T.C.
DOKUZ EYLUL UNIVERSITY
IZMIR INTERNATIONAL
BIOMEDICINE AND GENOME
INSTITUTE

OPTICAL VAGUS NERVE STIMULATION

OZAN YETİŞ

MOLECULAR BIOLOGY AND GENETICS

MASTER OF SCIENCE THESIS

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Supervisor: Assoc. Prof. Dr. Serhat TOZBURUN

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LIST OF ABBREVIATIONS

°: Degree

°C: Celsius degree

μ: micro- (10^{-6})

μa: Absorption Coefficient

μm: micrometer

μsec: microsecond

A: Ampere

AP: Arterial Pressure

ADN: Aortic Depressor Nerve

AMYG: The Amygdala

BD: Beam Diameter

cm: centimeter

CW: Continuous Wave

dB: Decibel

DBS: Deep Brain Stimulation

DRN: The Raphe Nuclei

DMN: The Dorsal Motor Nucleus

ECG: Electrocardiography

EEG: Electroencephalography

EMG: Electromyography

ENG: Electroneurography

ENS: Electrical Nerve Stimulation

GRIN: Gradient-Index

H&E: Hematoxylin-Eosin Dye

HF: High Frequency

HFOV: Horizontal Field of View

HIPP: The Hippocampus

HR: Heart Rate

HRV: Heart Rate Variability

Hz: Hertz

IFOV: Instantaneous Field of View

IR: Infrared

IU: International Unit

K/X: Ketamine/Xylazine

kHz: kilohertz

LC: The Locus Coeruleus

LF: Low Frequency

LF/HF: Ratio of Low Frequency to High Frequency

NA: Nucleus Ambiguus

NAC: The Nucleus Accumbens

NETD: Noise Equivalent Temperature Difference

nm: nanometer

NST: Nucleus of the Solitary Tract

NTS: The Nucleus Tractus Solitarius

MAP: Mean Arterial Pressure

MFOV: Measurement Field of View

mK: milliKelvin

ml: milliliter

mm: millimeter

mW: milliwatt

ONS: Optical Nerve Stimulation

OPD: Optical penetration depth

oVNS: Optical Vagus Nerve Stimulation

PAP: Pulsatile Arterial Pressure

PFC: Prefrontal Cortex

RMSSD: Root Mean Square of Successive Differences

s: Second

SDNN: Standard Deviation of NN intervals

SNT: Spinal Nucleus of the Trigeminal Nerve

STM: Stimulation

TEC: Thermoelectric Cooler

TRPV: Transient Receptor Potential Cation Channel Subfamily Vanilloid

UV: Ultraviolet

V: Volt

VFOV: Vertical Field of View

VN: Vagus Nerve

VNS: Vagus Nerve Stimulation

WD: Working Distance

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OPTICAL VAGUS NERVE STIMULATION

Ozan Yetiş, İzmir International Biomedicine and Genome Institute, Dokuz Eylul University

ABSTRACT

The Vagus nerve, the longest cranial nerve, innervates to the neck, abdomen, and colon. The vagus nerve is also a clinically significant nerve that controls parasympathetic actions in visceral organs like the heart, lungs. Moreover, electrical nerve stimulation of the vagus nerve is a treatment methodology for epilepsy.

Optical nerve stimulation is an alternative technique that uses infrared light to stimulate nerve cells. The relatively new technique has three main advantages over conventional electrical nerve stimulation: (1) a non-contact method of nerve stimulation; (2) improved spatial selectivity; (3) elimination of stimulation artifacts. Sciatic nerve and cavernous nerve are some examples of optical nerve stimulation. Despite all of the neural targets, optical vagus nerve stimulation has not been investigated in a rat model.

This thesis investigates the effect of optical vagus nerve stimulation in an in-vivo rat model. An infrared diode laser was used to stimulate the vagus nerve at a wavelength of 1505 nm in continuous-wave mode. Arterial pressure and electrocardiography signals were obtained to understand nerve stimulation's effectiveness using laser radiation on the vagus nerve.

Successful optical nerve stimulation of the rat vagus nerve was observed in terms of arterial pressure. Arterial pressure was significantly decreased during optical vagus nerve stimulation after the nerve surface reached a temperature of ~42 °C. However, optical nerve stimulation had no side effects on electrocardiogram signals, despite electrical nerve stimulation having side effects on the heart like bradycardia and arrhythmia. Histological analysis also showed that optical nerve stimulation is safe until ~46 °C.

Key Words: vagus nerve, optical nerve stimulation, infrared laser, arterial pressure, electrocardiography

VAGUS SİNİRİNİN OPTİK UYARIMI

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ÖZET

Vagus siniri boyun, toraks, karın ve kolonlara inerve olan en uzun kraniyel sinirdir. Vagus siniri, iç organlardaki parasempatik eylemleri kontrol eder. Vagus siniri klinik açıdan birçok uygulama alanı olan yüksek öneme sahip bir sinirdir. Ayrıca, Vagus sinirinin elektriksel uyarımı epilepsi hastalarında kullanılan bir yöntemdir.

Sinirlerin optik uyarımı, hücreleri aktive etmek için kızılötesi ışık kullanan alternatif bir tekniktir. Sinirlerin optik uyarımının, elektriksel sinir uyarımına karşı üç ana avantajı vardır: (1) Temassız sinir uyarımı, (2) uzaysal seçiciliğin iyileştirilmesi ve (3) uyarım artefaktlarının ortadan kaldırılması. Siyatik sinir ve kavernöz sinir, sinirlerin optik uyarımı uygulamalarında kullanılmıştır. Kullanılan farklı sinir dokularına rağmen sıçan modelinde vagus sinirinin optik uyarımı daha önce araştırılmamıştır.

Bu tez, sıçan vagus sinirinin optik uyarımını incelemektedir. Vagus sinirini uyarmak için 1505 nm dalga boyunda kızılötesi, diyot lazer kullanılmıştır. Vagus sinirinin optik uyarımının etkilerini anlamak için sistemik kan basıncı ve elektrokardiyografi sinyalleri toplanmıştır.

Bunun sonucunda, sıçan vagus siniri kızılötesi lazer kullanılarak başarı ile uyarılmıştır. Vagus siniri yaklaşık 42°Cye ulaştıktan sonra kan basıncı istatistiksel olarak anlamlı şekilde düşmüştür. Bununla birlikte, elektriksel sinir uyarımı bradikardi ve aritmi gibi problemlere neden olurken, vagus sinirinin optik uyarımının kalp üzerinde olumsuz etkisi gözlemlenmemiştir. Ek olarak, histolojik analizler de sinirinin optik uyarımı ~46 °C'ye kadar güvenli olduğunu göstermiştir.

Bu nedenle, Vagus sinirinin optik uyarımı, 42-46 °C arasında termal açıdan güvenli ve başarılı bir yöntemdir. Gelecekte vagus sinirinin elektriksel uyarımına alternatif olabilecek potansiyele sahiptir.

Anahtar Kelimeler: vagus siniri, optik sinir uyarımı, kızılötesi lazer, kan basıncı, elektrokardiyografi

1. INTRODUCTION AND AIM

1.1. Statement and Importance of the Problem

Optical nerve stimulation (ONS) is a unique technique that uses infrared laser to stimulate cells with electrochemical capacity without an extra agent or genetic modification [1]. On the other hand, a widely accepted methodology is electrical nerve stimulation (ENS) in clinics. ONS has three main advantages over conventional ENS. (1) A non-contact method of nerve stimulation, (2) Improved spatial selectivity, and (3) Elimination of stimulation artifacts [2, 3]. There is no remarkable ONS application in the vagus nerve until now, although it has been examined in various tissues like the sciatic nerve, cavernous nerve, cochlear, and heart.

The vagus nerve (VN) is the longest cranial nerve that controls parasympathetic actions in visceral organs like the heart, lungs, gastrointestinal system, and immune system [4]. Stimulation of the VN provides control of these parasympathetic events, which can be transferred to the clinic, such as heart rate (HR), arterial pressure (AP), and immune system control [5]. Moreover, electrical stimulation of the VN is a treatment methodology for epilepsy [6]. Even if vagus nerve stimulation is used for epilepsy patients in the clinic, it has serious side effects [7].

An infrared laser could bring the advantages of ONS to vagus nerve stimulation therapy for better stimulation and fewer side effects. Thus, the effects of ONS on the VN are examined in this study for the first time.

1.2. Aim of the Study

This study's primary purpose is to test ONS on the VN for the first time in an in-vivo rat model. The first aim is to understand whether the optical technique can successfully stimulate a VN. The second aim is to compare optical vagus nerve stimulation (oVNS) with a well-studied and clinically available technique (i.e., electrical nerve stimulation) regarding efficiency and side effects during vagus nerve stimulation. The final aim is to determine the VN's thermal damage threshold in ONS applications for future aspects.

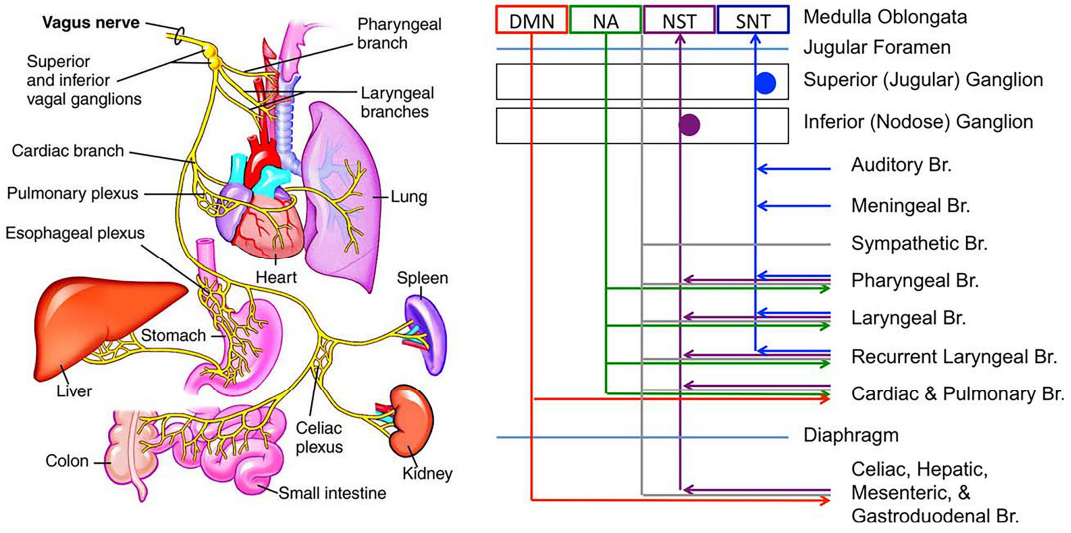
1.3. Hypothesis of the Study

The ONS will stimulate the rat VN like other peripheral nerves in a reliable, repeatable, and safe manner. Besides, this relatively new technique will provide a selective stimulation to potentially eliminate the side effects of conventional electrical vagus nerve stimulation, such as arrhythmia.

2. GENERAL INFORMATION

2.1. Vagus Nerve

The vagus nerve (VN, cranial nerve X), originating from the brainstem in the central nervous system as the longest cranial nerve, innervates to the neck, thorax, abdomen, and colon. The VN has a parasympathetic function on systemic regulations such as HR, AP, vascular resistance, and breathing. It can control and collect information from autonomic nervous, cardiovascular, immune, respiratory, gastrointestinal, and endocrine systems [4].



(a) (b)

Figure 1. (a) Illustration of the VN reaching various internal organs with various branches. (b) Schematic of the VN branches and their vagal nuclei connections thought The Dorsal Motor Nucleus (DMN), Nucleus Ambiguus (NA), Nucleus of the Solitary Tract (NTS) [5].

The VN contains both afferent and efferent fibers that transfer signals both ways. The VN's efferent fibers are responsible for some motor functions like the stimulation of muscles in the pharynx, larynx, and heart.

However, most of the fibers (80%) are afferent sensory fibers that transport information signals from internal organs to the brain. A-type, B-type, and C-type fibers classified for their fiber size and physiological function are also present in the VN. All types of fibers have an essential physiological function for the body. First, A-type fibers are the most prominent myelinated fibers with high conduction velocity (8-120 m/s). The primary function of A-type fibers is the transmission of visceral afferent information and motor input. Second, B-type fibers are tiny myelinated fibers with moderate conduction velocity (3-15 m/s).

Furthermore, parasympathetic inputs are carried by B-type fibers. Finally, type C vagal sensory fibers are tiny unmyelinated fibers responsible for mainly afferent visceral information like pain, stretch, chemical, and temperature signals with low conduction velocity (0.3-6 m/s) [5, 8]. A-type, B-type, and C-type fibers are physiologically and histologically different. Thus, they require numerous parameters to be stimulated. Thus, they require numerous parameters to be stimulated. A- and B-type fibers' lower stimulation thresholds are required for stimulation, while higher stimulation thresholds evoke C-type fibers [9, 10].

Afferent and efferent fibers are located in fascicles that reach or come from different body areas along with the VN. These separate fascicles have different targets like the laryngeal, heart, lung, stomach, and liver (Figure 1). At the moment, the organization of fascicles is almost entirely unknown in the VN bundle. If the VN is selectively stimulated in a short stimulation time, positions and functions of fascicles of the VN have to be determined [11].

Although many fascicles' exact organization is not fully known, some VN fascicles have begun to be studied. There are baroreceptors responsible for continuous monitoring of AP on the wall of the aortic arch. These baroreceptors carry AP information to the brain by the aortic depressor nerve (ADN), which contains afferent and unmyelinated fibers via the VN. In many

mammals, the ADN fascicle has a critical role in controlling the AP and baroreflex. Eventually, the ADN's selective stimulation could reduce AP without side effects like bradycardia and bradypnea, which might be side effects of stimulation of other fascicles in the VN [12]. This situation can only happen if ADN and other fascicles are fully understood.

Afferent neurons of the VN enter the brain, stem mainly from the nucleus tractus solitarius. The nucleus tractus solitarius is a critical neurological site with high interaction with the forebrain and brainstem, including projections into the locus coeruleus through important neurotransmitters [13]. Figure 2 shows the VN and nucleus tractus solitarius projection over hippocampus and amygdala important brain locations for epileptic seizures.

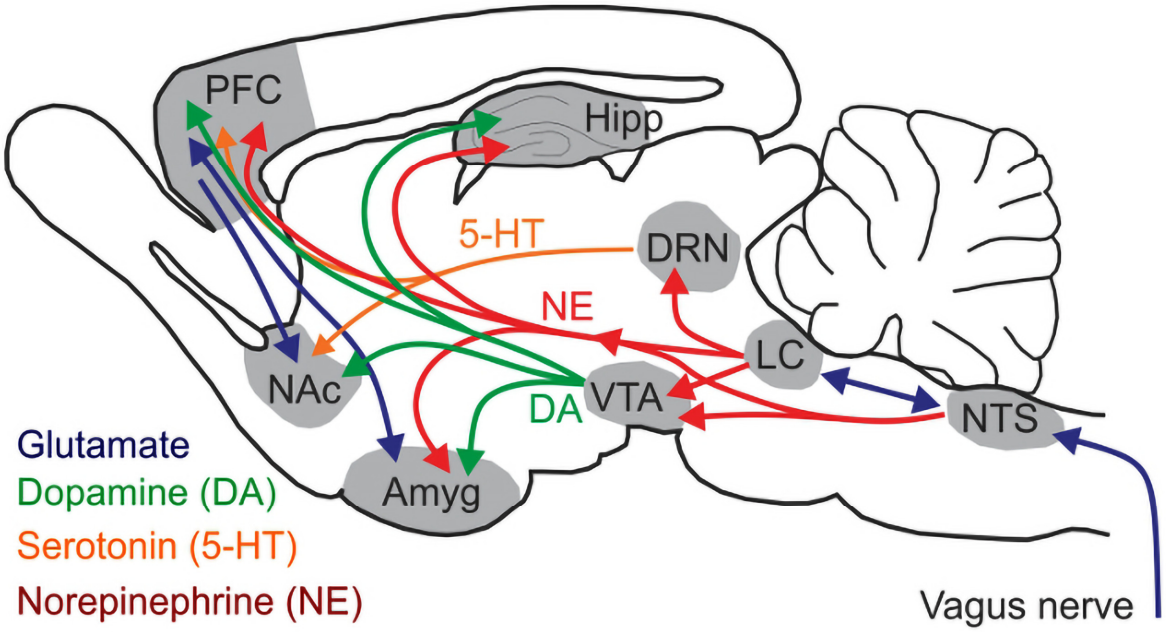


Figure 2. Illustration of the VN reaching different parts of the brain, including the locus coeruleus (LC), the raphe nuclei (DRN), the prefrontal cortex (PFC), the amygdala (Amyg), the hippocampus (Hipp), and the nucleus accumbens (NAc) over the nucleus tractus solitarius (NTS) [14].

These critical two-way communications of the VN with brain and body have become more clinically significant over the years. Thus, the development of improved methods for stimulation of the VN could provide better life quality for cardiac and epilepsy patients at the same time.

