Predictors of Diagnostic Yield of Implanted Loop Recorder in Patients with Cryptogenic Stroke: A Systemic Review and Meta-analysis

Udit Bhatnagar, MD

Hub Site: Las Cruces

Number of Students Accepted: 2

Efficacy and predictors of diagnostic yield of Implanted Loop Recorder in patients with cryptogenic stroke: a systemic review and meta-analysis

Of note – a preliminary study using this plan was already done and presented in poster format at Heart failure society of America and published in abstract form here:

https://www.ahajournals.org/doi/10.1161/str.49.suppl_1.WMP61

The plan is to expand and update the metanalysis and publish it in a leading cardiology journal. This will require students for data analysis, literature review, biostatistician.

Background: There is limited evidence of ECG monitoring (for detection of occult atrial fibrillation) in patient after cryptogenic stroke especially in determining the optimal duration of monitoring. Some guidelines suggest atleast 30 days of monitoring with event monitors, however newer studies show benefit of longer monitoring with implantable loop recorders (ILR)

We plan to systematically review prior available RCTs and observation studies studying ILRs in Cryptogenic stroke to look for efficacy of ILRs over time. We are specifically interested to assess the predictors of diagnostic yield. A future project may be to develop a scoring system to objectively assess higher predictive accuracy BEFORE implantation of ILR.

Plan:

Perform an extensive literature review and database search to come up with all studies with following criteria:

Inclusion Criteria for studies (PUBMED, COCHRANE):

- Studies of ILRs in cryptogenic stroke (with or without comparison to other modalities – ambulatory EKG/event monitor)
- >18 years of age
- >20 sample size of population in individual study
- English language
- Include studies that list baseline characteristics of patients with vs without AF

We will note:

- Study type
- Number of patients
- Mean age
- Mode of recording – ILR vs other

- Duration for monitoring
- Criteria for diagnosing AF
- % of people with AF detection
- Average time to detection of AF
- Baseline characteristics of people -- with AF vs without AF (predictors of diagnostic yield)
    - Age
    - Comorbidities
        - DM2
        - HTN
        - CHADS-VASC score
    - LA volume
    - Ectopy/APC burden on holter

Covariates among patients with vs without AF will be compared using various statistical tools to assess predictors of finding AF and time of detection of AF after implant.

High Fidelity Lateral Canthotomy

Camilo Mohar DO, Yasmany Cartaya MD, and Andy Little, MD,

Hub Site: Florida

Number of Students Accepted: 2

East Orlando

11/10/2021

Hello to those looking for potential projects for this upcoming summer.

We are hoping to recruit medical students to take part in our multi-center project involving training medical students, residents & attendings on how to perform a lateral canthotomy. We are planning on using low fidelity (LoFi) trainers, first devised by Kong, R et al., to demonstrate how to perform this high acuity low occurrence vision saving procedure.

We would love for medical students to assist in this study. This will include coordinating with residency programs and medical schools on how many LoFi trainers they would need for this multicenter study and placing together packages to sent them including a box with simple instructions and having anyone who is participating in the project to fill out the pre and post-survey using a QR code that will be provided.

The aim of this study is to demonstrate an economically alternative method of training healthcare professionals and trainees, on how to perform lateral canthotomy. This is an emergent procedure not often encountered in the emergency department but nonetheless paramount in understanding as it can save a patient's eye.

If you would like to take part in our project, we would love to have you. Our goal is to have the trainers sent out, survey completed and data compiled, and manuscript written by the end of 2022 summer. Your participation could involve co-authorship in a paper, conferences to present such work, and poster presentations on data with video demonstration.

Best
Camilo Mohar, DO
AdventHealth East Orlando EM Faculty

For more background information regarding our project, please read the article from EP Monthly.
https://epmonthly.com/article/train-yourself-lateral-canthotomy/

Clinical Efficacy of taVNS in Plaque Psoriasis

Harald Stauss, MD, PhD

Hub Site: Las Cruces

Number of Students Accepted: 4

Student Funding

For this project, we will allow one student to apply for a Summer Student Research Grant through the National Psoriasis Foundation (https://www.psoriasis.org/summer-student-research-grants/). If successful, this Summer Student Research Grant provides up to $4,000 as a stipend to the student. The application deadline is already on January 21, 2022. Thus, ***students interested in this possibility should contact Dr. Stauss as soon as possible***. Please note that this opportunity is highly competitive and only available to a single student.

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Significance

The prevalence of psoriasis in the US is estimated as 3.2% (about 10 million Americans) with slight variations in different ethnicities (1). This study explores a novel treatment strategy for plaque psoriasis based on transcutaneous auricular vagus nerve stimulation (taVNS). With this technique, afferent vagal nerve fibers in the ear are activated through a clip electrode attached to the ear lobe. Our previous studies (2) suggest that stimulation of these nerve fibers will activate the cholinergic anti-inflammatory pathway (3, 4) and reduce inflammation of the skin, which is the underlying pathophysiologic mechanism in plaque psoriasis. It is important to point out that taVNS is non-invasive and does not require any medication. Therefore, taVNS can potentially be combined with currently established standard of care pharmacologic treatments, including biologic drugs to achieve improved clinical outcomes. It is our expectation that taVNS (added to standard of care therapy) improves the clinical symptoms of plaque psoriasis. Thus, the study has the potential to improve the quality of life of millions of patients with plaque psoriasis.

Specific Aims

In previous (2) and ongoing studies, we demonstrated anti-inflammatory effects of taVNS in healthy study participants. taVNS is a neuromodulatory technique by which afferent vagal (parasympathetic) nerve fibers are activated through a clip electrode placed on the right or left auricle. These afferent vagal nerve fibers signal to the brain to elicit an anti-inflammatory reflex response (3, 4). Our previous data demonstrated that taVNS reduced salivary interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (2), two pro-inflammatory cytokines that have been demonstrated to correlate with clinical severity of psoriasis (5). Our intensive literature research identified only one study published in abstract form that reported a significant improvement of the Ankylosing Spondylitis Disease Activity Score (ASDAS) score in response to 5 days of taVNS in patients with psoriatic arthritis (6). However, we did not identify any studies on the efficacy of taVNS in patients with cutaneous manifestations of psoriasis. Therefore, the *Specific Aim* of this study is to investigate the clinical efficacy of taVNS in improving cutaneous manifestations of plaque psoriasis. To achieve this aim, we will test the *hypothesis* that taVNS - added to standard of care treatment - reduces the size of skin lesions in patients with plaque psoriasis through modulation of immune function.

