Indian J Physiol Pharmacol 2019; 63(1): 79–85

Medical Education / Method Paper

Study of Neuromuscular Transmission Under (i) Phenomenon of Fatigue, (ii) Site of Fatigue, (iii) Neuromuscular Blocking in an *in-situ* Rat Nerve Muscle Preparation: A Novel Approach to Nerve Muscle Physiology Experiment Teaching

Arani Das, Arpita Chakraborty, Shival Srivastav, Simran Kaur, Suman Jain and Geetanjali Bade*

Department of Physiology,
AIIMS, New Delhi

Abstract

Purpose of the study: Nerve-muscle physiology is a very basic and vital module in undergraduate physiology curriculum. The practical sessions on this system are demonstrated on amphibian (frog) nerve-muscle preparation, which becomes a limiting factor in most of the medical colleges of India. In this study, we propose an alternative approach by using mammalian (rat) model for nerve-muscle physiology undergraduate practical experiments.

Materials and methods: Rat *in-situ* sciatic nerve-soleus muscle preparation was used to study neuromuscular transmission. Stimulation of nerve and recording of muscle contraction (force) were done by using digital recording system. To demonstrate fatigue, repetitive electrical stimulation was applied to nerve-muscle preparation and muscle twitches were recorded. A reduction in amplitude of contraction to 50% of their basal recording was considered as onset of fatigue. To demonstrate site of fatigue, the muscle was stimulated directly after the fatigue was observed with nerve stimulation and muscle twitch recorded. To observe the effect of neuromuscular blocking drug Pancuronium bromide, on neuromuscular transmission, in a separate set up the drug was injected in muscle belly at multiple sites and nerve was stimulated to elicit muscle twitch. The response (amplitude of muscle twitch) was compared with control (injection of 0.9% saline).

Main findings: On repeated stimulation of nerve muscle preparation, initially there was an increase in amplitude of contraction but progressively amplitude went on decreasing. After development of fatigue on direct muscle stimulation, amplitude recorded was same as the initial twitch amplitude. This demonstrates that the site of fatigue is not the muscle. Further, as nerve is also non- fatigable, site of fatigue was neuromuscular junction.

Injection of Pancuronium bromide showed that the twitch amplitude decreased substantially than the control (saline injection) on stimulation of nerve, but on direct muscle stimulation amplitude of contraction remained same as initial, confirming the effect of drug on neuromuscular junction.

***Corresponding author :**

Dr. Geetanjali Bade, Department of Physiology, AIIMS, New Delhi, Email: geetanjalibade@gmail.com

(Received on July 1, 2018)

Conclusion: *In-situ* rat nerve-muscle preparation can be used as an alternative approach to amphibian experiments for effective demonstration of neuromuscular transmission.

Introduction

Nerve–muscle physiology constitutes a very important portion of undergraduate teaching curriculum which comprises of about 8-12 didactic lectures in most medical colleges. The practical sessions on this system are mostly on amphibian (frog) nerve-muscle preparation. The purpose of the practical teaching is to develop new psycho-motor skills as well as reinforcement of theoretical concepts. The concepts and practical skills on nerve-muscle physiology are indispensable part of undergraduate teaching that develops the basic understanding of electrophysiology. But the availability of frog is a limiting issue in most of the medical colleges. Here we propose an alternative approach by using mammalian (rat) model for nerve-muscle physiology practical experiments.

Several studies using rat's nerve-muscle preparation are available. By using nerve-muscle preparation of rat, Norenberg et al. in 2004 demonstrated that isotonic resistance exercise training increases muscle twitch tension in soleus and gastrocnemius muscles. Various physiological contractile properties of skeletal muscle (gastrocnemius) in rats, such as post-tetanic potentiation (MacIntosh et al., 1987), force-frequency relationship (Dormer et al., 2009), force-length relationship (MacNaughton et al., 2006), force-velocity relationship (Devrome et al., 2007) and fatigue (MacNaughton et al., 2006) were studied in *in-situ* nerve-muscle preparation.