2.2. Electrical Nerve Stimulation (ENS)

The ENS is one of the most practical methods for stimulating electrochemically capable cells in electrophysiology. ENS uses mild and repetitive electric currents to initiate action potentials on the cell membranes of neurons. The mechanism of ENS relies on an electrode, which transfers enough electrical current to depolarize the membrane of neurons, and is placed to tissue in contact. Eventually, electric current can easily penetrate the neural target and trigger voltage-dependent ion channels to propagate action potential when anode and cathode are close enough to the tissue [15, 16]. Figure 3 shows an illustration of a hook electrode that is attached to a nerve bundle.

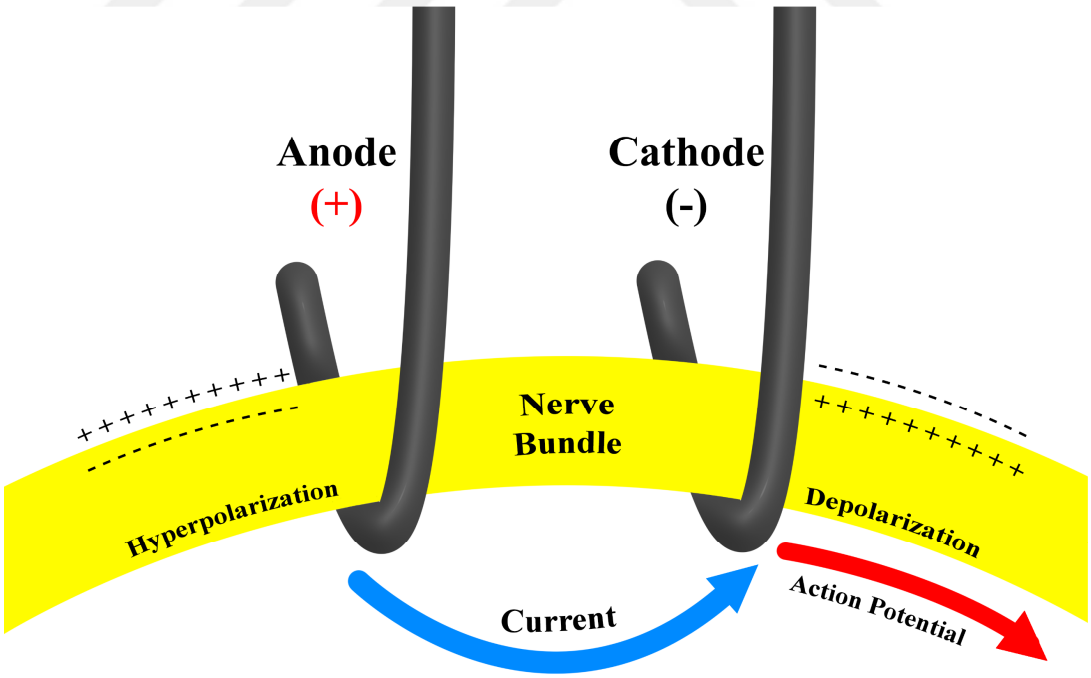


Figure 3. Illustration of a hook electrode and electrical current to initiate action potential in the nerve bundle.

Because ENS has been widely used for many years, the stimulation parameters are well known in the clinic. ENS has many significant examples in clinical approaches. ENS is used in many cases of acute and chronic pain in the clinic, such as muscle pain (muscle tension, post-exercise soreness), postoperative pain, orofacial pain, dysmenorrhea, low back pain, arthritic pain (osteoarthritis, rheumatoid arthritis), neuropathic pain (post-amputation, or trigeminal neuralgia) [17]. ENS can also help treat urinary incontinence cases, which is also called involuntary leakage of urine, by applying electric current to the posterior tibial nerve [18]. Parkinson's patients can also benefit from ENS with Deep Brain Stimulation (DBS). DBS device sends electrical stimuli to a particular part of the brain, responsible for the movement symptoms caused by Parkinson's disease. Electrical stimuli disrupt the abnormal activity in the brain's circuits, causing several symptoms [19]. Despite these significant examples of ENS, the most remarkable technological application is VNS.

2.2.1. Vagus Nerve Stimulation (VNS)

VNS is a non-pharmacological therapy used in patients with resistant epilepsy with no surgical chance. Many studies evaluate VNS's use of drug-resistant epilepsy in terms of seizure frequency and duration, neurocognitive effects, quality of life, tolerability, and safety. Moreover, VNS is limited to drug-resistant epilepsy, but it is also under investigation to treat hypertension, depression, Alzheimer's disease, migraine, and obesity.

The idea of stimulation of the VN was proposed first by an American neurologist, James L. Corning, in 1883. He believed that hyperemia in the brain causes epileptic seizures and can be controlled by decreasing cardiac output [20]. After about half a century, Bailey and Bremer also realized the VN's direct effect on the central nervous system using electroencephalography (EEG) in 1938 [21]. In 1985, Jacob Zabara realized that VNS treatment could control epileptic seizures from early studies exploring the relations between the brain and the VN [22]. Later, in 1992, he showed for the first time that VNS has inhibition effects on pentylenetetrazol-induced tremors and strychnine-induced motor seizures in dogs [23]. The FDA approved the VNS therapy for epilepsy patients aged 12 and older in 1997, after the first successful human trials reported by Penry and

Dean in 1988 for possible treatment of drug-resistant epilepsy [24, 25]. Figure 4 shows an illustration of the VNS device, which is attached to the VN surgically.

Epilepsy seizures are significantly reduced after the administration of VNS in certain patients. Half of the adult patients react to VNS in 4 months. Patients experienced a 50% decrease in seizure frequency during VNS therapy. However, only a few patients become totally seizure-free [26]. Every patient possibly reacts to VNS differently, and the reason lying behind this variety is still unknown.

A possible mechanism of the VNS is related to the stimulation of afferent fibers directly. Stimulated afferent fibers activate norepinephrine pathways over the nucleus tractus solitarius and prevent or reduce upcoming seizures. However, studies are indicating that various neurotransmitters are also involved in the pathways [27]. The general mechanism of the VNS is precise, but still, there are unknown parts of the mechanism.

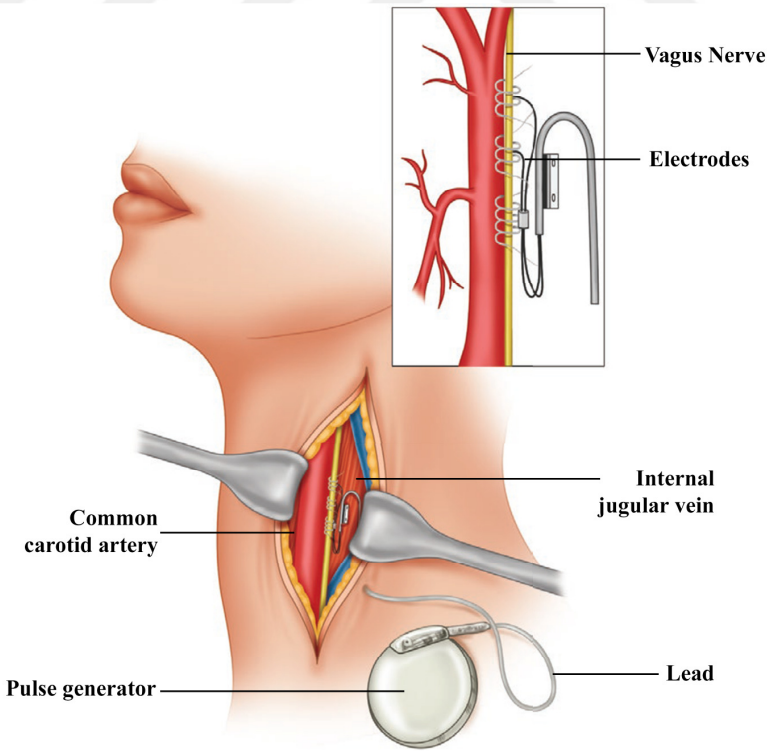


Figure 4. Illustration of the VNS device, including stimulation electrodes, pulse generator, and lead [28].

2.2.2. Stimulation Parameters

Amplitude, pulse width, frequency, and duty cycle of the device are the main parameters that can be adjusted for desired clinic application (Figure 5).

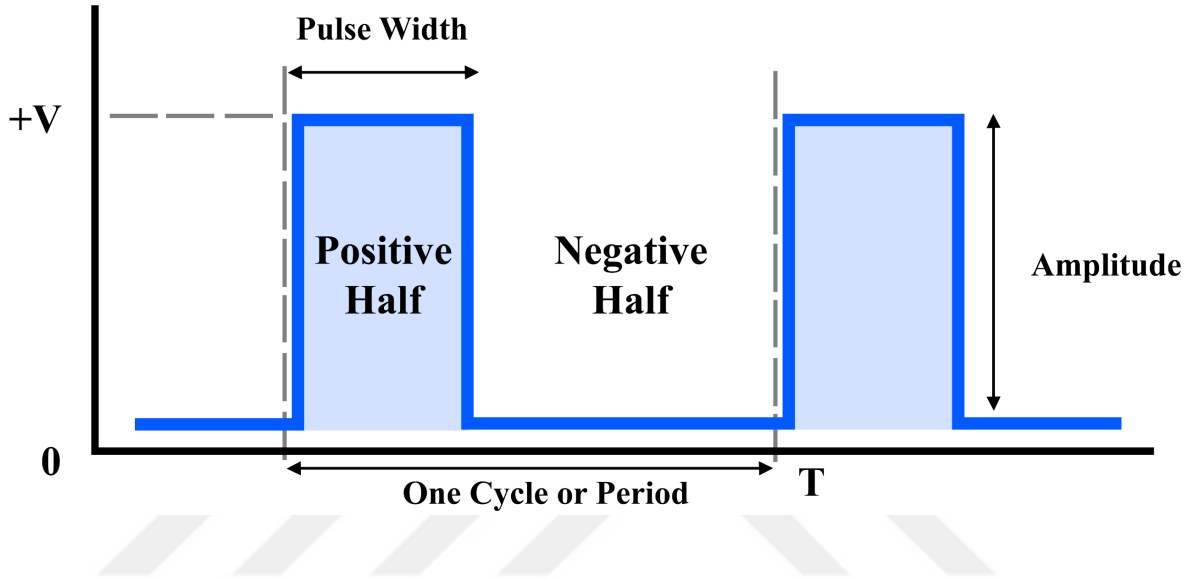


Figure 5. Drawing of stimulation parameters include amplitude, pulse width, and period.

Amplitude (current or voltage) determines the electrical current intensity that is transferred to the nerve tissue. The ability to initiate action potentials depends mainly on the amplitude of the electrical current. Large, A-type nerve fibers need low intensity to be initiated. On the other hand, small diameter C-type nerve fibers need higher intensity [10]. Due to different stimulation thresholds of various nerves, the ENS amplitude is critical for different clinic applications.

Amplitude is a very critical element in VNS therapy because of VN's fiber composition. Because of C type fiber majority, VNS requires high amplitude stimulations that lead to severe side effects, such as arrhythmia, vocal folds, headache, and shortness of breath. The approved range of VNS therapy is between 0.25 and 3.5 mA in current conditions [29].

Pulse width is the elapsed time of a single pulse required to transfer enough current to tissue. If pulse width duration is shorter than the nerve bundle's required activation time, there will be no initiation. If pulse width duration is longer than needed, the VN can be over stimulated and even get damaged (acute and/or chronic).

Frequency determines pulse count in one-second duration. Generally, 20 to 30 Hz stimulation frequencies have been found to provide better stimulation of the VN. Previous researches show that stimulation frequencies under 20 Hz produce less brain activation and high side effects because of the stimulation of slow conducting C fibers [30, 31].

The duty cycle (i.e., ON/OFF cycle) specifies active and inactive periods of stimulators. The typical duty cycle is 30 seconds ON and 5 minutes in epilepsy treatment. All these parameters can be adjusted for specific conditions in the clinic.

Table 1. Electrical parameters used for successful VNS therapy.

Parameter	Unit	Range	Suggested value
Current	Milliamperes	0-3.5	1.50–2.25
Pulse width	Microseconds	130-1000	250
Frequency	Hertz	1-30	20
ON time	Seconds	7-60	30
OFF time	Minutes	0.2-180	5

2.2.3. Side (Adverse) Effects of VNS

Despite the success of VNS, some limitations to this technique also exist. The available technology of the bipolar electrodes used in therapy cannot provide selective stimulation of the vagus nerve. Stimulation of the whole fascicles in the bundle decreases the effectiveness of the therapy and causes side effects. Most of the adverse effects may be related to the electric stimulation itself, and even VNS surgery can cause complications, including infections and pain in

the implantation site. Coughing, chest and throat pain, headaches, and most importantly, arrhythmia and bradycardia are examples of significant side effects that reduce the patient's life quality [7, 32].

Recent works of many research groups have attempted to solve the side effects of VNS therapy by selective stimulation using multiple electrodes [33]. However, although new electrode approaches have made valuable contributions, the electrical current induced constraints caused may persist until a fundamental change in stimulation methodology is achieved.

2.3. Optical Nerve Stimulation (ONS)

Optical nerve stimulation is a potential alternative that uses an infrared laser's heating capacity to evoke nerve cells instead of electricity without extra modification or genetic manipulation [34]. The relatively new technique offers three main advantages over conventional ENS:

1. ONS can provide a non-contact stimulation for target tissue because infrared light can be delivered in free space without any physical contact.

2. ONS's improved spatial selectivity can overcome the low precision of standard metal electrodes because the infrared laser can be focused on target tissue with the desired diameter.

3. Electric current tends to spread out through the tissue, which also reduces the spatial selectivity of ENS.

Figure 6 shows an illustration of the comparison of spatial selectivity in ONS and ENS. Red and blue lines indicate specific fascicles in the nerve bundle. ONS can stimulate the red fascicle without disturbing the blue fascicle with enhanced spatial selectivity. However, there is no defined selectivity in ENS, which causes stimulation of both fascicles. Besides, ONS avoids electrical artifacts that can interfere with electrophysiological measurements such as electrocardiography

(ECG), electromyography (EMG), electroencephalography (EEG), and electroneurography (ENG) and impair the function of other tissues and organs [1, 35].

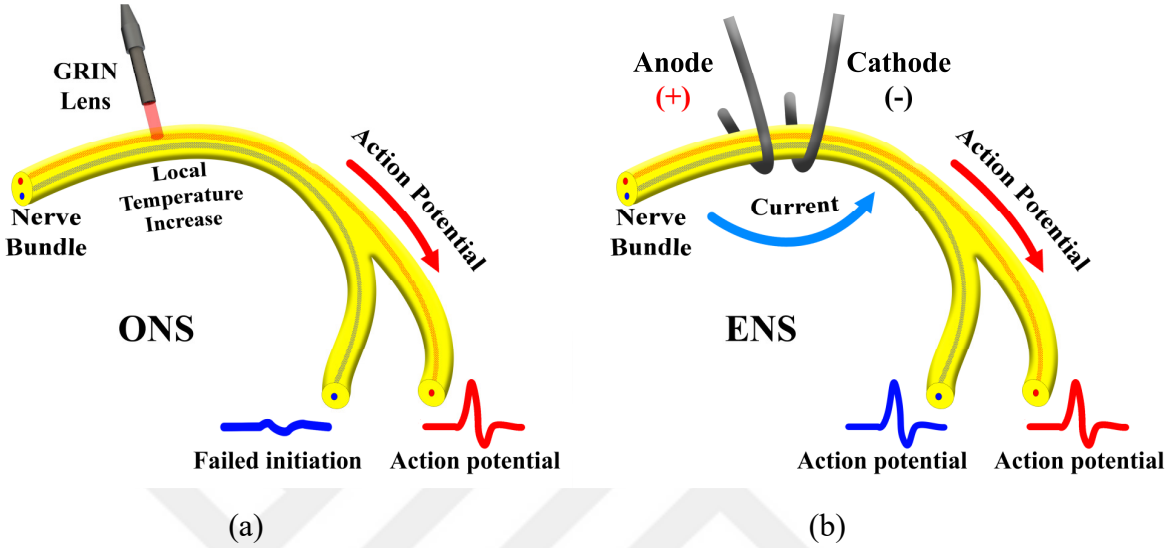


Figure 6. A representative comparison of spatial selectivity in optic nerve stimulation (ONS) and electrical nerve stimulation (ENS). (a) Spatial selectivity of ONS. (b) Spatial selectivity of ENS.

Wells et al. explored possible mechanisms, including photochemical, photothermal, and photomechanical interactions. The data suggested that ONS requires a temperature increase of 6-10 °C in the peripheral nerve and 3.8-6.4 °C at the axon level to generate direct or indirect action potentials [36]. This result proposed the possibility that the mechanism was photothermal interaction. One of the more in-depth explanations about the mechanism was that heat-sensitive ion channels respond to the photothermal interaction. Briefly, it has been suggested that transient receptor potential vanilloid (TRPV) channels are triggered using laser-induced heat, while triggered TRPV channels create an electrical potential that opens voltage-gated ion channels to maintain the action potential. Since TRPV channels are expressed in many tissues such as peripheral nerves, central nervous system, gastrointestinal tract, and lungs, TRPV channels have been considered to be the underlying mechanism of ONS [37]. Redistribution of cell membrane capacitance due to local temperature rise was another explanation that could justify the ONS mechanism in depth. Accordingly, the photothermal effect could change the cell membrane's electrical capacitance by affecting the 3-dimensional shape and fluidity. Thus, it was thought that

a change in capacitance could cause a change in the electric potential of the cell membrane, creating an action potential through voltage-gated ion channels [38]. While these studies highlight the possible mechanisms of ONS, we are still far from reaching a consensus view to end being highly controversial.