Innovation

The status quo in the treatment of plaque psoriasis relies on phototherapy and anti-inflammatory medications, including highly toxic chemotherapeutic agents. Our approach to the treatment of plaque psoriasis is innovative because it deviates from the status quo by proposing a non-pharmacologic treatment that has the potential to complement established pharmacologic treatment strategies, and therefore, may result in synergistic effects. This may potentially allow for lower doses of anti-inflammatory medication, resulting in reduced adverse effects at the same or perhaps even improved clinical outcomes.

Research Strategy

Experimental Protocol: Patients with plaque psoriasis will be recruited through collaborations with dermatologists and through advertisements (e.g., clinicaltrials.gov, flyers in the community). Written informed consent will be obtained from all study participants prior to official enrollment in the study. All enrolled subjects will participate in the study for a total period of 3 months. Throughout the 3-month study period, subjects will self-administer taVNS or sham taVNS on a daily basis and keep a daily diary to monitor compliance and potential off-target effects of taVNS as described below. All subjects are required to report for 5 visits throughout the 3-month study period:

Visit 1 (day 0): Assessment of potential exclusion criteria through Qualtrics survey. Training in keeping notes in the daily diary and in the use of the taVNS stimulator. Assessment of baseline body mass index (BMI, height and weight) and hemodynamic/autonomic parameters (blood pressure, heart rate, ECG, heart rate variability). Baseline clinical assessment of plaque psoriasis. Blood draw for basline assessment of immune function.

Visit 2 (day 7): Discuss potential questions about the use of the taVNS stimulator or the daily diary. Assess potential off-target effects of taVNS. Assessment of BMI and hemodynamic/autonomic parameters (blood pressure, heart rate, ECG, heart rate variability). Clinical assessment of plaque psoriasis. Blood draw for assessment of immune function.

Vist 3 (1 month): Check taVNS equipment (cables, battery). Discuss daily diary and assess potential off-target effects of taVNS. Assessment of BMI and hemodynamic/autonomic parameters. Clinical assessment of plaque psoriasis. Blood draw for assessment of immune function.

Visit 4 (2 month): Same as visit 3.

Visit 5 (3 month): Same as visit 3 and 4.

Daily diary: The diary is a bound paper notebook. Throughout the 3-month study period subjects are asked to maintain a daily diary to record: time and duration of taVNS application, stimulation intensity (based on dial setting that controls the stimulation current), beneficial/therapeutic effects (e.g., better sleep, smaller skin lesions, less itching, etc.) or adverse effects (e.g., worsening of skin lesions, pain at the stimulation site on the ear, etc.).

Adverse effects leading to discontinuation of study participation: During study visits 2-5, potential adverse effects of taVNS will be discussed with the study participants based on the entries in the daily diary. If any adverse effects are reported, the investigators will decide if the study participant will be excluded from continuing the study. Potential adverse effects that will lead to discontinuation of the

study include (but are not limited to): cardiac arrhythmia, hearing loss, tinnitus, seizures, erythema or inflammation at the stimulation site at the ear.

Study groups: Each subject will automatically be assigned a unique Participant ID between 1 and 999 by the Qualtrics survey administered during the first study visit. Subjects will be randomly assigned to one of two study groups (taVNS group or sham-taVNS group) based on the Participant ID (odd numbers taVNS, even numbers sham-taVNS). Subjects will be blinded to which group they are assigned to and subjects of both groups are instructed to use the taVNS stimulator on a daily basis. However, the electrode lead of the stimulators for the sham-taVNS group are tempered with so that no stimulation can occur. Subjects from both groups are instructed to not exceed a setting on the dial for the stimulation current of 2.5, even if they do not feel the tingling sensation on the ear. In our experience, the tingling sensation occurs at current settings between 2.0 and 2.3 in over 95% of subjects (when the electrode lead is not tampered with). All study participants from both groups will be told that they may or may not feel the tingling sensation. It is our expectation that this procedure will allow for a true single-blinded study where study participants are not aware of to which experimental group they are assigned to.

Inclusion criteria: 18 years of age or older, plaque psoriasis.

Exclusion criteria: below 18 years of age; pregnancy; signs of vestibulocochlear neuronitis or nerve damage (e.g., hearing loss or tinnitus); cardiac arrhythmia; epilepsy; anticipated change in any current medication during the 3-month study period. We do not exclude subjects based on any current medication use. But we will exclude subjects who change their medications during the 3-month study period.

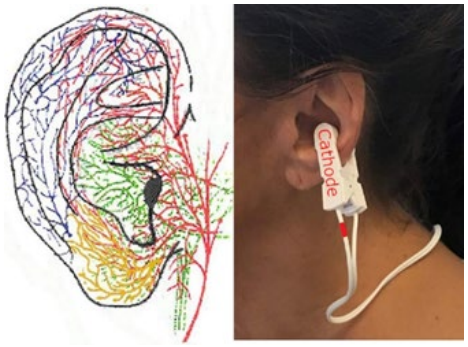

Fig. 1: taVNS

Transcutaneous auricular vagus nerve stimulation (taVNS): The auricular branch of the vagus nerve innervates the cymba conchae (7) as shown in Fig. 1, left. The clip electrode (Fig. 1, right) is connected to a transcutaneous electrical nerve stimulator (EMS 7500, Current Solutions, LLC, Austin, TX). This device is operated from a single 9V battery and not connected to any power outlets. The stimulation parameters will be 10Hz stimulation frequency and 300 µs pulse width. The stimulation current will be determined individually for each subject by slowly increasing the stimulation current until the subjects just barely feel a mild tingling sensation at the site of the electrode. This current will then be used for taVNS. In our previous studies (2, 8) this current was in the range of 20-30 mA. Subjects will be instructed to apply taVNS for 30 minutes in the evening before going to bed and to alternate the left and right ear every day.

Sham-taVNS: For sham-taVNS the electrode lead will be electrically interrupted so that no electrical current can reach the clip electrode.