Fatigue may be defined as a progressive loss of the ability to generate maximum force during (or following) repeated or sustained muscle contractions or the loss of force generation during a task (Davis et al., 2010). Fatigue is divided into central and peripheral fatigue. Central fatigue consists of impaired muscle performance due to central (CNS) cause where CNS drive is reduced or which prevents complete muscle group recruitment. Peripheral fatigue may be because of muscle fatigue or fatigue at neuromuscular junction because of depletion of neurotransmitter. Muscle fatigue predominately involves muscle bioenergetics or excitation-contraction coupling (Davis et al., 2010).

To study the effect of neuro-muscular blocking agent on neuromuscular transmission, Pancuronium bromide can be used. It is a long acting, non-depolarizing acetylcholine receptor blocking agent. It is used clinically to facilitate endotracheal intubation and to provide skeletal muscle relaxation during surgery (Larijani et al., 2011).

So the objective of this practical was to study of neuromuscular transmission under (i) phenomenon of fatigue, (ii) site of fatigue, (iii) neuromuscular blocking in an *in-situ* rat nerve-muscle preparation.

Material and methods

Material required

Dissection board, dissection instruments, glass seeker, dental spatula, povidone iodine solution, cotton, tissue paper, thread, mammalian ringer, normal saline, liquid paraffin, 1 ml syringe, Sodium Thiopental, Pancuronium bromide, stand, clamp, pulley, force transducer (MLTF500/ST), stimulating electrodes and digital recording system - Power Lab 26T with LabChart™ software version 8.1 (AD Instruments, Australia).

Experimental animals

Adult male wistar rats (body weight 200-250 gm) were obtained from institutional (All India Institute of Medical Sciences, New Delhi, India) central animal facility. They were housed in a temperature-controlled room at 24±2% C with a light:dark cycle of 14:10

hours and provided ad libitum food and water. Rats were food deprived overnight to avoid mucous and salivary secretion during experiment.

Ethics statement

The present study was approved by the institutional ethics committee, AIIMS, New Delhi ((65/IAEC-1/ 2018) and was performed in accordance with the Laboratory Animal Welfare Act, the Guide for the Care and Use of Laboratory Animal (National Institutes of Health, Bethesda, MD, USA).

Specific learning objective 1:

To demonstrate the phenomenon and site of fatigue in an *in situ* nerve–muscle preparation of rat.

Procedure

1. An adult wistar rat was anaesthetized by injecting Sodium Thiopental intraperitoneally (dose 50 mg/ kg). Depth of anesthesia was confirmed by tail pinch.

2. Rat was fixed on dissection table by tying all the four limbs with cotton thread as shown in Fig. 1.

3. One lower limb was shaved, cleaned with povidone iodine solution and a skin incision was given in midline between knee and ankle joint.

4. Soleus muscle is flat and red in appearance and lies with the surface of tibia. The soleus muscle was identified and detached from gastrocnemius and plantaris muscle by dental scalpel. As the tendons of three above mentioned muscles are attached, tendon of soleus muscle was isolated from them without damaging any blood vessels. The distal end (tendon) was isolated from ankle joint while the proximal end still remained attached.

5. One end of cotton thread was tied to the isolated tendon of soleus muscle and other end was attached to force transducer as shown in Fig. 1.

6. The skin incision was extended proximally in the thigh region to expose the gluteus muscle. Sciatic nerve was then located deep to the gluteus muscle and isolated using glass seeker.

