended by the ISO 10993-11.
   ii. Genotoxicity: by Ames test test [40] evaluates the mutagenicity in a bacterial reverse mutation system.
   iii. Enzymatic degradation: phage-born endosialidase [41].
   iv. Mass Spectrometric Analysis: using an Aquity UPLC. [41].
   v. The length of the neurite growth [42].
   vi. Cell proliferation and cell adhesions by fluorescence microscopy [41].
   vii. State of the nerve conduit combined with mesenchymal stem cells (MSCs) was assessed immunofluorescence [43].

I believe there are still elements of In-Vitro models that could be explored further to assist in development the optimal nerve conduit and in vitro nerve testing models are more easily more and standardized [44].

In conclusion, nerve regeneration with synthetic materials is challenging. The essential progression to human clinical use and trial had not been achieved despite 50 years of research.

The reason may be multifactorial, including the complexity of nerves, limited understanding of neurophysiology, and the vast diversity of clinical nerve injury. Over 70 types of nerve conduit materials have been tested over the last few decades, clearly highlighting the unsatisfactory results produced by these synthetic conduits.

The lack of progression may be contributed by the limited consensus and consistency on functional testing methods for nerve conduits, in particular, in vivo models. In vitro testing models were often focused on preliminary testing for conduit toxicity and compatibility, rather than functional outcomes, and appeared to have some standardized method. The concept of in vitro functional testing had recently faded, and had shifted towards in vitro models. We believe there are still areas of in vitro nerve testing that can be expanded and applied for functional testing use. In vitro testing is safe, experiences less ethical dilemmas, and if testing models were able to mimic human environment, the use of in vitro studies may become superior to in vivo studies.

For in vivo studies, there are many reasons why they had not been a uniform methodology for testing nerve conduit. First of all, studies varied in their experimental goal, i.e., the type of nerve required for regeneration and the gap required for bridging. Secondly, resources and financial implications, which will influence the choice of animal model and testing equipment used. Therefore, it is impossible and impractical to have a universal method to suit all. However, some standardization should be discussed and formulated by the leading research groups in this field of tissue engineering. This will greatly facilitate the growth in this field, as a more consistent method will allow greater cross examination amongst studies.

Finally, we suggest more studies are to be conducted to cross-examine testing methods and animals used, as there is currently no literature to compare the quality of different methods. We believe that tissue engineering can still be the answer to nerve regeneration.

Researchers would greatly benefit from a unified methodology of in vivo testing and exploration into functional testing models for in vitro testing would also be beneficial. The emerging use of stem cell and growth factors into nerve conduits are showing some promising results, and we hope this may accelerate progress in this field.

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Correspondence to Dr. Ali AlHamdi
E-mail: ali.alhamdi.13@ucl.ac.uk