Potential off-target effects of taVNS: erythema or mild ulceration at the site of the electrode placement on the ears (if this happens, subjects are instructed to terminate the protocol, inform the investigators, and not to continue with the taVNS application); improved sleep has been reported with taVNS (9); an

attenuation of weight gain has been reported with taVNS in a rat model of obesity and diabetes (10) (we will monitor BMI to assess this potential off-target effect).

Assessment of BMI: Body mass index will be monitored throughout the study by measuring height and weight.

Hemodynamic/autonomic assessment: Blood pressure and heart rate will be monitored during every study visit using an arm cuff blood pressure monitor (Omron 10 Series, Omron Healthcare, Inc., Lake Forest, IL). In addition, an ECG will be recorded for 30 min to assess autonomic function by heart rate variability analysis.

Clinical assessment of plaque psoriasis: Three widely-used and accepted methods for measuring psoriasis severity in clinical studies include the Psoriasis Area and Severity Index (PASI), the Static Physician's Global Assessment (sPGA), and the Lattice System Physician's Global Assessment (LS-PGA) (11). All three assessment tools have been demonstrated to by responsive to varying degrees of improvement in psoriasis induced by either placebo or active therapy. All three tools assess the extent of erythema, induration/thickness, and scaling of the skin lesions. However, the PASI is the most detailed assessment tool because it calculates individual scores for the head, trunk, and upper and lower extremities, while the other two indices assess the whole body as a single score. Therefore, we will utilize the PASI score to assess severity of plaque psoriasis. For this assessment subjects need to be partially undressed but will leave their underwear on. For assessment of the skin areas covered by the underwear, we will rely on the assessment of the study participants (by asking to which extent these skin areas are affected, without performing an inspection of these skin areas). In addition to assessing the PASI score, photographs of representive skin lesions will be taken for visual documentation of the potential effects of taVNS. For visual documentation, only skin lesions at the extremities (arms or legs) will be photographed. No photograph of other body parts, including the head and trunk will be taken. The same skin lesions will be photographed during all 5 study visits. The PASI and photographic assessments will be conducted in the clinic rooms at the Burrell College main building by an investigator of the same sex as the study participant or if the investigator is not of the same sex, a "chaperone" of the same sex will be present.

Blood draw: Blood (30 mL max.) will be drawn by venipuncture (antecubital vein) by an experienced phlebotomist. Immediately after the blood draw, the blood will be transported (on ice) from the Burrell main building to the Burrell laboratory (9035 Advancement Ave., Las Cruces, NM 88007) for analysis of immune cell function (extraction of plasma for cytokine analysis, isolation of white blood cells for flow cytometry and cell culture experiments). Blood draws will occur on all 5 study visits.

Assessment of immune function: Immune function will be assessed by measuring plasma concentrations of GM CSF, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10, and TNF-α using a Bio-Plex Immuno Assay kit ((#M 50000007A, Bio Rad, USA). While blood cells will be isolated and the relative abundance of T-helper cells, cytotoxic T-cells, B-cells, monocytes, and natural killer (NK) cells will be determined by flow cytometry. In addition, white blood cells will be cultured and incubated for 24 hours in the presence of lipopolysaccharide (TLR4 ligand) or placebo and cytokines release (GM CSF, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10, and TNF-α, Bio-Plex Immuno Assay kit #M 50000007A, Bio Rad, USA) will be determined in the cell supernatant.

Number of study participants: There are no data available on the effect of taVNS on plaque psoriasis. Therefore, a meaningful power analysis is not possible because the effect size and variability of these effects are unknown. In a recent study on cytokine responses to anti-TNF agents in psoriasis significant effects were observed in 46 patients (12). In another recent study significant effects of biologic drugs on PASI scores were found in 80 patients with plaque psoriasis (13). Based on these two studies, we plan to initially enroll a total number of 50 patients (25 patients in each of the two groups). Once data from these 50 patients are available, the effect size and variability of the taVNS intervention can be estimated and a power analysis can be performed. If the power analysis at this time reveals a too low statistical power, we will amend this IRB protocol to adjust the number of subjects accordingly.

Statistical data analysis: Date will be analyzed by 2-way analysis of variance (2-way ANOVA) for one repeated measure (study visits 1-5) and one independent measure (taVNS or sham-taVNS group). Post-hoc Fisher tests will be conducted to compare data obtained on visit 1 with data obtained on subsequent visits for both experimental groups.

Data handling: Identifiable data (name, address, phone number, e-mail address, date of birth, and gender) will be collected in the initial survey on visit 1 that is used to establish eligibility in the study based on inclusion and exclusion criteria. This survey will be conducted using the Qualtrics survey tool. Within this Qualtrics survey a subject ID number will be generated that will be used to identify all other collected data (blood samples, BMI, blood pressure, heart rate, ECG, heart rate variability, plasma cytokines and cell culture data). The diary will also be only identified by the subject ID number and will not contain the name of the subjects or any other identifiable information. Thus, the diary and all data other than the data on the Qualtrics server are considered as de-identified. The identifiable data collected in the Qualtrics survey will not be downloaded to any secondary data medium, but will remain on the Qualtrics server. The consent forms will be paper forms that contain identifiable data (name and signature). All consent forms will be stored in a locked file cabinet in the office of Dr. Stauss. All study participants maintain a daily diary in the form of a notebook to document the daily taVNS applications (time, duration) and any beneficial/therapeutic or adverse effects of taVNS. Study participants need to bring these notebooks to the study visits for discussion. At the end of the study, participants will keep the notebooks.

COVID-safety practices: COVID screening protocol will be in effect, specifically asking the subjects and investigators if they have had a fever in the past 14 days or need to quarantine because of COVID exposure within the past 14 days. The subjects' and investigators' temperatures will be taken on each study day along with screening for exposure to COVID. Masks will be worn by the investigators and subjects during the entire study period. The investigators will wear appropriate protective gear depending on the task during the study. All activities requiring interacting with study participants will be conducted at the clinic rooms at the Burrell College main building. To access the main building, all study participants have to sign in with the front desk security personell, allowing for contact tracing if needed. Investigators sign in with their Burrell College ID cards when entering the building to allow for potential contact tracing.

Subject Compensation: We will not compensate subjects for participation in the study. Participation in the study will not be associated with costs to the study participants other than costs for transportation to the Burrell main building. taVNS stimulators will be provided to the study participants for the duration of the study free of costs.