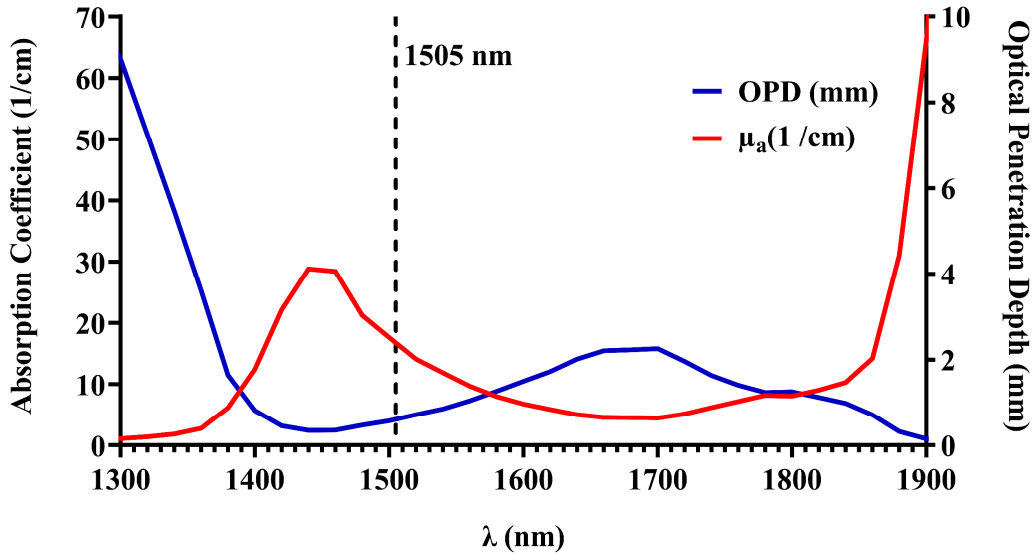


Figure 7. Plot of the absorption coefficient (μa) of water and the corresponding optical penetration depth (OPD) as a function of the optical wavelength.

Optical penetration depth (OPD) and absorption coefficient are the basic parameters that determine a given wavelength's efficiency in photothermal applications. The absorption coefficient, or the corresponding OPD, determines how deep the light travels at a particular wavelength in the tissue. Therefore, wavelength selection is very critical in ONS. For example, a high absorption coefficient can prevent the light from reaching the desired depth and eventually the targeted fascicles, causing the nerve tissue to overheat and be damaged within a specific power range. On the other hand, a low absorption coefficient can cause the neural tissue not to heat sufficiently. Figure 7 shows a plot of the absorption coefficient of water, the primary absorber in soft tissue, and the corresponding optical penetration depth (OPD) as a function of the optical wavelength.

Recent studies have mainly been conducted in wavelengths between 1875 nm and 2120 nm (Table 2). Even though researchers have used laser sources in a specific range of the infrared spectrum, as we have shown in this study, telecom wavelengths (1400 nm - 1600 nm) can be successfully applied with greater flexibility. On the other hand, the ONS technique has been successfully demonstrated in various samples with different neural targets, including central and peripheral nervous systems [39]. The sciatic nerve, cavernous nerve, auditory nerve, and visual cortex can be listed as some examples (Table 2). Despite all the primary targets, oVNS has not been investigated in a rat model comprehensively as in this study.

Table 2. Examples of ONS.

Author	Neural Target	Wavelength (nm)	Pulse Length (ms)
Wells [34]	Rat sciatic nerve	2120, 2000	0.25
Wells [40]	Rat sciatic nerve	2120	0.35
Wells [16]	Rat sciatic nerve	2120	0.35
Teudt [3]	Gerbil facial nerve	2120	0.25
Fried [41]	Rat cavernous nerve	1870	2.5
Fried [42]	Rat cavernous nerve	1850 – 1880	2.5
Jenkins [43]	Embryonic quail heart	1875	2
Jenkins [44]	Adult rabbit heart	1851	2.5 – 12
Cayce [45]	Rat somatosensory cortex	1875	0.25
Izzo [46]	Gerbil cochlea	2120	0.25
Izzo [2]	Gerbil cochlea	1844 – 1873	0.035 – 1
Izzo [47]	Gerbil cochlea	1923 – 1937	0.05 – 0.3
Richter [48]	Gerbil cochlea	1844 – 1873	0.03 – 1.6
Duke [49]	Rat sciatic nerve	1875	2
Duke [50]	Aplysia buccal nerve	1875	2 – 3
Tozburun [51-64]	Rat cavernous nerve	1455-1870	CW

3. MATERIALS AND METHODS

3.1. Type of the Study

This study is an experimental type.

3.2. Time and Location of the Study

The ONS setup for rat studies and optimization of the optical parameters were completed in Tozburun Laboratory at Izmir Biomedicine and Genome Center between May 2019 and September 2019. All animal experiments were carried out between September 2019 and November 2020 in Multidisciplinary Laboratory: Animal Experiments at Dokuz Eylül University in Pharmacology at Dokuz Eylül University. Histological preparation of VN samples was done in the histopathology unit at Izmir International Biomedicine and Genome Center in December 2020.

3.3. The Universe and Sample of Research

No human specimens were used in this study.

3.4. Working Materials

3.4.1. Animal Model

Male Wistar rats (n=14) weighing 250-400 grams were purchased from the Center for Research on Laboratory Animals (EGEHAYMER) at Ege University. Rats were housed in Multidisciplinary Laboratory: Animal Experiments at Dokuz Eylül University with controlled temperature and light periods. Ad libitum rat chow and water were provided. All animal studies

were carried out under an animal protocol (protocol number: 04/2019) approved by Dokuz Eylül University Multidisciplinary Laboratory Animal Experiments Local Ethics Committee. Rats received anesthetization with urethane (1200 mg/kg) by intraperitoneal injection [65]. and were secured in a supine position on a surgical table. After shaving the jugular area of the animal, it was sterilized with an antiseptic iodine solution.

A four to five centimeters incision was applied to the trachea far left from the hyoid clavicles. Salivary glands, mandibular glands, and connective tissue were cleaned until the sternomastoid, sternohyoid, and omohyoid muscles became visible. Meanwhile, the trachea of the rat was cannulated to provide better respiration. The small cleft between sternomastoid and sternohyoid muscles was expanded using blunt dissection to identify the carotid artery and the VN located just behind the sternomastoid muscle. The left VN and carotid artery were separated from each other. The right carotid artery was isolated to measure systemic blood pressure. The left and right VN were hanged to the hook stimulation electrode separately for ENS [14, 66, 67]. A GRIN lens targeted a red laser beam to the VN for ONS using infrared laser radiation. After the experiments were completed, all animals were euthanized by bilateral thoracotomy under deep anesthesia.

3.4.2. Electrical Stimulation of the Vagus Nerve

Following the standard methodology followed in the ENS, stainless steel stimulating electrodes (FGT35, Commat Ltd., Ankara, Turkey) was placed under the VN. Electrodes connected to an MP36 (Biopac Systems Inc., Santa Barbara, CA) data acquisition system incorporating a BSLSTMB stimulator (Biopac Systems Inc., Santa Barbara, CA). For a successful ENS, 3 V electrical pulses with a pulse duration of 1-millisecond at 20 Hz repetition rate for 30 seconds were applied to the VN (Figure 8). Similar to previous studies, the experiments used an amplitude set of 3 V as a threshold to generate an immediate and consistent bradycardic response. Considering the resistance applied by the electrodes (assuming negligible resistance of VN), it is estimated that 3

V electrical pulses provide an electrical current in the range of 0.75-1.5 mA [68]. Although current values are used instead of voltage in reporting of VNS, we preferred to note the voltage value since the BSLSTMB stimulator is set with voltage.

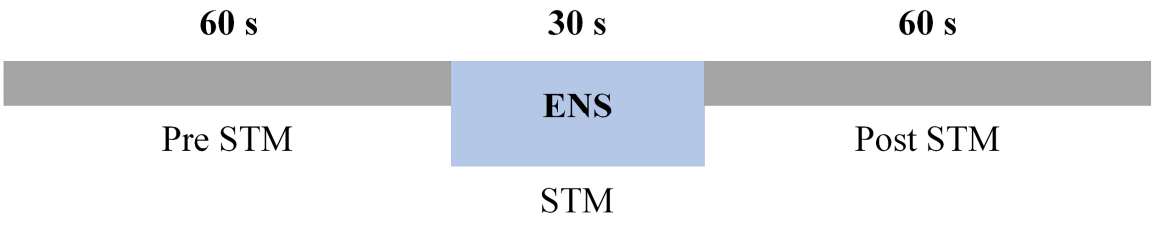


Figure 8. Experimental timeline of ENS sequence: 30-seconds stimulation, 60-seconds pre-stimulation, and 60-seconds post-stimulation.

3.4.3. Optical Stimulation

The all-single-mode fiber ONS system was designed to contain two different laser types, as shown in Figure 9. The first laser, a pigtailed, single-mode, diode infrared laser (BrightLock, QPC Lasers, Sylmar, CA), was used to stimulate the rat VN at a 1505 nm wavelength in CW mode. Two drivers (LD2.5CHA and LD5CHA, Wavelength Electronics, Inc, MT) controlled the near-infrared diode laser across the oscillator and amplifier sections. A thermoelectric cooler (TEC) provided thermal management of the diode (PTC5K-CH 5A, Wavelength Electronics, Inc, MT). The second laser, a 20-mW red diode laser (BT-VFL650-20, Beek Electronics, Ankara, Turkey) at a wavelength of 661 nm, was used as an aiming laser to confirm the location of the infrared laser beam on the surface of the nerve. A single-mode fiber coupler (TN1310R1A2, Thorlabs, Newton, NJ) with a compound ratio of 1:99 was used to combine both lasers into one fiber output. Infrared and red laser beams were delivered to the surface using a GRIN lens (50-1310-APC, Thorlabs, Newton, NJ), which provides a Gaussian laser beam with a diameter of approximately 0.5 mm on the nerve (Figure 9). Figure 10 shows a representative photograph of the ONS system developed for rat VN studies.

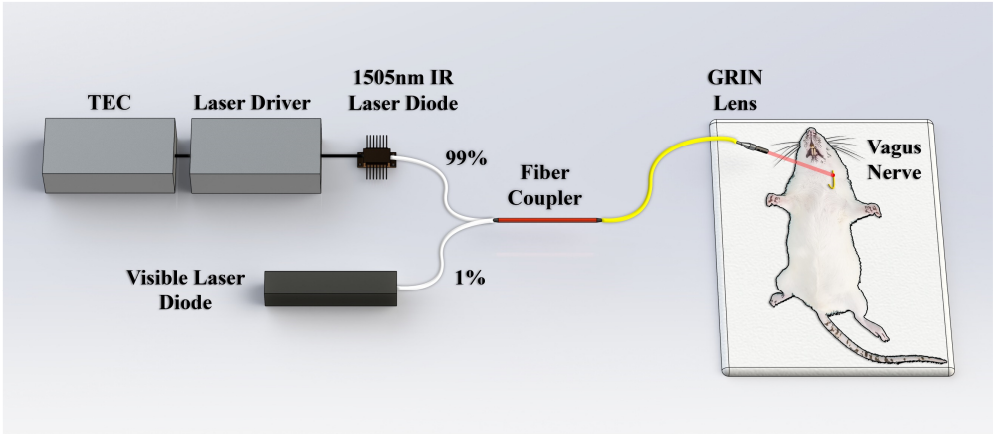


Figure 9. Schematic of a custom-build ONS system consisting of a laser driver, a 1/99 fiber coupler, laser diodes, and a GRIN lens.

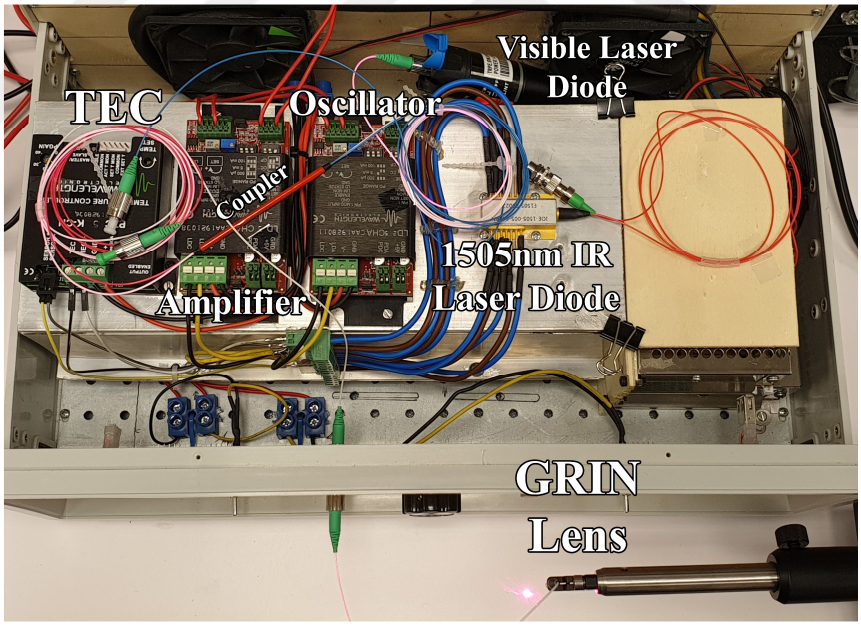


Figure 10. Photograph of custom built ONS system with TEC, amplifier, oscillator, fiber coupler, 1505 nm laser diode, visible laser diode, and GRIN lens.

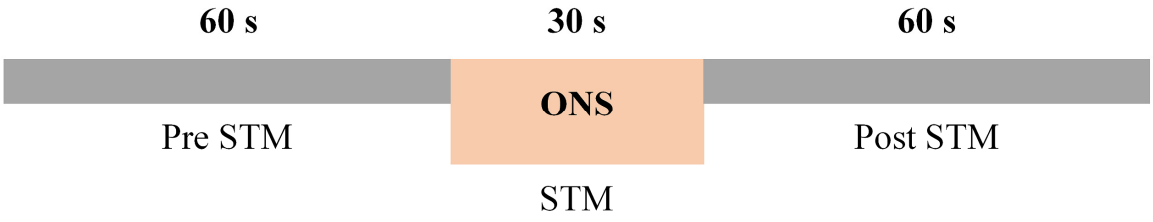
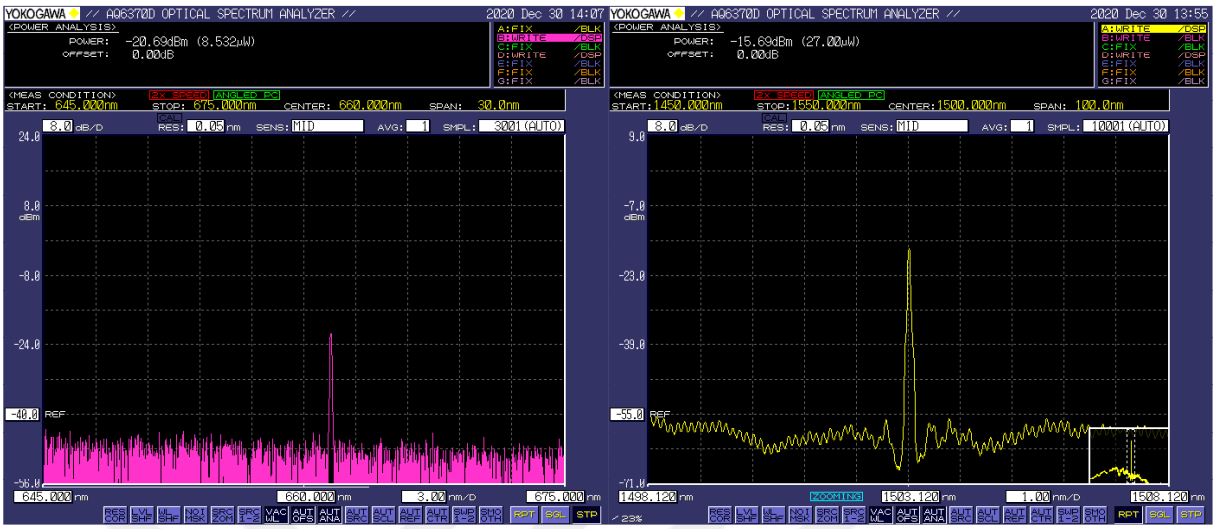


Figure 11: Experimental timeline of ONS sequence: 30-seconds stimulation, 60-seconds pre-stimulation, and 60-seconds post-stimulation.

Figure 11 shows an experimental timeline of the ONS sequence. The IR laser was applied to the VN with an irradiation time of 30 seconds. The laser power was adjusted by gradually increasing to reach the nerve's temperature above the stimulation threshold level. Optimization and characterization of laser parameters (i.e., laser wavelength, output power, beam diameter) were carried out to produce reliable and repeatable oVNS results.