7. Stimulating hook electrodes were applied to the sciatic nerve and used to stimulate the nerve (Fig. 1).

8. Liquid paraffin was used to prevent drying of muscle and nerve.

9. Force transducer (MLTF500/ST) and stimulating

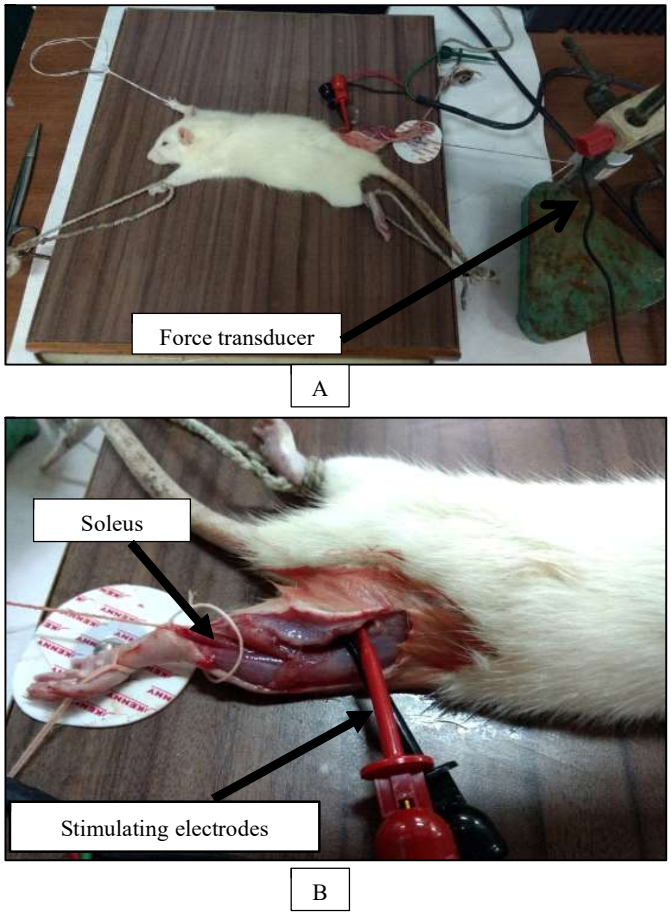

Fig. 1 : Experimental set up used for the study: (A) Limbs of rat are tied on a dissection board. Fixing of rat on a dissection table and placement of force transducer are shown.Tendon of muscle is attached to force transducer using a thread passed over a pulley.

(B) Sciatic nerve and soleus muscle along with tendon are exposed (marked in diagram). Stimulating hook electrodes are placed over sciatic nerve and tendon of muscle is tied using a thread.

electrodes were connected to the digital recording system. Power Lab 26T with LabChart™ software version 8.1 (AD Instruments, Australia) were used for real time digital data acquisition and analysis.

10. Since the force transducer measures contraction strength in Volts, it was essential to calibrate the same before the start of the experiment to get values in grams. Transducer channel was identified and zeroing was done before commencement of calibration. Two-point calibration was done by applying weights from 10-50 g in a step up and step down manner. Unit conversion was done to get the subsequent values in Newton.

11. A simple muscle twitch was recorded by applying supramaximal strength of stimulus. The amplitude of contraction and total twitch duration were measured.

12. For demonstrating the phenomenon of fatigue, the nerve-muscle preparation was stimulated with supramaximal stimuli at a frequency [(1/total twitch duration) per second] till the amplitude of contraction reached to 50% of the first recording.

13. The changes in latent, contraction and relaxation periods after the onset of fatigue were analyzed. Further the phenomenon of beneficial effect during initial phase and contraction remainder were observed.

14. The stimulating electrodes were applied directly to the muscle belly immediately after fatigue sets in and a muscle twitch was recorded. The amplitude of contraction was observed.

15. After the data collection, the rat was sacrificed with the over dose of anaesthesia or cervical dislocation while still under anaesthesia.

Specific learning objective 2:

To demonstrate the neuromuscular blocking effect of Pancuronium bromide in an *in situ* nerve-muscle preparation of rat

Procedure

1. Two nerve-muscle preparations were prepared in both hind limbs of a rat as per the procedure mentioned earlier.

2. Simple muscle twitch was recorded from both the preparations by stimulating the sciatic nerve. The amplitude of contraction and total twitch duration were measured.

3. 0.5 ml of 0.9% saline was injected into one muscle (control), and 0.5 ml of Pancuronium bromide (10 mg/ml) was injected into the center and periphery of the other muscle belly by multiple punctures.

4. Sciatic nerves of both the preparations were stimulated every minute with supramaximal stimulus strength and the muscle twitch responses were recorded.

   The muscles in both the preparations were also stimulated directly and twitch responses were recorded.

6. After the data collection, the rat was sacrificed with the over dose of anesthesia or cervical dislocation while still under anesthesia.

Results

The representative records show the muscle twitch responses before and after fatigue followed by direct muscle stimulation and after injection of normal saline or Pancuronium bromide in Fig. 3 to 7.