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Endocrine disrupting chemicals and placental dysfunction

Vanessa De La Rosa, PhD

Hub Site: Las Cruces

Number of Students Accepted: 2

RESEARCH PLAN

Significance

Endocrine disrupting chemicals (EDCs) are a class of chemicals ubiquitous in the environment and products that we use in our daily lives.[1] EDCs can interfere with normal endocrine function and pose a risk to human health, particularly during windows of susceptibility, such as pregnancy.[2] Pregnant women are exposed to EDCs as evidenced by biomonitoring studies and some EDCs have even been detected in the placenta, suggesting the potential for placental dysfunction and fetal exposure.[3] However, there is a limited understanding if and how EDCs contribute to placental dysfunction.[3] The placenta is a dynamic tissue that facilitates transport of essential nutrients and oxygen to the fetus and has a high level of steroid hormone receptor expression. The placenta also secretes vesicles known as exosomes into the maternal circulation to deliver molecular signals that regulate the activity of target cells. Thus, exosome signaling represents an integral pathway mediating intercellular communication.

This project proposes to evaluate the effects of EDCs on exosome signaling using an *in vitro* placental model.[4] Profiling the changes in the molecular cargo (microRNAs and protein) of exosomes will help us to understand how EDCs affect placenta function. These insights provide a biological foundation for further investigating the association between endocrine disruptor exposure and pregnancy complications.[5–8]

Specific Aims

The goal of this project is to address knowledge gaps in our understanding of EDC exposure and placental dysfunction using an *in vitro* model.

Aim 1: Identify changes in placental exosome profiles exposed to EDCs
Exosome content will be analyzed in JEG-3 cells exposed to EDCs

Innovation

-While placenta-derived exosomes have be studied in the context of biomarkers of disease[7], they have not been profiled to understand the effects of environmental exposures on placenta function.
- The approach proposed in this study will contribute to filling a knowledge gap on endocrine disruptor exposure and placental dysfunction.

Research Strategy

Aim 1: Identify changes in placental exosome profiles exposed to EDCs
JEG3 is a choriocarcinoma cell line cloned from primary choriocarcinoma used as an in vitro model of the placental extravillous trophoblast. It has been previously reported that JEG-3 cells secret exosomes into cell culture media that can be isolated and profiled.[4] Exosome content will be analyzed in JEG-3 cells exposed to 3 different EDCs: arsenic, BPA, and PFOA at 3 doses. These doses will be determined from cell viability studies using the XTT assay. Initially untreated cells will be utilized to determine the feasibility of isolating exosomes prior to EDC exposures. Exosomes will be isolated and prepared for downstream RNA and protein analyses using commercially available kits. Exosome profiles will be evaluated by measuring the exosome markers CD63 and/or PLAP using western blot or flow cytometry.[4,9] Changes in total exosomal protein content will be measured using a colorimetric ELISA assay. Exosomes range generally between 30–150 nm in size and changes in size distribution can be measured using nanoparticle tracking analysis. This analysis is pending a collaborative effort with the University of Florida. Recent studies have identified a small subset of exosome miRNAs associated with gestational diabetes.[8] Expression of these miRNAs (n=6) will be measured using real-time PCR.

Exosomes collected from maternal serum under pathological conditions have shown changes in exosome profiles and we expect protein and miRNA expression in response to EDCs to be robust endpoints. However, this may not occur under *in vitro* conditions, which lack complex tissue dynamics. While miRNA and protein expression changes have been demonstrated in exosomes from complicated pregnancies, these targets may be not be altered in response

to EDCs and an untargeted approach using small RNA sequencing or proteomics may need to be utilized in the future. Future directions include evaluating the bioactivity of EDC derived exosomes on other cell types.

Endocrine disrupting chemicals should be handled with care (full PPE) in the designated chemical hood. These chemicals should **not** be disposed down the drain and should be disposed in a designated waste bottle.

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Neurological responses to stress in females: The role of ovarian hormones

Kristin Gosselink, PhD

Hub Site: Las Cruces

Number of Students Accepted: 2

RESEARCH PLAN
Neurological responses to stress in females: The role of ovarian hormones

Significance

The significance of this project is that it will provide a comprehensive assessment of neuronal activation patterns in the female rat brain following acute or repeated exposure to restraint stress. Furthermore, it will begin to evaluate the role that ovarian hormones play in the female response to stress. The majority of published data on stress-induced brain activation from this laboratory and others has been generated from male rats. While valuable, this approach fails to address the fact that affective disorders such as depression are more common in females, can result from chronic stress exposure, and have been shown to decrease after menopause when estrogen levels are low. In contrast, weight gain is often associated with stress, and this effect may be exacerbated in women as they undergo an age-related decrease in ovarian hormone secretion. Animal studies have shown that anxiety-like behaviors were decreased in female rats during estrus; these animals also had reduced levels of glucocorticoid receptor expression in their hippocampal tissue (Ramos-Ortolaza et al., 2017). Circulating estrogen levels can also influence the neurological and behavioral response to stress (Ter Horst et al., 2009), highlighting the importance of understanding how sex and sex hormones contribute to stress-related health issues.

Specific Aims

It is well-established that significant exposure to stress can precipitate, worsen, or predispose for a wide array of disease states and health challenges. Whether through repeated or extended activation of the sympatho-adrenal medullary system or the hypothalamic-pituitary-adrenocortical axis, stress can impact multiple body systems and has been implicated in cardiovascular, neurological, metabolic, and immune dysfunction. Responses to stress at the level of the brain have been heavily studied, but vary by stressor type, severity, and duration as well as the age at which stress exposure occurs. Connections between stress-sensitive brain regions and the mechanisms that ultimately lead to poor(er) health conditions or outcomes are more difficult to draw, but gaining knowledge in this area is critical to the development of treatment strategies for stress-related illness.

The hypothalamus and, specifically, the paraventricular nucleus of the hypothalamus (PVN), is the main site in the brain from which systemic stress responses are generated. As such, in animal models, PVN activation can serve as a proxy for stress, and the timing and patterns of neuronal activation in this nucleus can provide important information about the stressor in lieu of repeated blood sampling to test for glucocorticoid levels. Extensive mapping has identified neuronal subpopulations within the PVN that respond to acute versus repeated restraint stress (Viau and Sawchenko, 2002), and numerous other brain regions have been evaluated for their role in mediating or modulating the stress response. Few studies, however, have provided a more comprehensive neuroanatomical assessment, and almost none have done so in female animals despite the fact that sex differences in stress responses and stress-related illness manifestations clearly exist. One recent article (Kim and Chung, 2021) looked at neuronal activation in 18 brain regions in acutely stressed female and male mice, and showed sexually dimorphic responses in some areas but similarities in what they refer to as the generalized “core” response machinery. This study did not examine responses to repeated or chronic stress.