ONS will need safe and repeatable results. Therefore, laser parameters' optimization and characterization of the system has a critical role in nerve stimulation due to the nerves' sensitive structure.

First, the stimulation laser's wavelength and the aiming laser's wavelength were verified with a spectrum analyzer (AQ6370D, Yokogawa Electric, Tokyo, Japan). Figure 12 shows the transient results of the spectrum measurements. The wavelength measured as 1503-nm was very close to the intended stimulation wavelength. With the help of Figure 7, it was estimated to provide >0.55 mm optical penetration depth (the corresponding absorption coefficient was ~ 17.6 1 / cm) in water. The estimated depth matched closely with the rat VN's diameter (300 μm - 500 μm).



(a) (b)

Figure 12: The emission spectrum images of (a) 1505 nm IR laser diode and (b) visible laser diode in spectrum analyzer.

Second, measurements of the output power transmitted through the GRIN lens were performed. Figure 13 shows a plot of the output power as a function of applied electrical current. The maximum output power of the 1505 nm laser was 500 mW. However, the power measurement was limited as the GRIN lens's optical damage threshold was about 300 mW. As described in the literature [34, 51, 69], the optical power required to create the stimulation temperature window (40° C-50 °C) for ONS was determined to be between 10.6 mW and 42.5 mW. This range was easily accessible with the 1505-nm laser, as shown in Figure 13. Besides, the total optical loss of the ONS system was calculated as <20%.

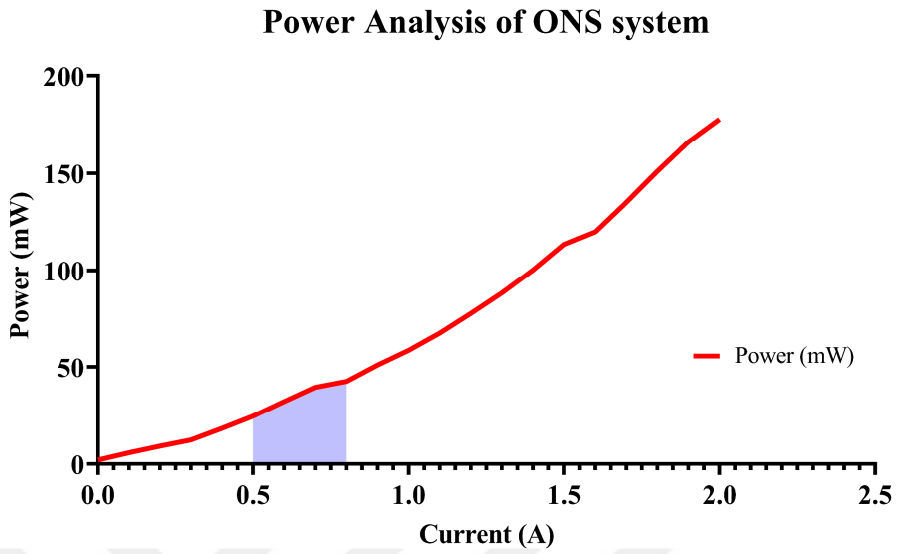


Figure 13. Plot of the output power of the ONS system as a function of applied electrical current. The blue area indicates the required power range during ONS studies.

In the third study, the diameter of the Gaussian laser beam produced by a GRIN lens was defined by the knife-edge method at different working distances (WD). Figure 14 shows the estimated diameter of the Gaussian beam as a function of the working distance. The GRIN lens provided a 0.5-mm-diameter, collimated laser beam at a distance of 15 mm to 50 mm. The distance between the GRIN lens and the VN was at most 20 mm throughout the animal experiments.

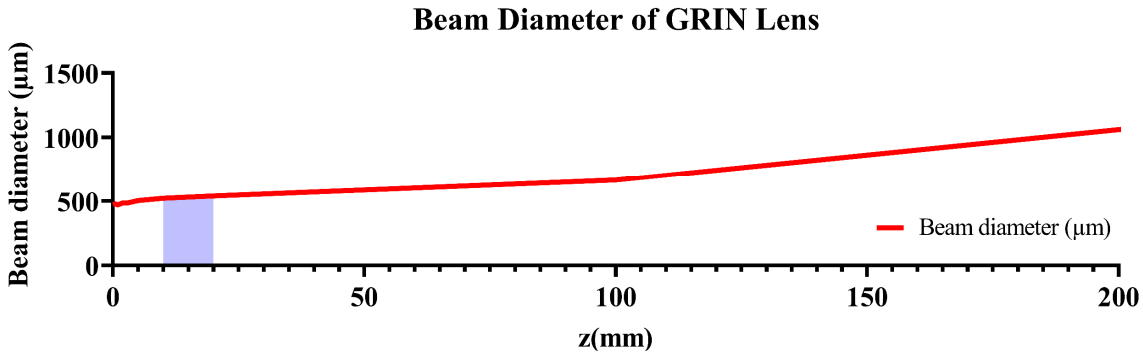


Figure 14. Estimated diameter (1/e2) of the Gaussian beam produced by a GRIN lens as a function of the working distance. The blue area indicates the working distance between the GRIN lens and the VN during experiments.

Temperature measurements from the rat VN's surface were critical for detecting acute thermal damage occurring during ONS and maintaining the nerve's healthy structures. Therefore, a thermal camera (Xi400, Optris GmbH, Berlin, Germany) was integrated into the set-up to provide real-time temperature mapping of the VN during laser irradiation. Table 3 summarizes the technical specifications of the Xi400 thermal camera.

Table 3. Technical Specifications of the thermal cameras.

Optical resolution	382 x 288 pixels
Detector	FPA, uncooled (17 µm pitch)
Spectral range	8 – 14 µm
Temperature ranges	–20 ... 100 °C, 0 ... 250 °C, (20) 150 ... 900 °C
Optics (FOV)	18° x 14° (f = 20)
Thermal sensitivity (NETD)	80 mK
Optical resolution (D:S)	390:1 (18° optics)

The thermal camera provided sufficient optical and digital resolution to capture the rat VN's small structure at 2.5 pixels. Horizontal field of view (HFOV) was measured as 68.8 mm, vertical field of view (VFOV) as 51.1 mm, and instantaneous field of view (IFOV) as 0.18 mm. The field of view (MFOV) was reduced to 0.54 mm at a 200 mm working distance (Figure 15).

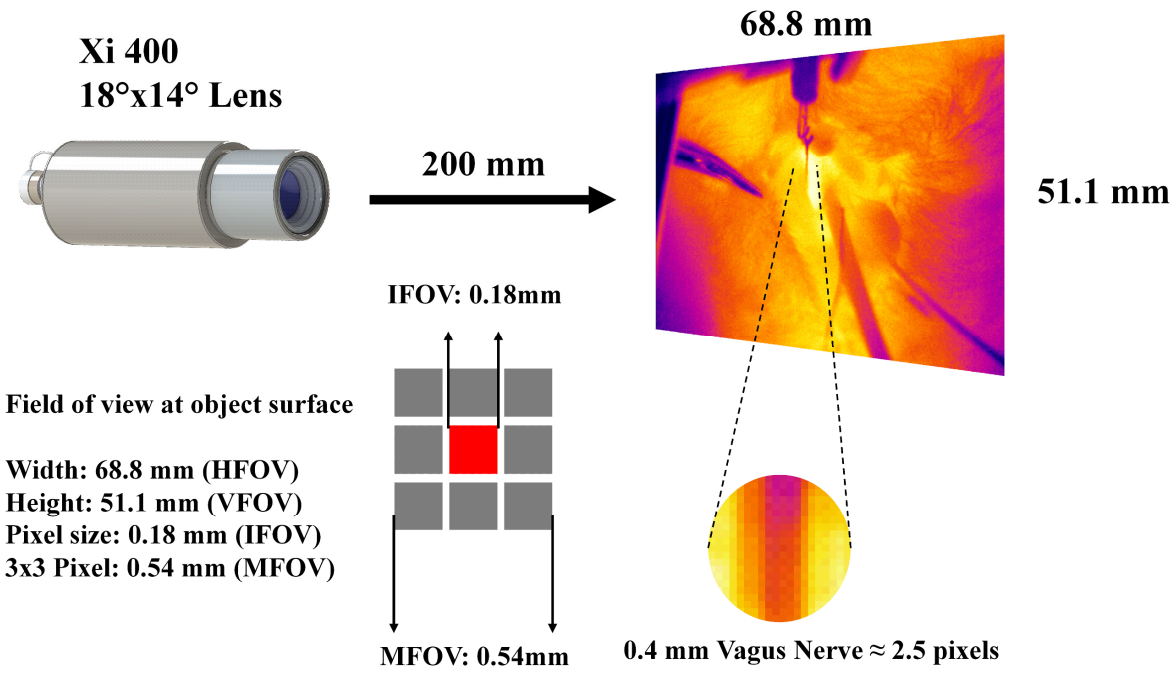


Figure 15. The camera provided a 0.18 mm pixel size. The vagus nerve could be imaged by around 2.5 pixels at a 200 mm working distance.

Visual and auditory alarms were set from thermal camera software (PIX Connect, Optris GmbH, Berlin, Germany) to prevent overheating VN and possible thermal damage. Diagram and photograph of this study's experimental setup, including stimulation and recording systems, are shown in Figure 16 and Figure 17, respectively.

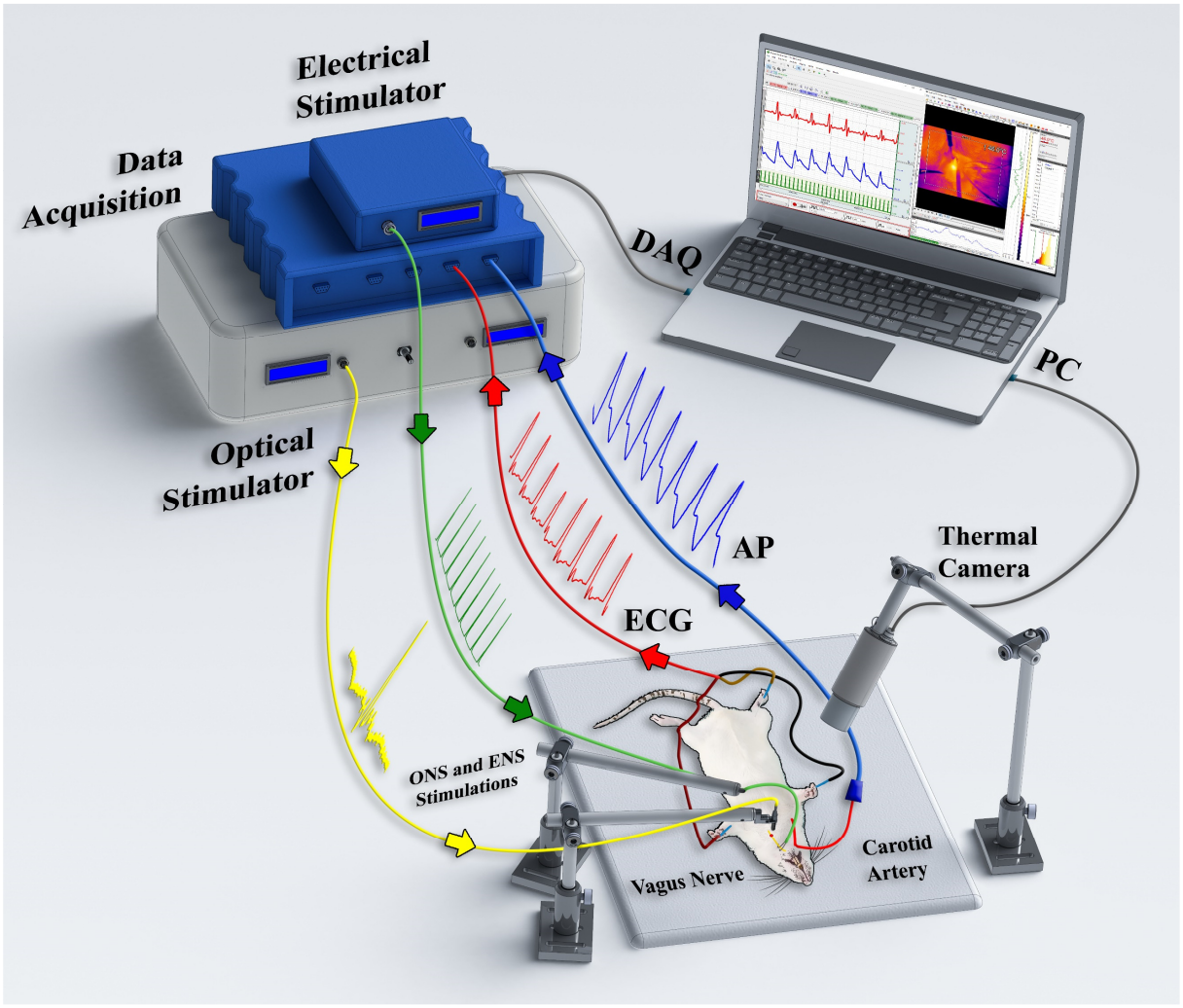


Figure 16. Diagram of experimental setup with ONS, ENS, and measurement tools.

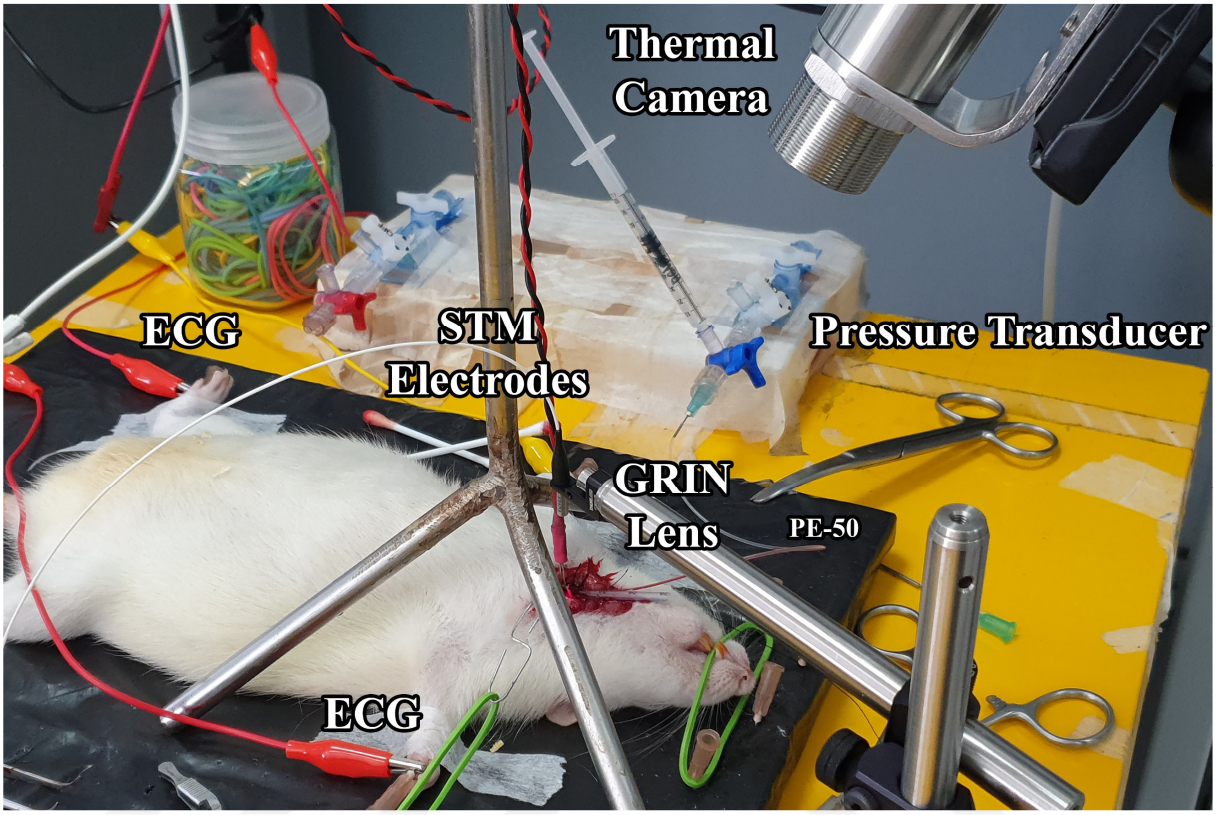


Figure 17. Photograph surgical preparation of rats for ENS and ONS, including a thermal camera for real-time temperature measurements, electrodes for ECG measurements, and a pressure transducer for AP measurements.

3.5. Variables of the Study

The study's variables decrease arterial pressure (AP) and changes in electrocardiography (ECG) pattern during ENS and ONS of the VN.