On repeated stimulation of nerve muscle preparation, for the first few contractions, increase in amplitude was recorded which is termed as beneficial effect (Fig. 2). As stimulation was continued, there was progressive increase in latent period and decrease in amplitude (Fig. 3). As the fatigue started developing relaxation became incomplete and there

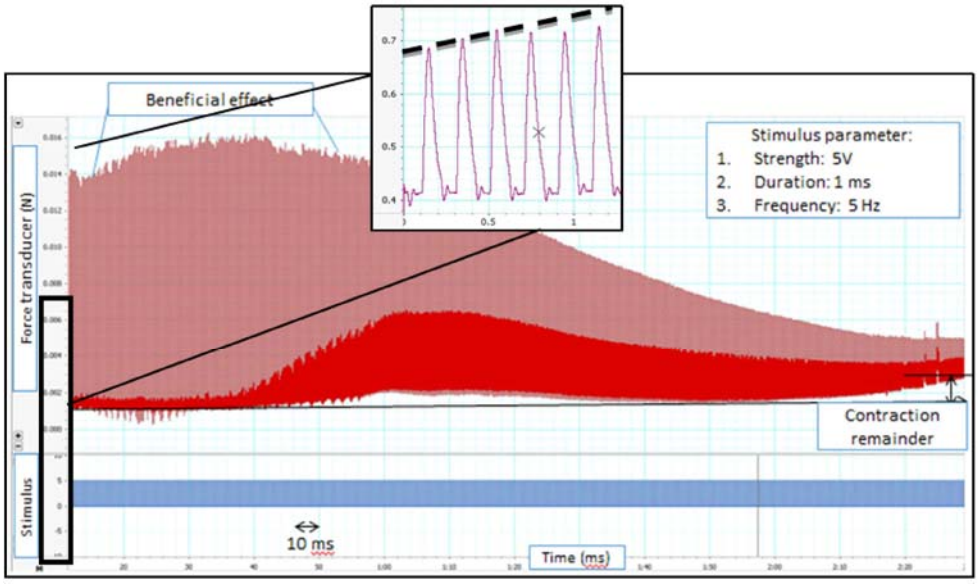

Fig. 2 : Phenomenon of fatigue with beneficial effect and contraction remainder X- axis shows time recording. Y- axis shows force of contraction (upper panel) and stimulus strength (lower panel).

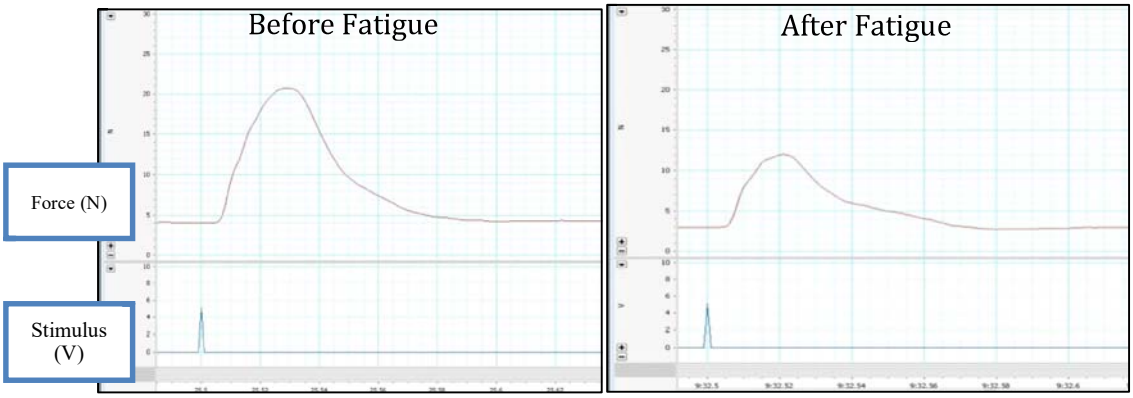

Fig. 3 : Simple muscle twitch response obtained on stimulation of sciatic nerve before and after development of fatigue (Stimulus parameter: 5 V for 1 ms)
X- axis shows time recording. Y- axis shows force of contraction (upper panel) and stimulus strength (lower panel)

was shifting of baseline, which is called as contraction remainder as shown in Fig. 2. After development of fatigue (twitch amplitude decreased to 50% of initial) when muscle was stimulated directly amplitude recorded was the same as the initial twitch amplitude (Fig. 4).