Previous work in the PI’s laboratory has shown that sex-specific stress responses are evident at the level of neuronal activation, which may contribute to female-male differences in the consequences of chronic

stress exposure (Zavala et al., 2011). It was further suggested that females may be more sensitive to negative feedback from secreted glucocorticoid hormones, possibly impacting how stress responses are initiated or terminated. In male rats, we have shown that acute or repeated restraint stress both lead to increased orexigenic signaling in the brain (Chagra et al., 2011), which may underlie the propensity for stressed individuals to carry excess body weight. In females, the interaction between stress and estrogenic hormones may exacerbate this problem.

The goal of this project is to identify stress-sensitive regions in the adult female rat brain, and potential mechanisms through which repeated stress might contribute to the increased incidence or enhanced progression of depression, metabolic imbalance, or neuroinflammation. The Specific Aims are:

Aim 1. To determine the activation and distribution of neuronal populations in the female rat brain under conditions of acute and repeated restraint stress.

Aim 2. To assess the impact of ovariectomy, with or without estrogen replacement, on responses to stress in the female rat brain.

Innovation

The research question and methods to be employed in this project are not particularly innovative, but seek answers to important questions about how the female brain processes stressful stimuli.

Research Strategy

Methods

Note: the animal studies described herein were conducted at another institution, under an approved animal care and use protocol. The tissue to be stained and analyzed in this project, coronal sections of rat brain, have been transferred to the laboratory at Burrell and are available for study. This project will not involve the further use of vertebrate animals.

<u>Experimental animals</u>
Adult female Sprague/Dawley Rats were housed individually in standard cages and maintained on a 12:12 h cycle with food and water ad libitum. Rats were allowed to acclimate for one week before being used for experiments. All animals used in this study were cared for in accordance with the Guide for the Care and Use of Laboratory Animals, and all procedures were approved by the UTEP Institutional Animal Care and Use Committee (IACUC protocol A-201006-1).

<u>Ovariectomy and hormone replacement</u>
Bilateral ovariectomy was completed before 60 days of age when normal cycling typically begins in females. Rats were anesthetized with ~ 0.3 ml of Rompun mixture [ketamine/xylazine/acepromazine, 25:5:1 mg/kg, s.c.], with inhaled isoflurane gas anesthesia available as a supplement. Skin incisions were made bilaterally on the dorsal surface below the ribcage, and blunt dissection was used to isolate the ovaries in the abdominal cavity. Forceps were used to gently crush the fallopian tubes at the tip of the dorsal horn of the uterus, sutures were wrapped tightly underneath to cut off blood supply, and the ovaries were removed by cutting above the clamped area. Care was taken not to crush or otherwise

disturb the ovary during excision. The remaining uterine and fat tissue was returned to the abdominal cavity, muscle tissue was sutured, and skin incisions were closed using wound clips. Rats were allowed 6 days for recovery before being subjected to the restraint stress paradigm. Control animals underwent sham surgery in which the same incisions were made but no tissue was excised. Some OVX females were replaced with subcutaneously-implanted 17β-estradiol pellets (Innovative Research of America) at high (1.5 mg/pellet, 120-day release) or low (0.5 mg/pellet) doses.

Restraint stress

Intact and OVX rats were randomly assigned to one of three stress conditions: Control (Con), Acute restraint (Acu), or Repeated restraint (Rep). Physical restraint was used as an emotional stressor, with the rats placed inside an acrylic restraining device (Kent Scientific) for 30 min. All restraint and exposure was done near the beginning of the light cycle, between 0900 and 1100 h. Acu rats were exposed to open restraining devices in their home cages for 30 min/d for 13 consecutive days, and then restrained for 30 min on the 14th day only, while Rep rats were restrained for 30 min on each of the 14 days. Con rats were exposed to the restraining device daily for 30 min but never restrained.

Perfusion and tissue collection

At the end of the restraining treatments, the animals were deeply anesthetized with 100 mg/kg of sodium pentobarbital, i.p. (Nembutal®; McKesson), followed by perfusion through the ascending aorta with ~100 mL of 0.9% saline, and 400–500 mL of 4% paraformaldehyde (JT Baker) at pH 9.5 in 0.1 M borate buffer. Brains were dissected, post-fixed for 5 h at 4 °C, and cryoprotected overnight at 4°C in KPBS with 10% sucrose, then serially sectioned in 30 µm sections on a freezing microtome (Model SM 2000R; Leica) and stored in antifreeze (30% ethylene glycol, 20% glycerol) at −20°C until used for immunohistochemical analysis.

Note: Methods prior to this point have already been completed. Subsequently-described methods will be carried out as part of the current proposed project.

Nissl staining

Brain sections will be stained for Nissl material to aid in the anatomical localization of specific brain regions for future, comparative, immunohistochemical analyses.

Immunohistochemistry and cell counting

Brain sections will be immunohistochemically stained for Fos, the protein product of the immediate early gene *c-fos*, as an indicator of neuronal activation in response to stress. Fos expression will be localized using an avidin-biotin-immunoperoxidase technique. Tissue sections will be incubated overnight at room temperature in primary antiserum against Fos (1:10,000; Abcam), and incubated on the following day for 1 h at room temperature in secondary antibody (biotinylated goat anti-rabbit IgG, 1:200; Vector). An avidin-biotin-complexing solution (Vectastain Elite kit; Vector) will applied for 1 h, and a peroxidase method using diaminobenzidine (DAB) as a chromogen will be used to visualize specific binding. Separate series of brain sections will be immunostained for markers of oxidative stress and/or neuroinflammation, such as Iba-1, cyclooxygenase-2 (Cox-2), and nitric oxide synthase, using a similar method. Stained sections will be mounted on gelatin-coated slides, dehydrated through a graded series of ethanol and xylene, and coverslipped with DPX mountant (Electron Microscopy Sciences).