3.6. Data Collection Tools

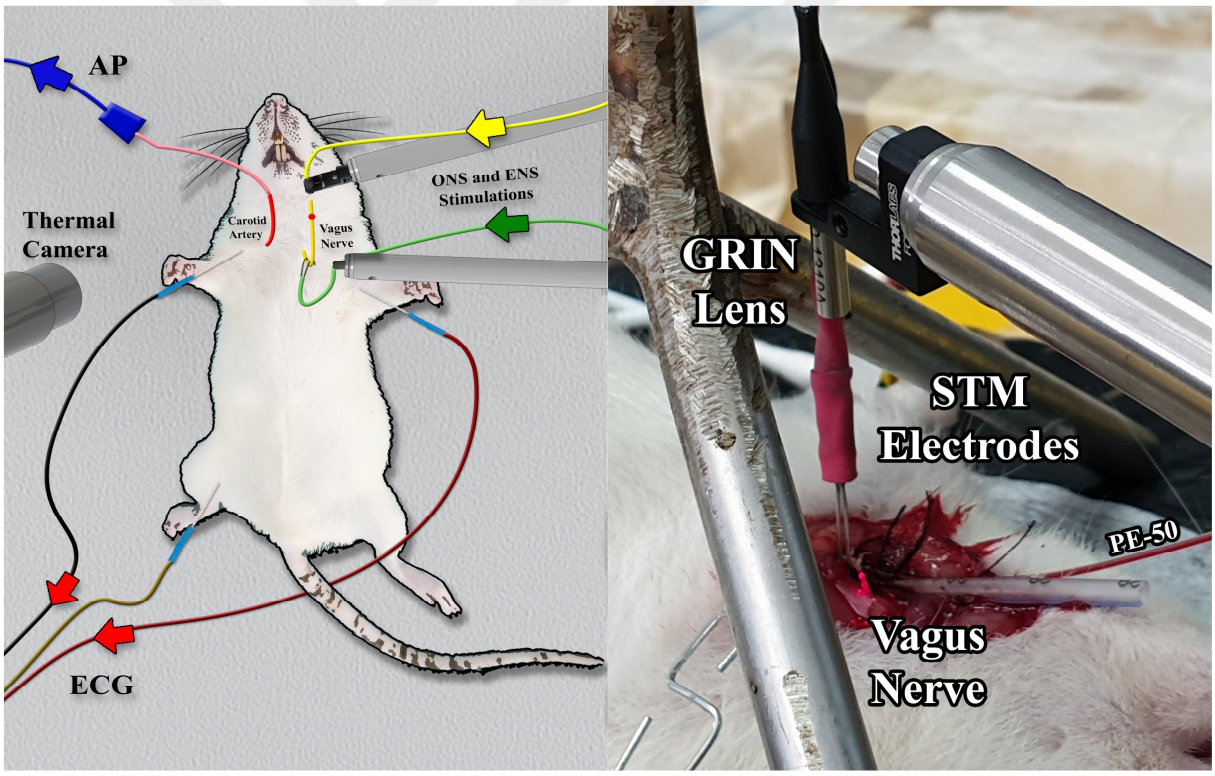
The MP36 (Biopac Systems Inc., Santa Barbara, CA) data acquisition system was used to obtain AP and ECG measurements. Table 4 shows the general characteristics of the system designed for physiological measurements.

Table 4. Technical Specifications of MP36.

Number of Channels:	Isolated human-safe universal input amplifiers 4 Channels
A/D Sampling Resolution:	24-bit
Gain Ranges:	5x to 50,000x (13 steps)
Pulse Output:	Width: variable, 50 µsec 100 msec Repetition: variable. 100 µsec 5 seconds
Pulse Level:	Adjustable from -10 V to +10 V With BSLSTMB Stimulator: 0 100 V
Signal to Noise Ratio	> 89 dB min
Hardware Filters:	Low pass 20 KHz (MP36); 8 KHz (MP45) High pass DC, 0.05 Hz, 0.5 Hz, 5 Hz
Sample Rate:	100,000 samples/sec each channel
Certification:	Complies with IEC 60601-1 EMC complies with IEC 60601-1-2 CE Marked

3.6.1. Arterial Pressure (AP)

As shown in Figure 18, systemic blood pressure was obtained in the right carotid artery. P-50 intravenous cannula (Commat Ltd., Ankara, Turkey), three-way valve to a pressure transducer for measuring the arterial pressure (SS13L, Biopac Systems Inc., Santa Barbara, CA) were ligated. An MP36 system provided a 1-kHz channel sampling rate, 1000X amplification, and DC high pass filtering. Both intravenous cannula and pressure transducer were filled with heparinize containing saline solution (100 IU/mL) to prevent clotting, and the pressure transducer was calibrated before each experiment. Pulsatile arterial pressure (PAP) and mean arterial pressure (MAP) were determined using BSL Analysis 4.1 software (BioPac Systems Inc., Santa Barbara, CA).



(a) (b)

Figure 18. (a) Close up diagram and (b) photograph of the experimental setup with ONS, ENS, and measurement tools.

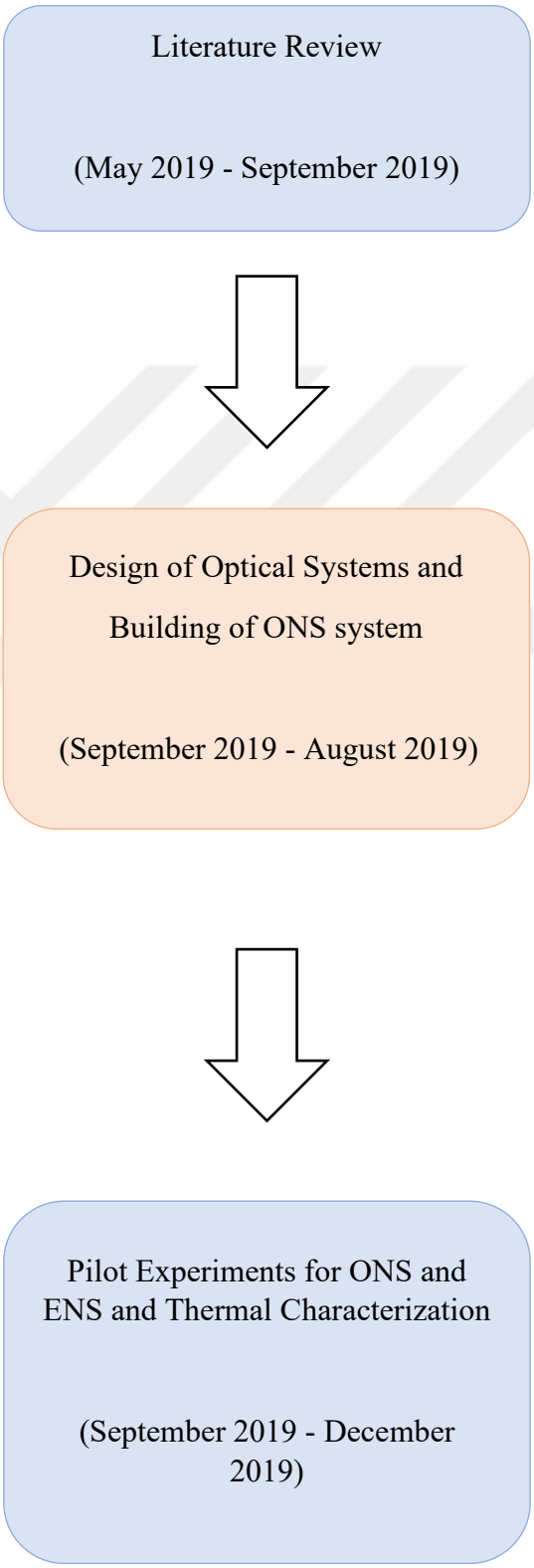
3.6.2. Electrocardiography Recordings and Heart Rate Variability Analysis

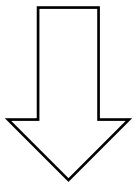
ECG measurements were recorded with three stainless steel electrodes (Natus Neurology Inc., Middleton, WI), inserted subcutaneously into the right foreleg, left foreleg, and right hindleg (Figure 18). An MP36 system acquired ECG signals with a 1 kHz channel sampling rate, 1000X amplification, and 0.05-35 Hz notch band-pass filtering. Analysis of heart rate variability from raw ECG signals was performed in Kubios software (Kubios HRV. 3.4.2, Biosignal Analysis and Medical Imaging Group, Kuopio, Finland). Kubios software offered a wide variety of analysis options in the time domain, frequency domain, and nonlinear results. The standard deviation of all R–R intervals (SDNN), root-mean-square of successive differences (RMSSD), and the ratio between low and high frequencies (HF/LF) were thought to make a direct comparison between electrical and optical rat vagus nerve stimulation.

3.6.3. Histological examination of Vagus Nerves for thermal damage

Animals were prepared for ONS by the previously described surgical procedure. Each VN was stimulated with different laser power until it reached the stimulation threshold temperature (40° C, 42 °C, 44 °C, 46 °C, 48 °C, 50 °C). The stimulation time (ON-time) was 30 seconds, and the post-stimulation time (OFF-time) was 5 minutes. All VN bundles were stimulated 12 times at the same VN for approximately 1 hour to test for possible long-term optical stimulation. The stimulation area was ensured with a visible red aiming laser. The VN bundle was harvested following euthanasia as control samples from an untreated animal and as samples after ONS at different surface temperatures. Both the right and left VN were fixed with 10% buffered formaldehyde and embedded in paraffin to prepare the sectioning process. Paraffin blocks were then cut into 4-5 μm longitudinal sections, and the specimen were stained with hematoxylin-eosin (H&E) dye for further microscopic examination. Bright-field and UV fluorescence images of the sections were acquired using a BX61 Fluorescence Microscope (Olympus Corporation, Tokyo, Japan).

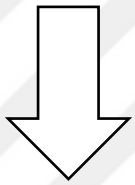
3.7. Research Plan





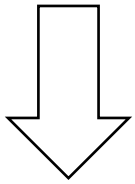
Covid-19 Restrictions

(February 2020 – July 2020)



ONS and ENS experiments of the VN

(July 2020– November 2020)



Histological Analysis of the VN

(November 2020 – December 2020)

3.8. Data Evaluation

Ordinary one-way analysis of variance (ANOVA) for multi-grouped data and unpaired t-test for two-group data was performed to assess statistical significance. Values of $P < 0.05$ were taken into account. Data were analyzed and plotted using GraphPad software (GraphPad Prism version 8.4.2 for Windows, GraphPad Software, La Jolla, California USA).

3.9. Limitations of the Study

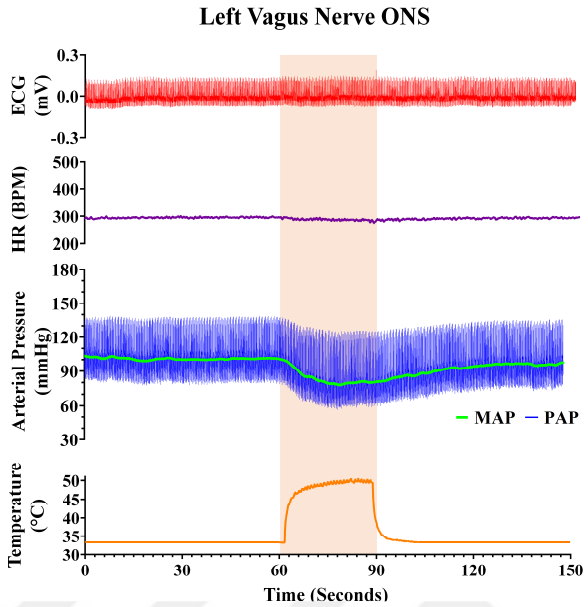
This study has some limitations. First, the effect of anesthesia on oVNS is not well known. Results could be varied using different anesthetized or non-anesthetized rats. Preliminary experiments of this study were initiated with Ketamine/Xylazine (K/X) anesthesia. However, K/X anesthesia had adverse effects on cardiac responses and prevented successful and reliable oVNS due to an unknown pharmacological mechanism. For these reasons, urethane anesthesia was preferred. Second, rats should be euthanized after the procedures due to arterial pressure measurement surgery and carcinogenic urethane anesthesia. This limitation restricted the chronic investigation of oVNS and its possible adverse effects. Third, only cardiac responses were evaluated in this study. Various measurements such as electroencephalogram or electromyogram and measurements of the bowel system could provide another aspect of the study. Also, various measurement techniques can broaden the application of oVNS.

4. RESULTS

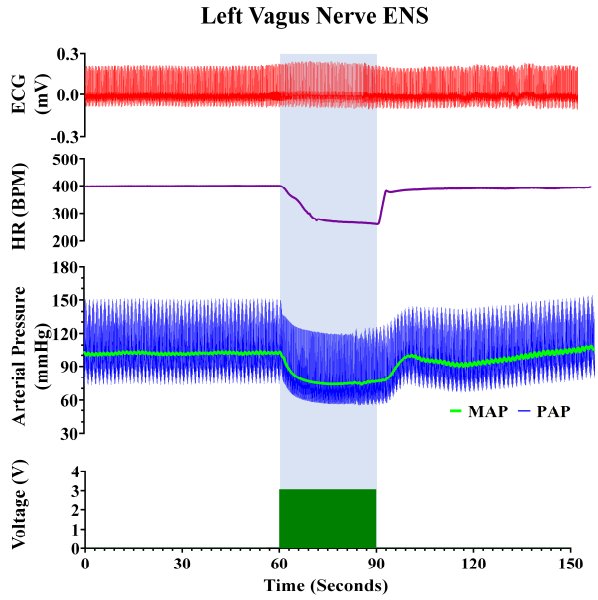
In this thesis, optical nerve stimulation of the rat VN was investigated and compared with electrical nerve stimulation in terms of arterial pressure, heart rate, and heart rate variability. Histological analysis was done to examine the laser-induced thermal damage threshold. The results were obtained from 188 ONS and 54 ENS experimental tries in 14 rats.

4.1 Effect of ONS and ENS on the VN

Both CW infrared laser at 1505 nm wavelength and electricity were able to stimulate the rat VN. Figure 19 and Figure 20 show a representative example of ECG, HR, PAP, and MAP results during electrical and optical stimulation of the right- and left-sided rat VN. The results show the effectiveness of both techniques. As shown in Figure 19 and Figure 20, when the VN was irradiated with a 40 mW infrared laser, the VN's temperature exceeded the stimulation threshold and caused a significant decrease in arterial pressure without altering ECG pattern and arterial pressure. A significant decrease in arterial pressure was observed in ENS (3V, 20Hz repetition rate, 1-milliseconds pulse width). However, ENS caused bradycardia and arrhythmia as expected.

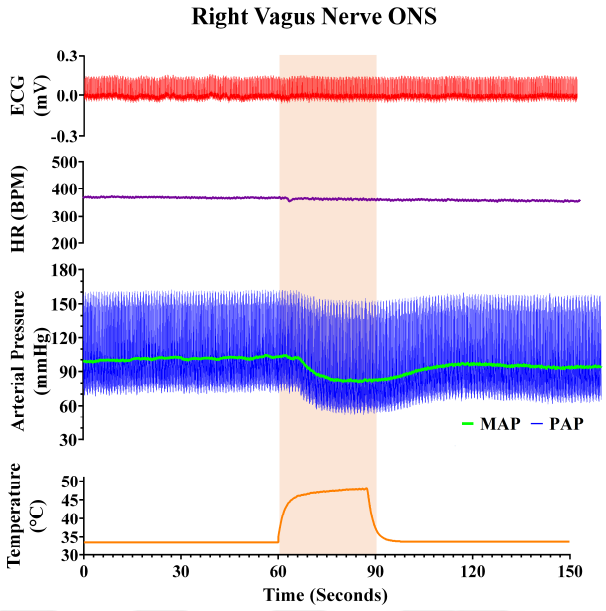


(a)

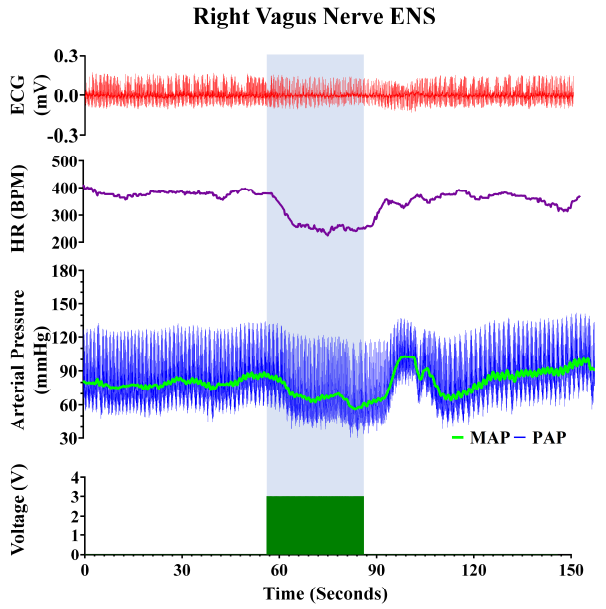


(b)

Figure 19. Typical responses to (a) ONS and (b) ENS of the left-sided rat VN. ECG, HR, PAP, MAP recordings, and simulation signals are shown from top to bottom. Orange and blue areas indicate stimulation periods.



(a)



(b)

Figure 20. Typical responses to (a) ONS and (b) ENS of the right-sided rat VN. ECG, HR, PAP, MAP recordings, and simulation signals are shown from top to bottom. Orange and blue areas indicate stimulation periods.