Injection of Pancuronium bromide decreased twitch amplitude substantially as compared to control (0.9% saline injection) secondary to nerve stimulation, but on direct muscle stimulation, twitch amplitude recorded was the same as initial (Fig. 5 to 7).

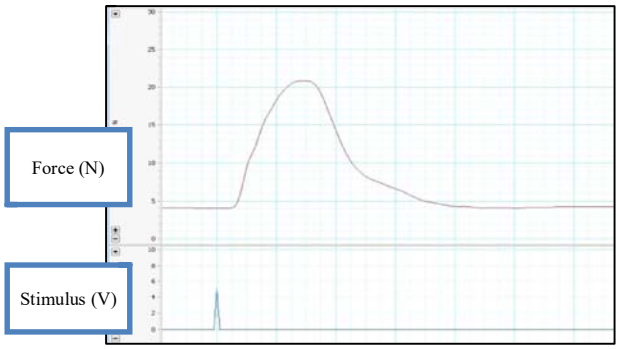

Fig. 4 : Simple muscle twitch response obtained on direct muscle (soleus) stimulation after fatigue (Stimulus parameter: 5 V for 1 ms)
X- axis shows time recording. Y- axis shows force of contraction (upper panel) and stimulus strength (lower panel)

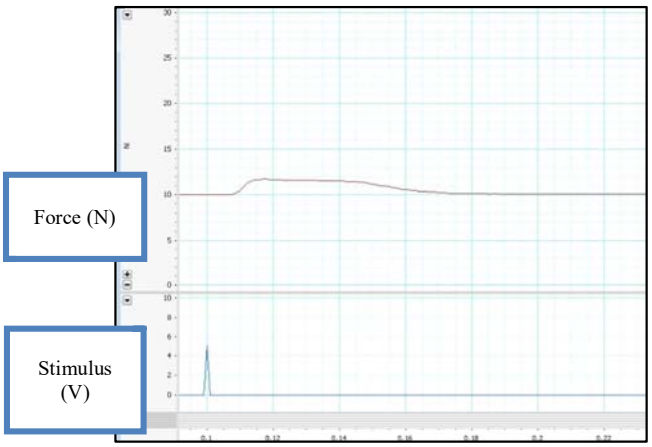

Fig. 5 : Simple muscle twitch response obtained on stimulation of sciatic nerve 3 minutes after injection of Pancuronium bromide in the soleus muscle (Stimulus parameter: 5 V for 1 ms)
X- axis shows time recording. Y- axis shows force of contraction (upper panel) and stimulus strength (lower panel)

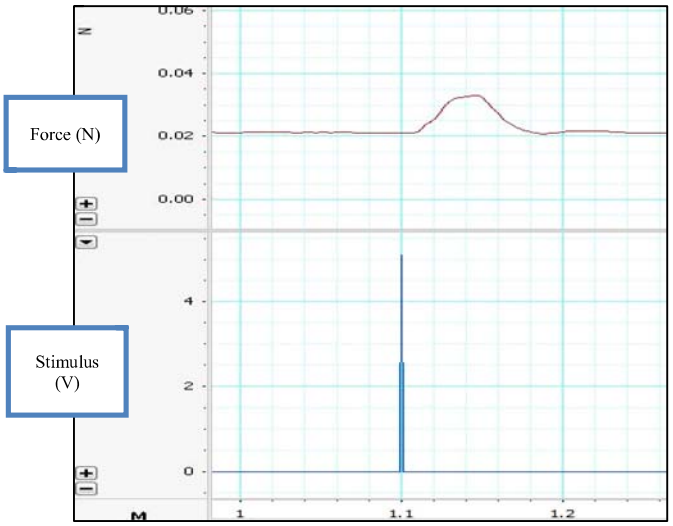

Fig. 6 : Simple muscle twitch response obtained on direct muscle (soleus) stimulation 3 minutes after injection of Pancuronium bromide (Stimulus parameter: 5 V for 1 ms)
X- axis shows time recording. Y- axis shows force of contraction (upper panel) and stimulus strength (lower panel)

Discussion

The present study proposes an innovative practical teaching experiment to demonstrate the phenomenon of fatigue and site of fatigue. In this *in-situ* nerve-muscle preparation there are three possible sites of fatigue: the nerve, neuromuscular junction and the muscle. Nerve is practically unfatiguable. Direct