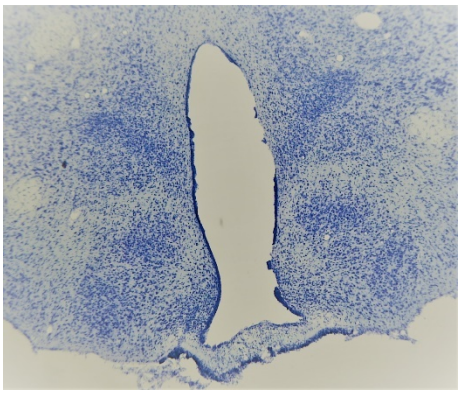
Quantification of Staining

The neurological response to stress will be assessed by counting the number of Fos-positive cells in the paraventricular hypothalamic nucleus (PVN). A light microscope coupled to a digital camera will be used

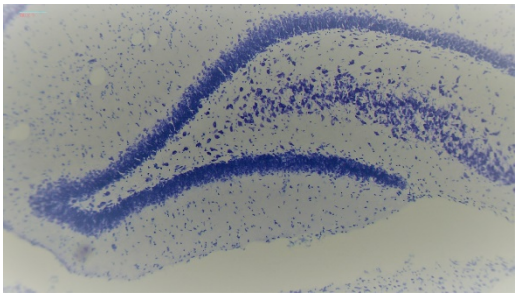
to capture images of this brain region, taken unilaterally from 4-5 sections throughout the rostrocaudal extent of the PVN, and cells will be quantified using ImageJ software. Addition brain regions that have been associated with stress responsivity, feeding and energy balance, or affective disorders, will also be quantified for Fos expression. Counts from all sections within a single brain region will be summed for each animal. Group averages will then be calculated, and compared statistically using two-tailed t-tests with a p value of ≤0.05 considered significant.

Preliminary images and results (Male rats)

Through Nissl staining, we can identify specific neuroanatomical regions in the rat brain; this will aid us in localizing and quantifying our immunohistochemical staining signals.

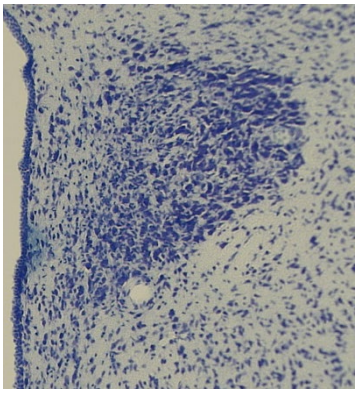


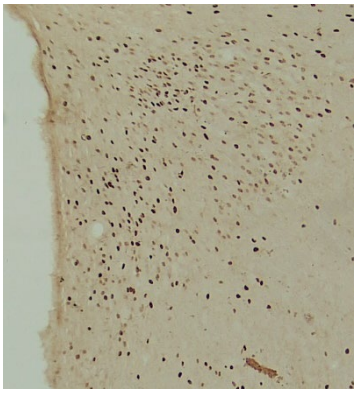
Caudal Hypothalamus



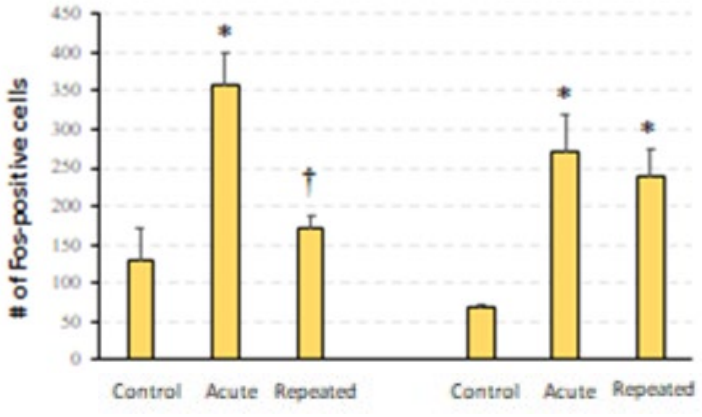
Hippocampus / Dentate Gyrus

A Nissl-stained paraventricular nucleus of the hypothalamus (PVN), and an adjacent section stained for Fos expression which shows PVN neurons activated by an acute restraint stress exposure.





The number of Fos-positive cells in the PVN (or other regions) can be quantified and compared across treatment groups. Below, the graph shows the level of PVN activation in response to acute or repeated restraint stress (versus Control, no stress) in two different strains of male rats.



Wistar-Kyoto Spontaneously Hypertensive

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Physiol Behav. 2011 Aug 3;104(2):215-21. doi: 10.1016/j.physbeh.2011.03.022. Epub 2011 Mar 29.
Female responses to acute and repeated restraint stress differ from those in males. **Jaidee K Zavala**, Almendra A Fernandez, Kristin L Gosselink

Optimization of Splenic Pump to Induce Translocation of Immune Cells from the Spleen to the Systemic Circulation

Adrienne Kania, DO, FAAO and Harald M. Stauss, MD, PhD

Hub Site: Las Cruces

Number of Students Accepted: 4

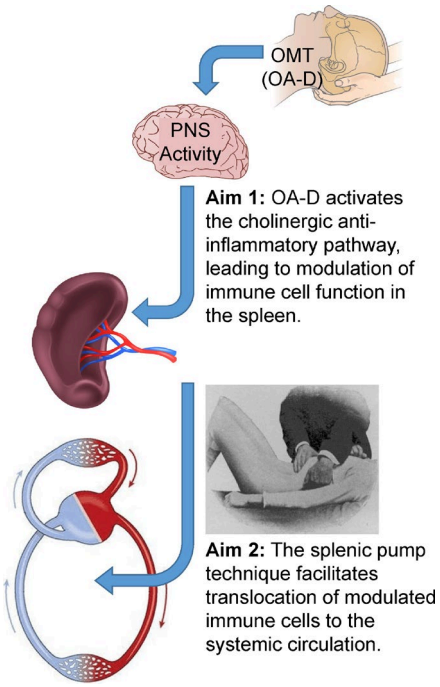

Fig. 1: Working hypothesis.