Figure 21 shows representative MAP responses during ONS more detailed in terms of time. ONS caused a significant decrease (19.04 mmHg) in MAP after about 4 - 6 s after laser irradiation began. 40 mW laser irradiation was elevating the VN above a threshold temperature of ~ 42 °C. The VN reached the maximum surface temperature of 47 °C at the end of the optical stimulation, 30 s. After the laser was off, MAP returned to baseline level.

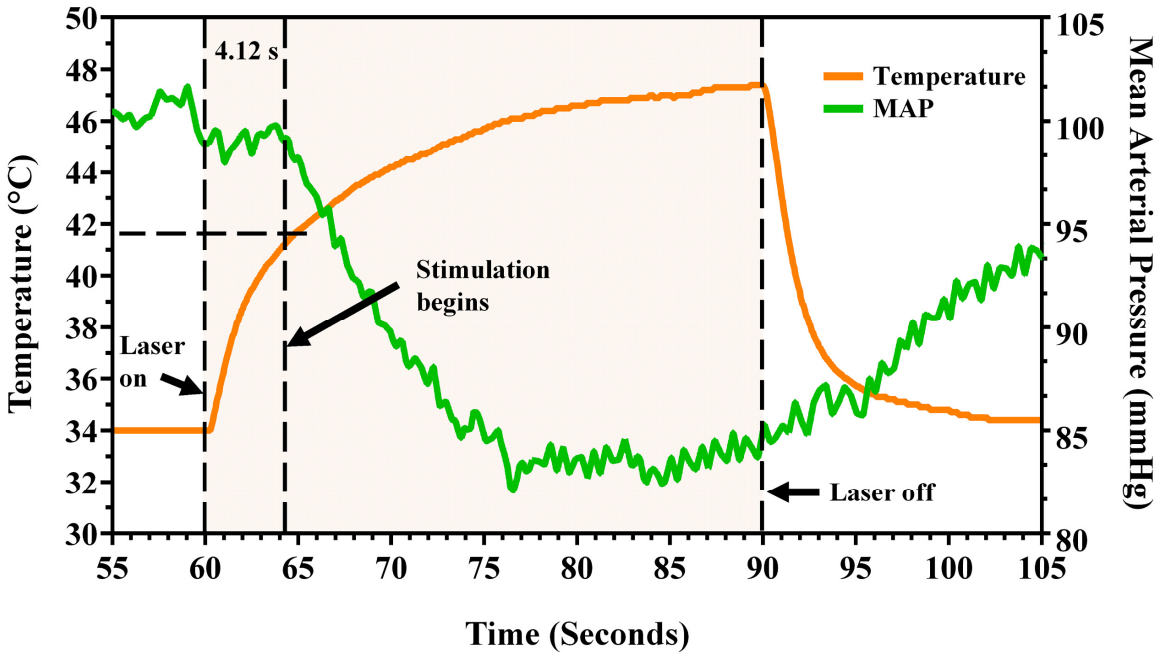


Figure 21. Typical MAP response for ONS of the rat VN. The Orange area indicates the stimulation period.

When ONS and ENS are compared in terms of change in MAP, there is no significant difference between them (unpaired t-test; $p = 0.1498$, $n = 59$). MAP decreased from a baseline of a maximum of 29.7 mmHg and meant 11.23 mmHg during ONS. The standard deviation of the distribution was 5.052. Furthermore, a maximum of 24.62 mmHg and a mean 12.84 mmHg decrease from baseline in MAP were observed in ENS. The standard deviation of the distribution was 5.026. Therefore, it is not unexpected to see a difference between ONS and ENS (Figure 22).

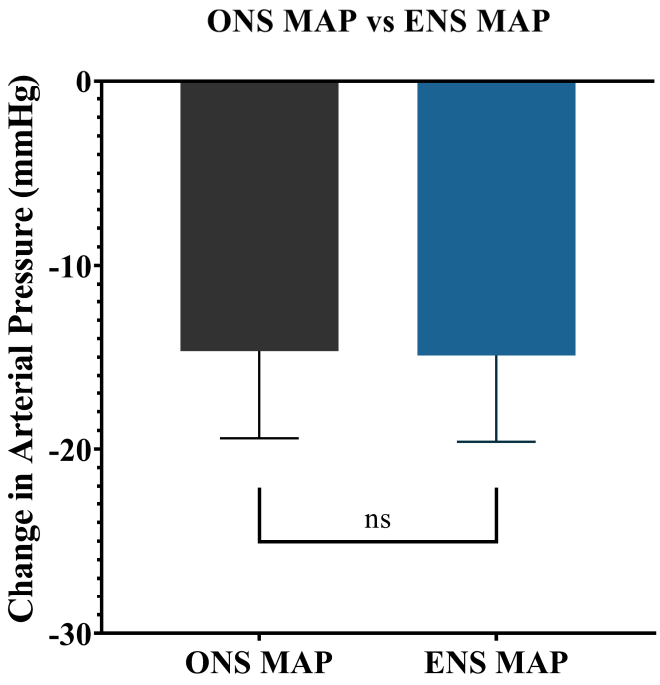


Figure 22: Comparison of ONS and ENS in terms of change in MAP during stimulation periods.

The effect of ENS and ONS on HR is quite different despite MAP. ONS does not affect HR, while ENS causes a maximum 283 bpm and mean 65 bpm drop in HR (unpaired t-test; $p < 0.0001$, $n = 120$). The standard deviation of the distribution was 61.39. It is quite a surprise to find that ONS does not affect HR (Figure 23).

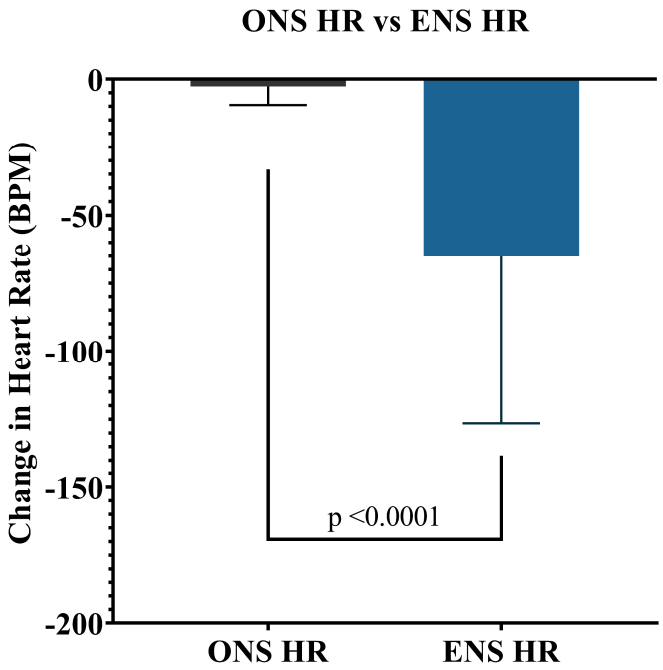
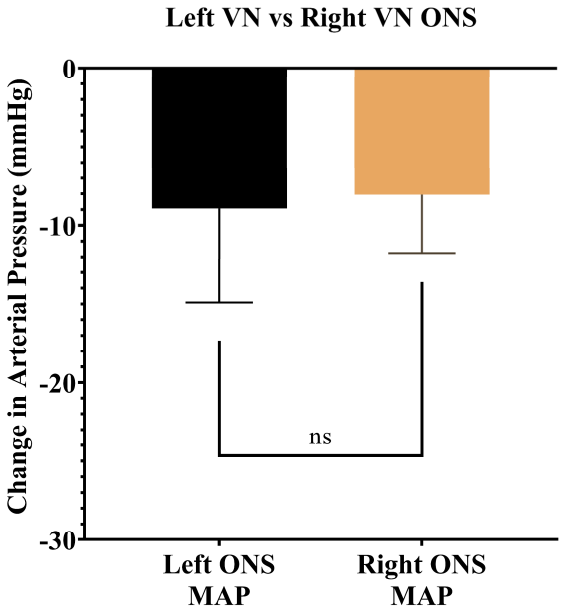
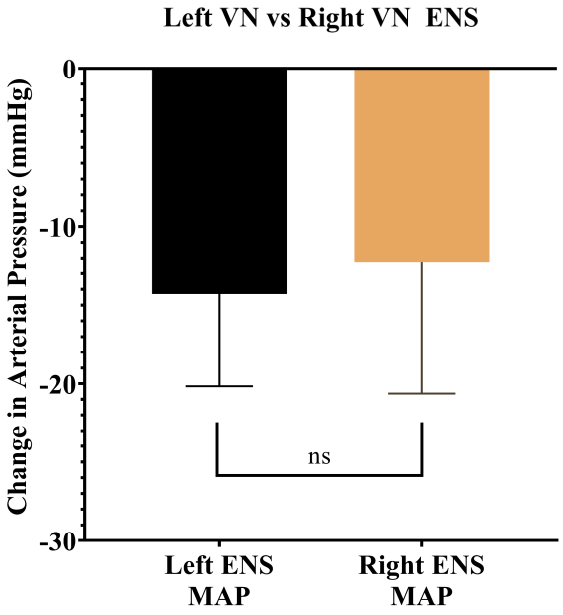


Figure 23. Comparison of ONS and ENS in terms of change in HR during stimulation periods.

Left VNS is generally chosen to prevent seizures because of safety concerns on the heart. The VN's left and right sides are thought to innervate to the heart in many species asymmetrically. Because of this, left and right-sided ONS and ENS of the VN were compared to understand this statement. However, ONS and ENS were able to stimulate both left and right sided equally in rats. There was no significant difference between left-sided versus right-sided in terms of change in MAP (unpaired t-test; $p = 0.3673$, $n = 53$ for ONS, unpaired t-test; $p = 0.4348$, $n = 25$ for ENS) (Figure 24). The standard deviations of the distribution were 5.880 and 8.339, respectively.



(a)



(b)

Figure 24. Comparison of the left and right-sided, (a) ONS and (b) ENS in terms of change in MAP during stimulation periods.

Additionally, HRV analysis was applied to ECG for both ONS and ENS. HRV analysis with Kubios Software was performed to raw ECG data. SDNN, RMSSD, and LF/HF were considered analyses of stimulation for the VN to understand ENS and ONS's effect on the heart.

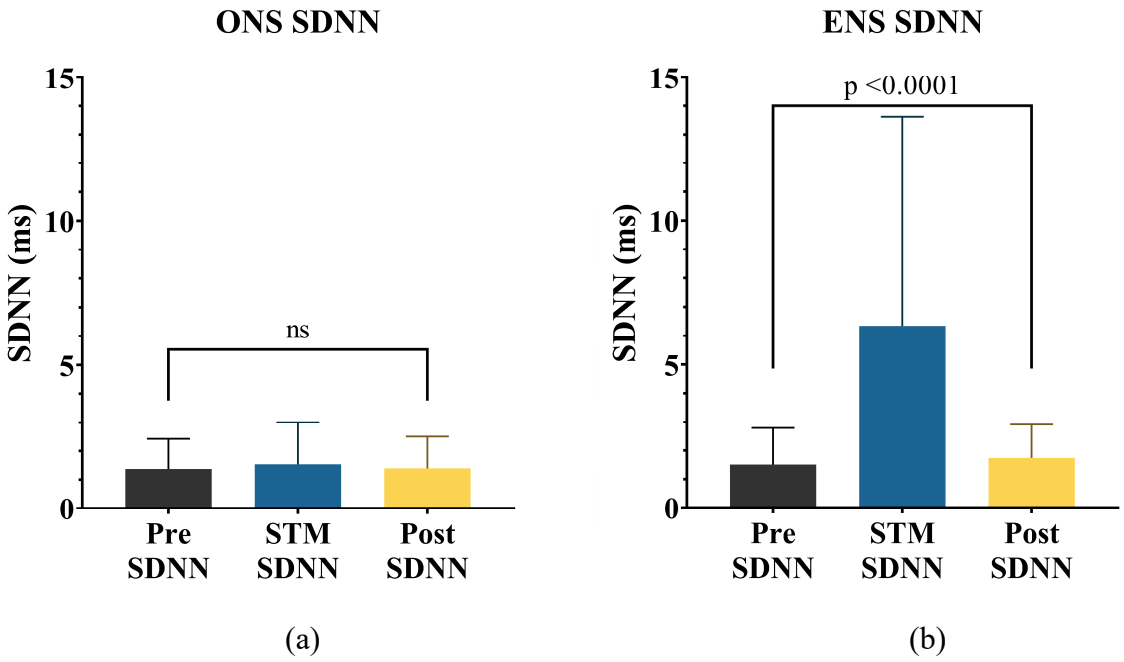


Figure 25. Comparison of (a) ONS and (b) ENS in terms of change in SDNN during stimulation periods.

SDNN, one of the time-dependent parameters, shows the general state of autonomic nervous system balance [70]. This number indicates whether HRV is generally within the normal range. Figure 25 shows a comparison of SDNN during pre-stimulation, stimulation, and post-stimulation of ONS and ENS. SDNN did not change while the infrared laser was applied to the VN (one-way ANOVA, $p = 0.4471$, $n = 147$). The standard deviation of the distribution was 1.475. However, SDNN significantly increased during ENS (one-way ANOVA, $p < 0.0001$, $n = 40$). The standard deviation of the distribution was 7.278.

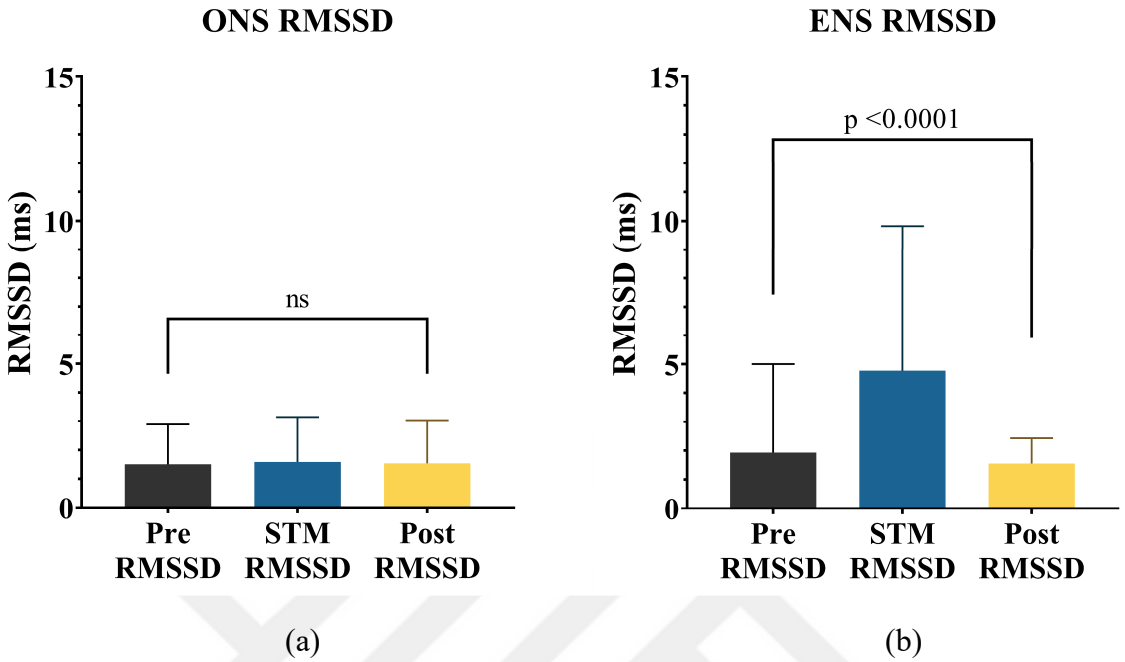


Figure 26. Comparison of (a) ONS and (b) ENS in terms of change in RMSSD during stimulation periods.

RMSSD predominantly reflects parasympathetic activity [70]. Figure 26 shows RMSSD results of both ONS and ENS. ONS did not cause any change in RMSSD (one-way ANOVA, $p = 0.09779$, $n = 147$). The standard deviation of the distribution was 1.567. However, RMSSD also significantly increased during ENS (one-way ANOVA, $p < 0.0001$, $n = 40$). The standard deviation of the distribution was 5.050.

LF/HF generally reflects sympathovagal balance. The ratio of LF and HF was also analyzed for ONS and ENS. There was no significant effect of ONS on LF/HF during stimulation (one-way ANOVA, $p = 0.1853$, $n = 147$). The standard deviation of the distribution was 1.382. In contrast, LF/HF ratio significantly increased during ENS (one-way ANOVA, $p = 0.0033$, $n = 40$) (Figure 27). The standard deviation of the distribution was 9.351.