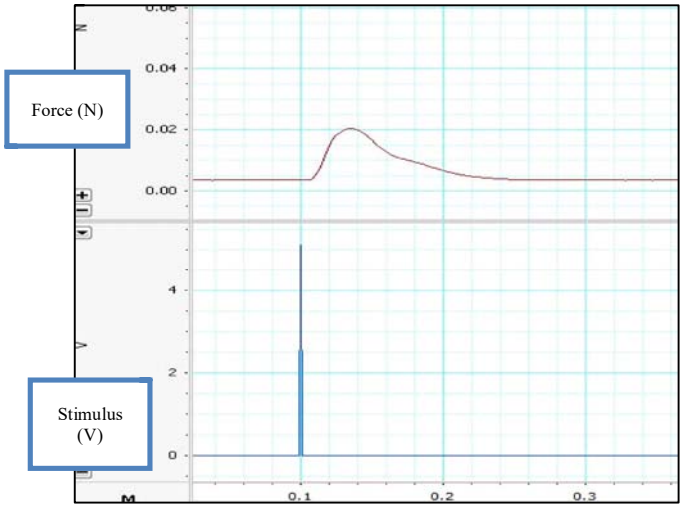

Fig. 7 : Simple muscle twitch responseobtained on nerve stimulation 3 minutes after injection of 0.9% saline solution in Soleus muscle (Stimulus parameter: 5 V for 1 ms)
X- axis shows time recording. Y- axis shows force of contraction (upper panel) and stimulus strength (lower panel)

muscle stimulation after development of fatigue showed same muscle twitch amplitude as initial, which confirms that muscle is not the primary site of fatigue. So by exclusion it can be demonstrated that in a nerve-muscle preparation, neuromuscular junction is the site of fatigue. With further extension, recording of compound action potential from sciatic nerve in a fatigued nerve-muscle preparation can demonstrate that nerve is not the site of fatigue.

We also demonstrated the effect of neuromuscular blocker, Pancuronium bromide, on muscle twitch amplitude, which decreased 3 to 4 minutes after injection. As Pancuronium bromide is a known neuromuscular blocker, twitch amplitude remained same as initial when muscle was stimulated directly.

By demonstrating this practical, students can easily understand the phenomenon of fatigue, the site of fatigue, phenomenon of beneficial effect, contraction remainder and the effect of neuromuscular blocker on muscle twitch responses of a nerve-muscle preparation.

In conclusion, this novel approach of using mammalian nerve-muscle preparation for demonstrating the phenomenon and site of fatigue and effect of neuromuscular blocking is

an effective undergraduate practical teaching module in physiology. The recordings from the present experiment can be modified as 'objective structured practical examination' (OSPE) questions for undergraduate practical assessment examinations.

Further, these experiments can be included as an individual practical module in post graduate assessment examination as it requires dissection skills, arrangement of stimulating and recording instruments and demonstration of several physiological phenomena in nerve-muscle physiology.

References

1. Norenberg KM, Fitts RH. Contractile responses of the rat gastrocnemius and soleus muscles to isotonic resistance exercise. *J Appl Physiol* 2004; 97(6): 2322–2332.
2. MacIntosh BR, Gardiner PF. Posttetanic potentiation and skeletal muscle fatigue: interactions with caffeine. *Can J Physiol Pharmacol* 1987; 65: 260–268.
3. Dormer GN,Teskey GC, MacIntos BR. Force-frequency and force-length properties in skeletal muscle following unilateral focal ischeaemic insult in a rat model. *Acta Physiol* 2009; I197: 227–239.
4. MacNaughton MB,MacIntosh BR. Reports of the Length Dependence of Fatigue are Greatly Exaggerated. *J Appl Physiol* 2006; 101: 23–29.
5. Devrome AN, MacIntosh BR. The biphasic force-velocity relationship in whole rat skeletal muscle in situ. *J Appl Physiol* 2007; 102: 2294–2300.
6. Davis M.P, Walsh D. Mechanisms of fatigue. *J Support Oncol* 2010; 8: 164–174.
7. Larijani GE, Gratz I, Silverberg M, Jacobi AG. Clinical pharmacology of the neuromuscular blocking agents. *Dicp* 1991; 25: 54–64.