Research Proposal

Significance

Chronic inflammatory diseases such as psoriasis are difficult to treat, and newer highly effective biological pharmaceuticals, such as TNF-α inhibitors, are not available to all patients because of the high costs or contraindications. Thus, there is a need for affordable and effective treatment options. In an ongoing research study, we test the hypothesis illustrated in Fig. 1. According to this hypothesis, the osteopathic manipulative treatment (OMT) technique of occipitoatlantal decompression (OA-D) would activate the cholinergic anti-inflammatory pathway[1] through its well-documented effect on the parasympathetic nervous system (PNS).[2] Activation of this pathway would then shift the function of immune cells in reticular organs such as the spleen, from a pro-inflammatory to an anti-inflammatory state.[3,4] Finally, the subsequently applied splenic pump technique would facilitate translocation of these spleen-derived immune cells to the systemic circulation, from where they can reach the site of potential inflammatory insults.

Indeed, our pilot data obtained from flow cytometry in healthy study participants demonstrate a 60% increase in circulating monocytes following three consecutive days of application of the splenic pump technique compared to control (Fig. 2). Importantly, monocytes (isolated by magnetic cell separation) from subjects who underwent three consecutive days of splenic pump expressed less interleukin-6 (IL-6) mRNA (RT-PCR) then monocytes isolated from control subjects (Fig. 3). This finding suggests that circulating monocytes originating from cell pools in the spleen differ in their phenotype from other circulating monocytes originating from the bone marrow. It is important to note that the spleen is the largest reservoir for monocytes and contains seven-times more monocytes than the blood.[5] Thus, if only 10% of the monocytes stored in the spleen are translocated into the systemic circulation by the splenic pump, the monocyte concentration in the blood would increase by 70%, which is close to the observed increase in circulating monocytes of 60% (Fig. 2).

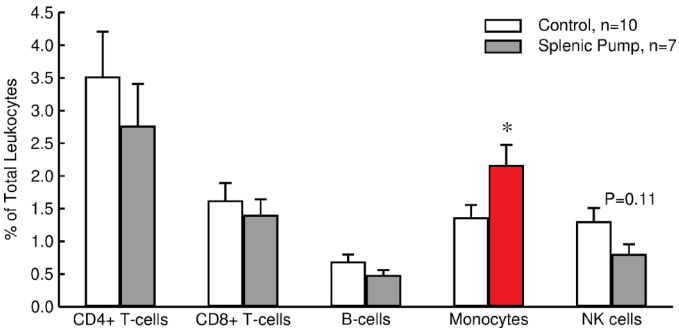

Fig. 2: Flow cytometry analysis of leukocytes from study participants that underwent three consecutive days of splenic pump (n=7, filled bars) or control intervention (n=10, open bars). *: P<0.05 vs. control.

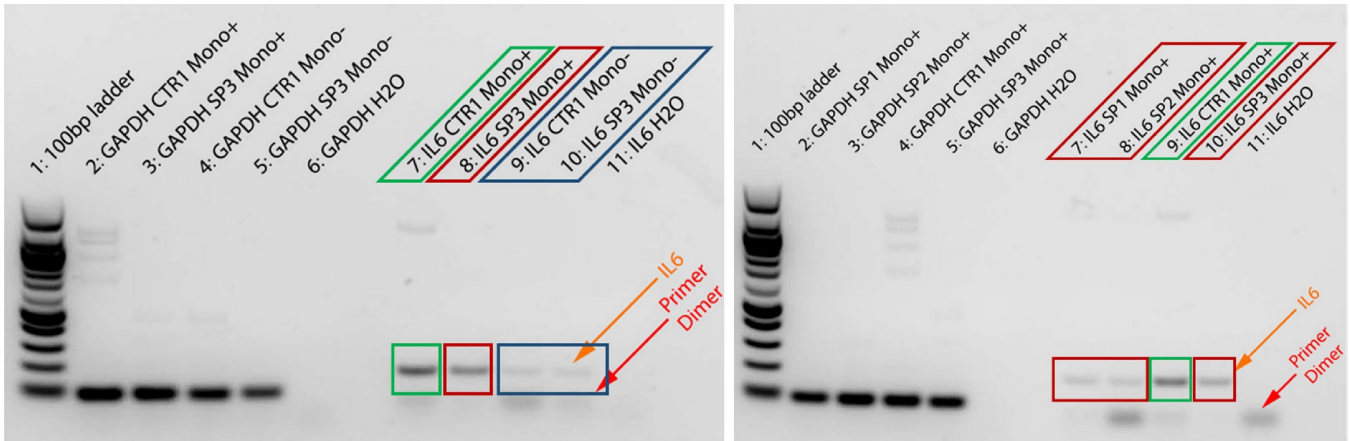

Fig. 3: RT-PCR of IL-6 mRNA expression in isolated monocytes (Mono+) from subjects who underwent three consecutive days of splenic pump (SP, red) compared to control subjects (CTR, green). IL-6 mRNA expression was less in monocytes from subjects who underwent SP (red, n=4) compared to control subjects (green, n=2). The monocyte depleted (Mono-, blue, n=2) cell fraction expressed very little IL-6.

These preliminary data are significant, because they potentially imply that the splenic pump technique results in an abundance of spleen-derived circulatory monocytes. These spleen-derived monocytes are characterized by a less inflammatory phenotype than the other circulating monocytes that are derived from the bone marrow, as indicated by the lower mRNA expression of the pro-inflammatory cytokine IL-6 in monocytes from subjects who underwent the splenic pump technique compared to those from control subjects (Fig. 3).

For the effect of the splenic pump on the relative abundance of circulating spleen-derived monocytes it is critical that a maximum number of monocytes are mobilized from the spleen during the application of the splenic pump. In our currently ongoing study, we standardized the splenic pump technique by applying compressions of 2-4 mmHg (measured with a partially inflated blood pressure cuff placed under the left lower ribcage) at a rate of 20 compressions per minute for a duration of 10 minutes on each of the three consecutive study days. Even though, we did not attempt to optimize these parameters, a 60% increase in circulating monocytes was observed following application of the splenic pump technique (Fig. 2). It is possible that an even larger effect of the splenic pump technique may be achieved if compressions at greater pressures or at different rates may be applied. The *objective of the proposed study* is to identify optimal parameters for the splenic pump technique that are most effective in translocating immune cells from the spleen to the systemic circulation. *Optimizing these parameters is significant, because a maximal response to the splenic pump technique is required for the next step towards achieving our long-term goal, which is to apply this OMT technique to patients with chronic inflammatory diseases (e.g., psoriasis)*, which will be the topic of a future NIH-NCCIH application.