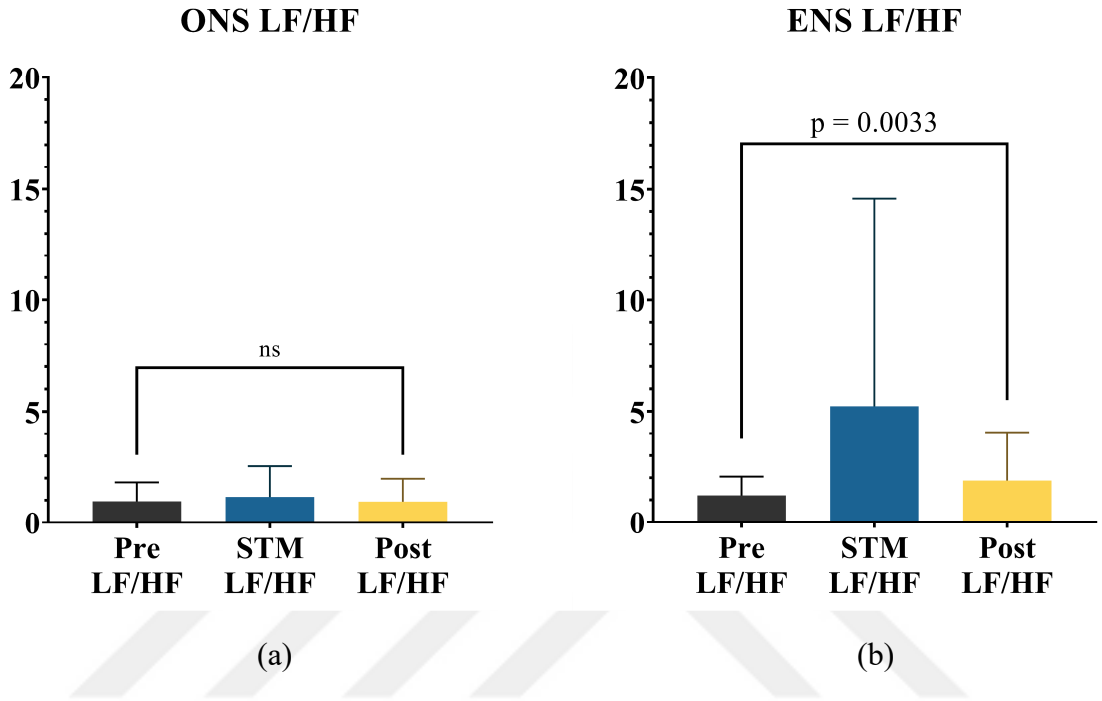


Figure 27. Comparison of (a) ONS and (b) ENS in terms of change in LF/HF during stimulation periods.

4.2. Determination of Stimulation Threshold

Different levels of ONS were compared in terms of temperature to determine the stimulation threshold of ONS.

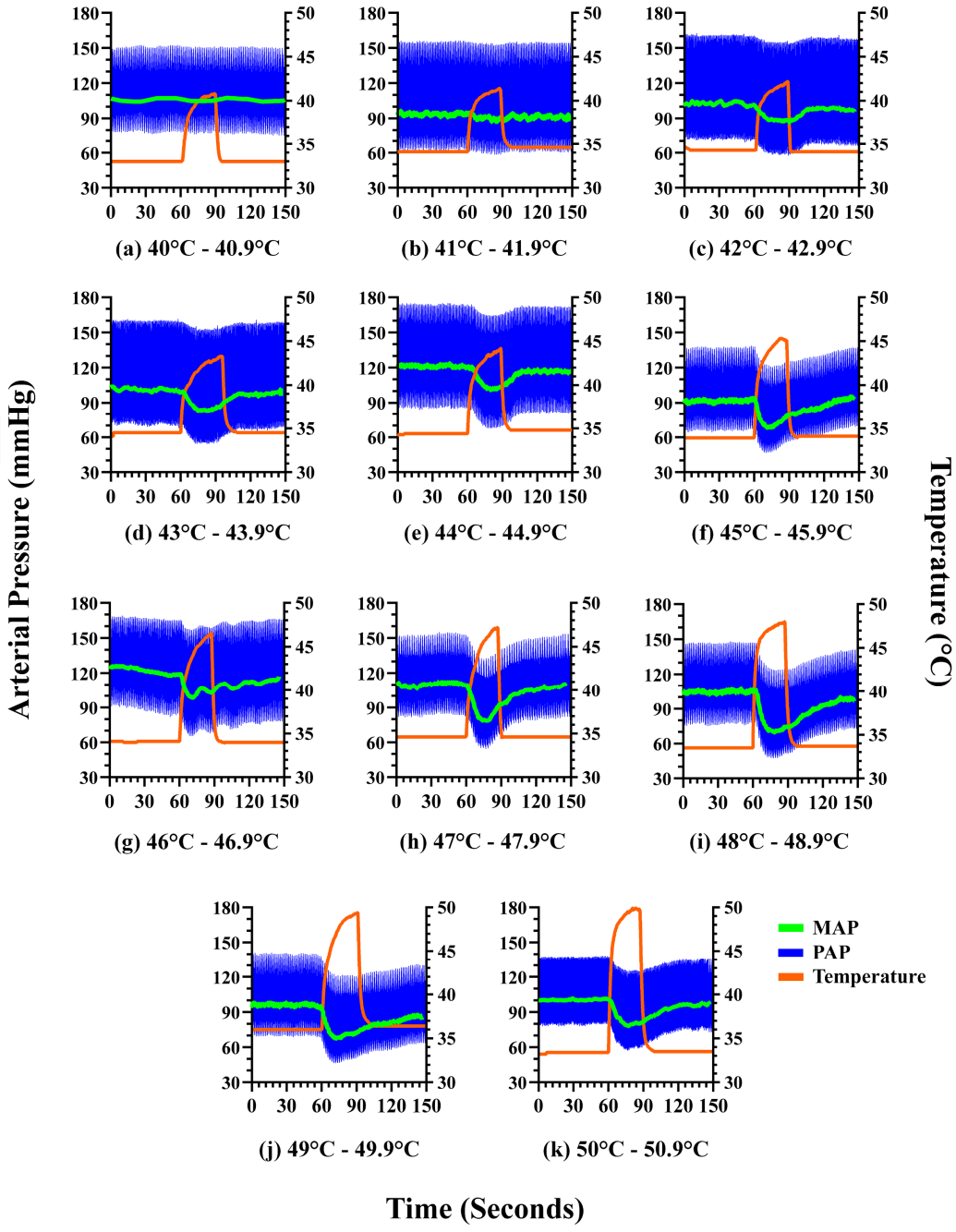


Figure 28. Representative responses to ONS in terms of PAP and MAP at different levels of temperature (a) 40-40.9 °C, (b) 41-41.9 °C, (c) 42-42.9 °C, (d) 43-43.9 °C (e) 44-44.9 °C, (f) 45-45.9 °C, (g) 46-46.9 °C, (h) 47-47.9 °C (I) 48-48.9 °C, (j) 49-49.9 °C, (k) 50-50.9 °C. ONS significantly decreases MAP after 42-42.9 °C.

The infrared laser power was gradually increased until the surface temperature was reached and grouped with a difference of 1 °C to determine the stimulation threshold of ONS. As seen in Figure 30, the VN was irradiated to the threshold temperature range during stimulation. Thermal imaging of the VN was critical to preventing overheating. It should be noted that the VN's initial temperature was 34 °C (below than body temperature, 37 °C) since it was an open procedure.

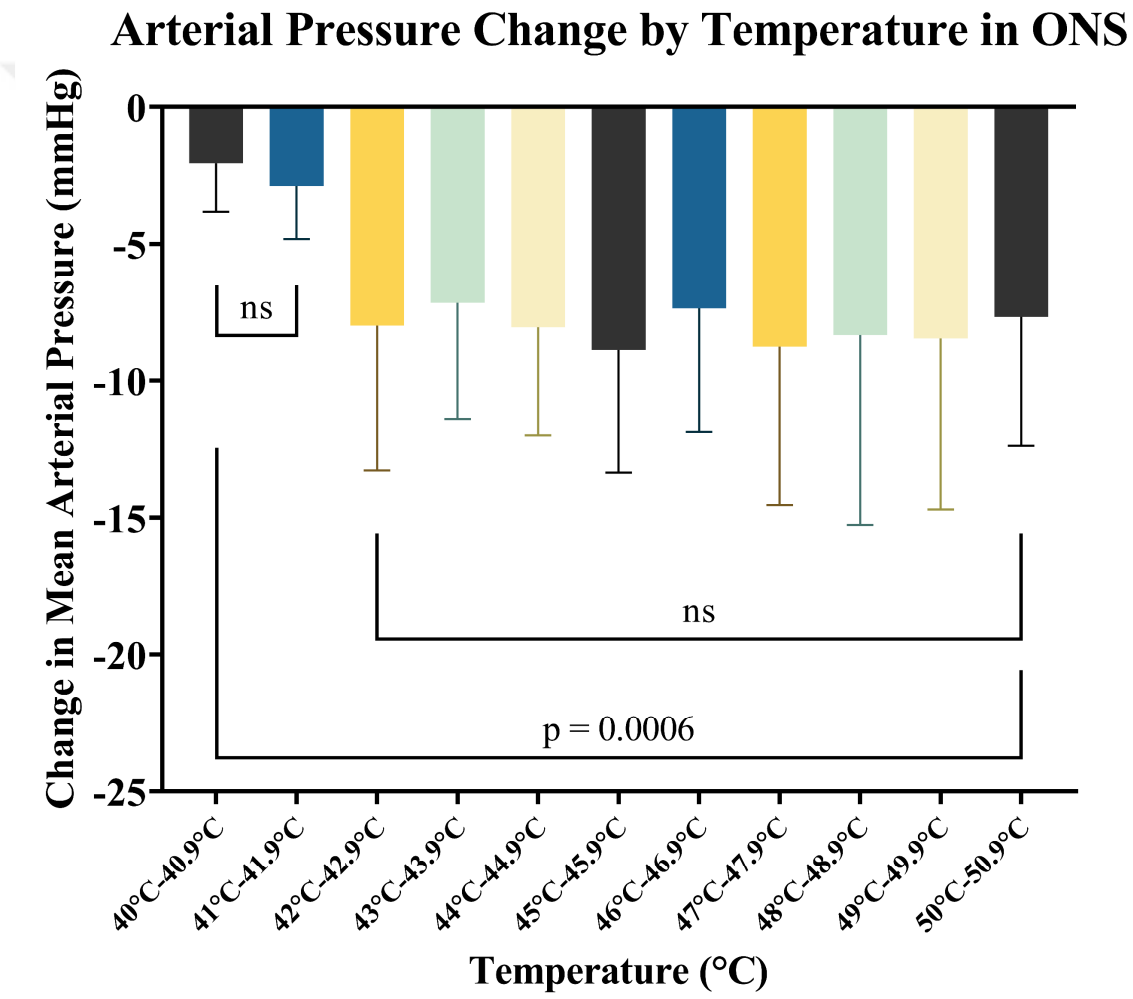
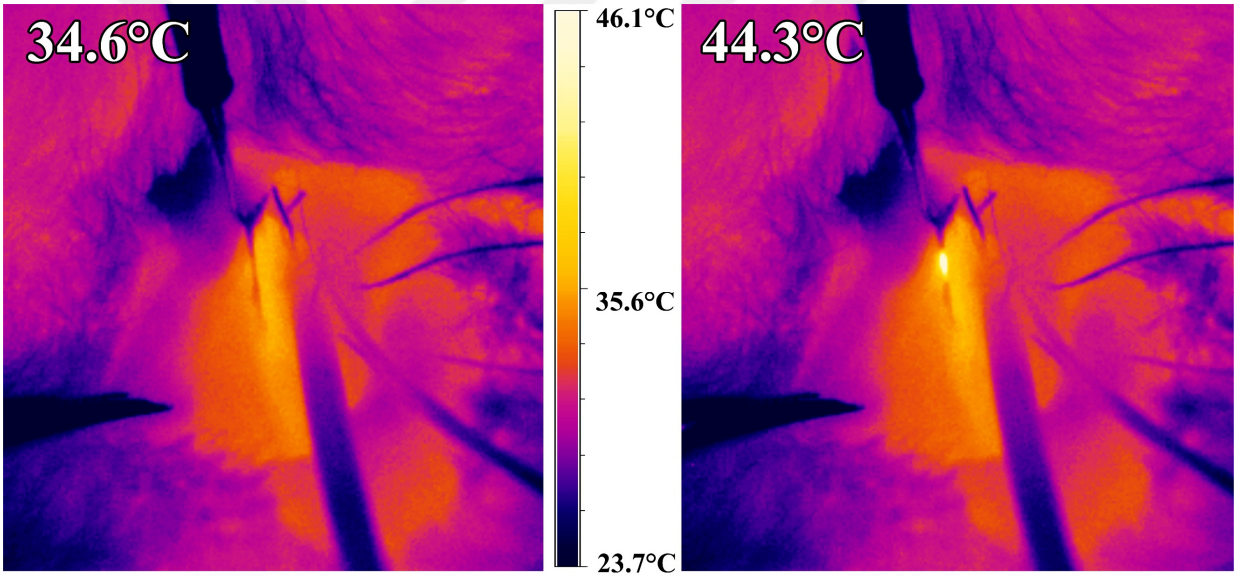


Figure 29. MAP changes at different temperatures in ONS.

After rigorous examination, it was discovered that ONS successfully stimulated the VN after 42 °C. There was no significant decrease in MAP until 42 °C, which is also the activation temperature of TRPV1 channels. However, MAP started to decrease significantly after 42 °C, which indicates the stimulation threshold (one-way ANOVA, $p = 0.0006$, $n = 147$) (Figure 29). Representative responses of different temperatures can be seen in Figure 28. The ONS performance was increasing until 48 °C, but it started to decrease after that point. This result can be an indication of that ONS could heat more bundles until 48 °C. However, since VN may have started to be damaged after 48 °C, performance loss may have occurred.



(a) (b)

Figure 30. Thermal images of the VN. (a) Baseline temperature of the VN before stimulation. (b) The peak temperature of the VN during stimulation.

4.3. Determination of Damage Threshold by Histology Analysis

Acute thermal damage thresholds were determined by histological analysis of the VN with different temperatures.

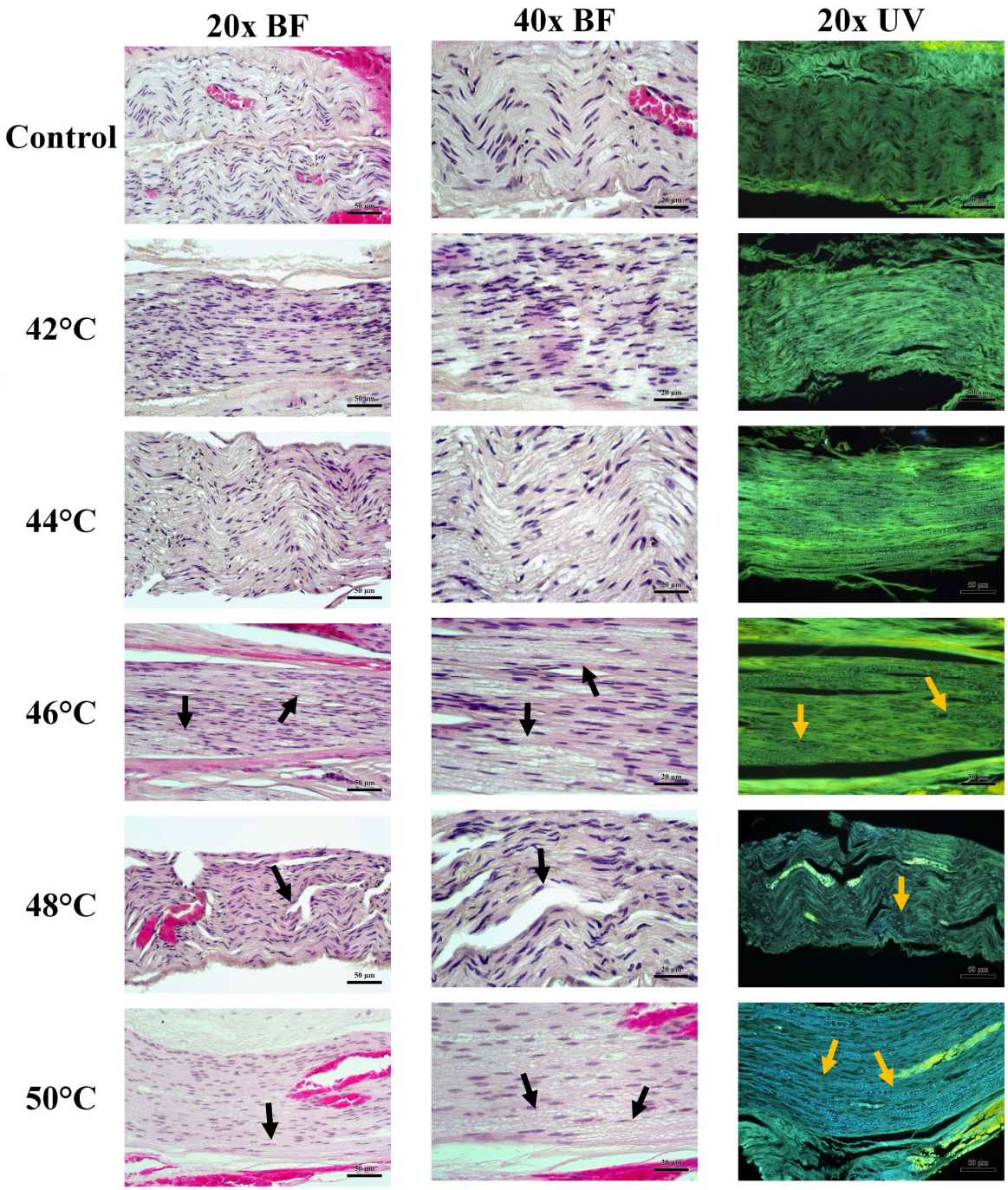


Figure 31. Histological analysis of the VN at different surface temperatures. 20x BF, 40x BF, and 20x UV fluorescence images reveal thermal damage after 46 °C. Black and orange arrows indicate small bubbles, swelling, and granules which are signs of neuropraxia due to thermal effect.

Acute thermal damage thresholds were determined by histological analysis of the VN with different temperatures. Hyalinization of collagen proteins, swelling of collagen proteins, deformation of the nucleus, and cytoplasm can indicate thermal damage in nerve tissue. There is no thermal damage sign until 46 °C. However, small bubbles, swelling, and granules were observed inside the VN bundle after 46 °C (Figure 31). Small bubbles, swelling, and granules may indicate neuropraxia, which causes temporary function loss due to thermal damage in this study.

4.4. Summary of ONS Results

Table 5 summarizes the fundamental results of ONS at 1505 nm wavelength for the rat VN.

The fastest stimulation time (s) is ~ 3 s, which is the shortest duration that ONS requires to stimulate the VN.

Threshold stimulation response time (s) is determined as ~ 6 s. This latency period indicates that the infrared laser needs to heat the nerve to stimulate and baroreflex feedback activates AP reduction. This period is directly related to power and maximum temperature on the nerve. The faster the nerve heats up, the less time it will take to stimulate the VN.

Power to stimulate (mW) is the minimum required power to increase heat and stimulate the VN. The VN needs ~ 28 mW laser power to reach stimulation temperature and get stimulated.

Temperature to stimulate (°C) is the temperature that the nerve must reach to be stimulated. ~ 42 °C surface temperature is needed to stimulate the VN.

Power to damage nerve (mW) is the minimum power that causes thermal damage in the VN. Damage threshold power was determined as 41.8 mW. The VN will get more damage after damage threshold power, which can cause irreversible damage after that power.

Temperature to damage nerve (°C), which is determined by damage threshold power, is the minimum temperature that causes thermal damage in the VN. Temperature to damage nerve was identified as 46 °C after histological analysis.

From these results, it is seen that ONS, which is both effective after 42 °C and safe until 46 °C, is an alternative technique of ENS after AP and histology experiments.

Table 5. Summary of optical vagus nerve stimulation results in an in-vivo rat model.

Parameter	
Fastest stimulation response time (s)	**3.07**
Threshold stimulation response time (s)	**5.95±2.88**
Power to stimulate (mW):	**28.27±9.97**
Temperature to stimulate (°C):	**41.72±2.49**
Power to damage nerve (mW):	**41.8**
Temperature to damage nerve (°C):	**46**

5. DISCUSSION

The potential applications and possible side (i.e., adverse) effectiveness of vagus nerve stimulation (VNS) have been widely described in literature before. Stimulation of the vagus nerve can be used as a clinical approach to treat epilepsy, obesity, migraine, hypertension, and Alzheimer's disease. However, the conventional technique using electrical current has severe side effects causing coughing, chest and throat pain, headaches, and most importantly, arrhythmia and bradycardia.

On the other hand, optical nerve stimulation (ONS) can be considered as an alternative stimulation technique using an infrared laser that has been investigated at different neural targets, including sciatic nerve, cavernous nerve, auditory nerve, somatosensory cortex, and visual cortex. Besides, ONS demonstrates the potential to reduce the side effects of the VNS with three main advantages: (1) a non-contact method of nerve stimulation; (2) improved spatial selectivity; (3) elimination of stimulation artifacts. Multiple previous studies have explored cardiac outputs and epilepsy of the VNS, although ONS has no remarkable application on the vagus nerve (VN) yet [68, 71-77].

This thesis has demonstrated that ONS is applicable to stimulate VN in a rat model for the first time. In the studies, cardiac outputs (i.e., arterial pressure and heart rate variability) were evaluated to prove that ONS could stimulate the rat VN in a reliable, repeatable, and safe manner. With further studies (e.g., including comprehensive electrophysiological and systemic measurements), vagus nerve stimulation using laser irradiation as a therapy method can be considered an essential step in preclinical trials.

The experimental outcomes obtained from the studies conducted in the rat model within the scope of this thesis are as follows:

Optical vagus nerve stimulation (oVNS) causes a significant decrease in arterial pressure (AP), proving successful stimulation. The results of ENS are similar to ONS in terms of change in MAP. Both ENS and ONS cause a significant decrease in the mean arterial pressure (MAP) during the stimulation. Moreover, MAP is quickly recovered when the stimulation is over. The only difference explored is the stimulation response time between ENS and ONS. ONS requires much

more time to initiate the action potential (5.95 s) due to the photothermal effect's nature, even though ENS is almost instant. Therefore, a smoother decrease in AP can prevent systemic problems too.

In addition to AP response, ONS and ENS produce dramatically different responses on electrocardiogram (ECG) parameters. There is no change in heart rate (HR) and heart rate variability, including time-domain (i.e., the standard deviation of NN intervals and the root mean square of successive differences - RMSSD) and frequency-domain (i.e., the high-frequency power - HF and the low-frequency power - LF) analysis during the laser irradiation. However, HR significantly decreased, and SDNN, RMSSD, and LF/HF parameters increased during the vagus nerve's electrical stimulation. As noted, analysis of SDNN, RMSSD, and LF/HF reflect different physiological events in the heart. For example, SDNN indicates the general state of autonomic nervous system balance. RMSSD indicates parasympathetic activity. HF is associated with respiratory sinus arrhythmia, and it is predominantly indicative of parasympathetic activity. LF is associated with baroreceptor control of sympathetic activity. LF/HF ratio is generally thought to reflect the sympathovagal balance. Increasing all HRV parameters, including both sympathetic and parasympathetic, is scientifically meaningless. It may be an indication of artifacts on the heart during ENS.

Every single parameter, which is changed during stimulation, turns back to pre-stimulation base levels in the post-stimulation period in both ONS and ENS. There is no permanent effect in the post-stimulation period. HR decrease and HRV change were only observed in three ONS trials. Thus, this can be an indication that ONS can also change HR with different parameters or positions. This finding suggests that oVNS could potentially control AP without electrical side effects on the heart.

Three possible mechanisms might explain the HR and HRV difference between ENS and ONS. First, selective stimulation of the VN may have been achieved by ONS physiologically. Both afferent and efferent fibers are located in the VN. As already known, A, B, and C type fibers are physiologically and morphologically different. A-type fibers are large-diameter fibers that require low stimulation threshold electricity to be stimulated.

On the other hand, C-type fibers are small diameter afferent fibers that require high current. A similar logic may be applied to ONS. Different diameter axons can reach different temperature levels in a microstate during laser irradiation. For instance, small diameter afferent axons can reach 42 °C in this ONS system. Thus, small diameter afferent axons can be stimulated, and large-diameter axons may require either higher or lower temperatures. Eventually, small diameter afferent sensory fibers are stimulated, and they create feedback to decrease AP instead of stimulation of large diameter efferent fibers to motor response on HR and HRV.

Second, ONS may provide selective stimulation of the VN. In the animal studies, all rats were secured in a supine position on a surgical table. After the VN was isolated from the carotid artery, it was hanged to a hook electrode. Generally, the ventral side of the VN was irradiated with infrared laser unintentionally. It may explain this mechanism if ADN or the other arterial-pressure-associated fascicles are located in the VN's ventral side. Unfortunately, the experimental setup did not allow to figure out the location of the fascicles. Furthermore, locating the fascicles was beyond the scope of the thesis topic.

Third, the elimination of electrical artifacts might be playing a role in this mechanism. Electric current is hard to control in tissue. Researchers have been trying to develop smaller electrodes to keep the electrical field in a defined area [12]. However, a common type of hook electrode was used in the ENS experiments. Due to electricity's nature, the current could move through the VN and reach to heart without actually stimulating the VN. This artifact might affect the heart's internal electric system and cause a drop in HR and irregularity in HRV. For instance, ENS causes a more considerable HR decrease when a higher electric current or voltage is applied. Because the electricity reaching the heart may be so high that it creates irregularities in functioning. Consequently, all these mechanisms require specific experimental designs to resolve the underlying explanation.

The vagus nerve stimulation therapy is often inserted to the left-sided VN in clinical settings because the right-sided VN has more innervation in the heart than the left-hand side, potentially causing severe adverse effects. Moreover, it has experimentally shown that right-sided VNS is also useful as a left-sided VNS for reducing seizures [71]. However, there was no significant difference between right and left-sided optical stimulation of the VN, as shown. Anatomical differences

between rats and humans may explain these results. Therefore, demonstrating that right-sided VNS is as effective as left-sided VNS indicates that right-sided VNS may have new applications in cardiac and epilepsy patients.

Optical stimulation of both left and right-sided VN could turn into a remarkable technology in many clinic applications like controlling cardiac outputs for hypertension, reducing seizures for epilepsy patients, and treating obesity. Some problems have to be resolved to achieve these clinic goals. First of all, the physiology and location of each fascicle of VN should be determined using different laser wavelengths (defining optical penetration depth) and laser spot positions along the nerve surface. Second, as obesity, chronic epilepsy, hypertension, and behavior studies require a longer time to explore, an optical prosthetic attached to the VN can be designed for long-term clinical applications. Third, optical parameters, including laser power and spot diameter, have to be optimized. The wavelength-dependent optical penetration depth, absorption coefficient, beam diameter, power, and working distance can change the performance of ONS. Selective stimulation of the desired fascicles can be achieved by understanding optical parameters and their photothermal outputs.

Male wistar rat models were used in these experiments. The rat model was suitable for the VNS therapy as shown in many other preclinical studies [78, 79]. However, it is hard to handle in surgical operations due to the rat VN's delicate structure. Because of their anatomical and physiological similarities to humans, using a pig model should be considered as a pre-clinical animal model.

The choice of anesthesia can critically affect the result. For example, it has been previously shown that ketamine–xylazine (K/X) anesthesia had more side effects than other anesthetics on heart functions [37]. An early iteration of the experiments was conducted with K/X anesthesia, reducing the animal's cardiac responses to the ONS. Therefore, relatively stable urethane anesthesia was used in the next phase of the thesis, and thus, successful oVNS results were obtained in the rat model. However, despite urethane anesthesia's success, it is carcinogenic and requires immediate euthanasia of the rat. The development of ONS prostheses can overcome anesthesia problems.

Combination of a 1505 nm infrared diode laser and visible red laser was used in this study to build the ONS system. The most cost-effective approach would be the one which uses telecommunication wavelengths. ONS system, which uses 1505 nm wavelength diode laser, is more cost-effective than other ONS systems in the literature. Holmium:YAG lasers and tunable thulium fiber laser are likely to be more expensive because of the manufacturing process and cost of the components.

One of the most critical aspects is protecting nerve tissue from laser-induced thermal damage. The VN was irradiated until it reached a specific temperature to investigate the thermal effect of ONS. After a total of 12 attempts, the VN tissue sample was collected and histological analysis performed. Histological examinations of the VN showed that thermal damage (small bubbles, swelling, and granules) became more pronounced after 46 °C. The visible (color change) thermal damage of VN was determined after 48 °C. Moreover, the VN's performance also decreased after 48 °C. Therefore, the safe therapeutic window for oVNS was determined between 42-46 °C.

Early studies showed that the unmyelinated sensory neurons were more vulnerable to hyperthermia [80]. The unmyelinated neurons are reversibly blocked at 47 °C [81]. The decrease in the performance of the VN may be an indication that ONS stimulates small sensory neurons. Further studies must maintain this safe temperature level.

6. CONCLUSION AND FUTURE DIRECTIONS

In this thesis, the optical nerve stimulation (ONS) technique's effects on the rat vagus nerve were investigated and evaluated for the first time in arterial pressure and electrocardiogram. A direct comparison between optical vagus nerve stimulation (oVNS) and electrical vagus nerve stimulation (VNS) was performed in the in-vivo rat model. Histological analysis was conducted to define the safe therapeutic window for reliable and repeatable oVNS. The major findings of the study are listed below.

First, reliable, reproducible, successful optical stimulation of the rat vagus nerve has been demonstrated at the nerve temperature of ~42 °C. In particular, a significant decrease in arterial pressure was observed during ONS. Second, conventional electrical and optic vagus nerve stimulation pointed to a similar drop-in effect (>10 mmHg on average) on arterial pressure. Third, the optical technique did not significantly change heart rate and heart rate variability, despite the conventional technique had a strong effect on heart functions as expected. Heart rate dramatically decreased while all heart rate variability parameters (i.e., SDNN, RMSSD, and LF/HF) increased during electrical stimulation. These outcomes are considered as adverse effects of electrical techniques in many clinical applications. Last but not least, histology analysis of the rat vagus nerve confirmed that acute thermal injury has occurred at a surface temperature of 46 °C or higher. In an in-vivo rat model, the therapy window of ONS was noted to be 42-44 °C.

The future aspects of this work should be considered from two main aspects:

1. Examination of the rat vagus nerve's anatomy and physiology: The exact location and functions of the vagus nerve's fascicles can be resolved. Selective stimulation of the VN can be effective and efficient when the precise locations and functions of fascicles within the bundle are determined. It can also help study the optical vagus nerve stimulation mechanism by controlling the rat model's stimulation response.
2. Development of the subsequent iteration of the optical nerve stimulation and measurement systems: In this study, the ONS system requires open surgery to stimulate the vagus nerve. This optical technique will be an advanced technology with the development of an implanted stimulator for chronic studies. Moreover, better optimization of laser parameters could produce well-defined

laser-induced nerve stimulation. Different infrared laser wavelengths, beam diameters, and laser power can be adapted to increase the efficiency and selectivity of ONS. Additionally, this study only considers cardiac responses during the stimulation. However, electroencephalogram or electromyogram and bowel system measurements potentially provide better information about optical stimulation of the vagus nerve.

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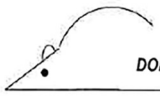
8. APPENDIX

8.1. Research Ethics Committee Approvals



DOKUZ EYLÜL ÜNİVERSİTESİ MULTİDİSİPLİN LABORATUVARI HAYVAN DENEYLERİ YEREL ETİK KURULU

Gündem No/ Toplantı No/Yıl : 02/03/2019
Toplantı Tarihi : 13 Şubat 2019

Sayın, Öğr.Üyesi Dr.Serhat TOZBURUN
Dokuz EylülÜniversitesi İzmir Uluslararası Biyotıp ve Genom Enstitüsü

04/2019 Protokol No'lu: yürütücüsü olduğunuz **"Vagus Sinirininn Optik Uyarımı ve Epileptik Sıçan Modelinde İncelenmesi,"** isimli; Mehmet Ensari Güneli, Aslı Çelik, Ozan Yetiş, H.Alper Bağrıyanık, Ayşe Semra Hız, İbrahim Akkaya'nın araştırıcı olduğu, 35 adet Sprage Dawley erkek sıçan talep edilen projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilmiştir.

Bilgilerinizi ve gereğini rica ederiz.

DOKUZ EYLÜL ÜNİVERSİTESİ MULTİDİSİPLİN LABORATUVARI HAYVAN DENEYLERİ YEREL ETİK KURULU

Gündem No/Toplantı No/Yıl : 01/07/2020
Toplantı Tarihi : 08 Temmuz 2020

Sayın, Öğr.Üyesi Dr.Serhat TOZBURUN
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04/2019 Protokol No'lu: yürütücüsü olduğunuz **"Vagus Sinirininn Optik Uyarımı ve Epileptik Sıçan Modelinde İncelenmesi,"** isimli; Mehmet Ensari Güneli, Aslı Çelik, Ozan Yetiş, H.Alper Bağrıyanık, Ayşe Semra Hız, İbrahim Akkaya'nın araştırıcı olduğu, 35 adet Sprage Dawley erkek sıçan talep edilen projenin uygulanmasında etik açıdan sakınca olmadığına oy birliği ile karar verilen projede; gönderilen belgeler incelenerek, projede kullanılanacak hayvan türünün Sprage Dawley erkek sıçan yerine, 35(otuzbeş) adet Wistar Albino erkek sıçan kullanılması uygun bulunmuştur.

Bilgilerinizi ve gereğini rica ederiz.

8.2. Curriculum Vitae

ÖZGEÇMİŞ

Adı Soyadı: Ozan Yetiş

TC Kimlik No / Pasaport No:	
Doğum Yılı:	
Yazışma Adresi:	
Telefon:	
Faks:	
e-posta:	

EĞİTİM BİLGİLERİ

Ülke	Üniversite	Fakülte/Enstitü	Öğrenim Alanı	Derece	Mezuniyet Yılı
TR	Dokuz Eylül Üniversitesi	İzmir Uluslararası Biyotıp ve Genom Enstitüsü	Moleküler Biyoloji ve Genetik	Yüksek Lisans	2021
TR	Orta Doğu Teknik Üniversitesi	Fen-Edebiyat Fakültesi	Biyoloji	Lisans	2016

UZMANLIK ALANLARI

Uzmanlık Alanları
Nerve stimulation techniques, Electrophysiology measurements, Laser-Tissue interactions, Confocal imaging, Serial histological sectioning, 3-dimensional reconstructions.