Specific Aims

The *long-term goal* of our research is to establish a novel approach to use OMT techniques as alternative or adjuvant treatment modalities for chronic inflammatory diseases, such as irritable bowel syndrome or psoriasis. The idea is to utilize a mechanistic approach that combines two OMT techniques that elicit complementary actions (Fig. 1). First, OMT techniques, such as OA-D, are known to increase vagal tone[2,6-9] and, therefore, are likely to modulate the inflammatory reflex[1] that involves the cholinergic anti-inflammatory pathway.[10] In our ongoing study, we test the hypothesis that OA-D changes the phenotype of immune cells in the spleen through activation of the cholinergic anti-inflammatory pathway.[10] Second, we propose to use the splenic pump technique to translocate these immune cells from the spleen to the systemic circulation and to potential sites of inflammatory insults. As shown above, our preliminary data suggest that monocytes are translocated from the spleen to the systemic circulation by the splenic pump technique and that these splenic cells express less mRNA for the pro-inflammatory cytokine IL-6 compared to the other circulating (bone marrow-derived) monocytes. Thus, a highly effective splenic pump technique is essential to achieve our long-term goal to utilize OMT to treat chronic inflammatory diseases. However, *it is not known* how repetitive compressions need to be applied when performing the splenic pump technique to achieve a maximal translocation of immune cells. To our knowledge, there is no literature

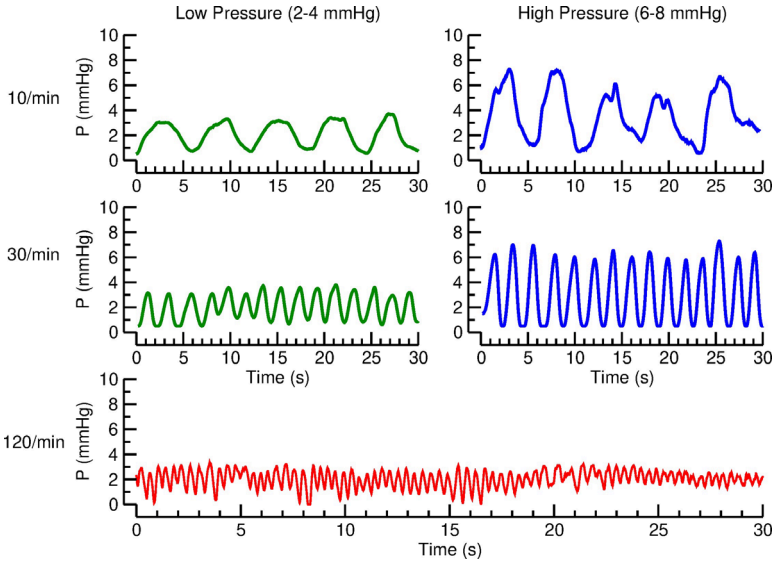

Fig. 4: Different pressures (P) and rates of compressions applied when performing the splenic pump technique. Pressures were monitored using loadpad sensors (Novel Electronics Inc., Saint Paul, MN).
Top: splenic pump at a rate of 10 compressions/min.
Middle: splenic pump at a rate of 30 compressions/min.
Green: splenic pump using a low peak pressure of 2-4 mmHg;
Blue: splenic pump using a high peak pressure of 6-8 mmHg.
Red: splenic pump performed using a low peak pressure of 2-4 mmHg applied at a vibratory compression rate of 120 compressions/min.

comparing the effects of different

pressures or rates of compressions when performing the splenic pump technique on physiologic parameters, such as translocation of immune cells from the spleen. Thus, *there is critical need to establish the optimal parameters for the splenic pump technique* (pressure applied and rate of compressions) that result in a maximal translocation of immune cells from the spleen to the systemic circulation. The lack of this knowledge constitutes an important problem because it prevents the optimal utilization of the splenic pump technique in an OMT-based treatment approach for chronic inflammatory diseases. Thus, *the objective of this study* is to establish these optimal parameters by testing the *hypothesis* that translocation of immune cells from the spleen to the systemic circulation in response to the application of the splenic pump technique depends on the rate of compressions and the pressure applied. *We are well-prepared to undertake the proposed research* because the expertise of the two primary investigators, Dr. Kania and Dr. Stauss complement each other to aid in the success of the research. Dr. Kania is an osteopathic physician with over 30 years of experience practicing OMT, while Dr. Stauss is physician-scientist who has conducted basic science research in the areas of physiology and pharmacology for over 30 years. Furthermore, our preliminary data demonstrate that the splenic pump technique indeed results in translocation of immune cells from the spleen to the systemic circulation. Finally, we have established all required experimental methods in our laboratories, including flow cytometry, Bio-Plex assays, and technologies to monitor the pressure and rate of compressions when performing the splenic pump technique (Fig. 4).

Specific Aim: Maximize translocation of immune cells in response to the splenic pump technique. *The working hypothesis*, based on our preliminary data, is that translocation of immune cells from the spleen to the systemic circulation induced by the splenic pump technique depends on the rate of compressions and the pressure applied. Furthermore, we hypothesize that an optimal combination of pressure and rate of compressions exists that results in a maximal translocation of immune cells. At the completion of these studies, we anticipate the following *expected outcomes*. First, the pressure and rate of compressions resulting in maximal translocation of immune cells when applying the splenic pump technique will be revealed. Second, it will become possible to most effectively utilize the splenic pump technique to elicit anti-inflammatory actions. These outcomes are expected to have *important positive impact on osteopathic medicine*, because they will allow optimization of the splenic pump technique in patients with chronic inflammatory diseases.

Innovation

The status quo as it pertains to the use of OMT techniques for chronic inflammatory diseases is based on the original ideas of Andrew Taylor Still from over 100 years ago and largely depends on personal opinions and clinical experience of practicing osteopathic physicians. A recent literature review on the use of osteopathy in chronic inflammatory diseases, demonstrated that osteopathic physicians apply a large variety of totally different OMT techniques for the same chronic inflammatory diseases, suggesting that the current osteopathic approach is largely based on individual opinions and personal experience.[11] In addition, there is a paucity of mechanistic research studies exploring the physiologic, cellular and molecular effects of OMT techniques. *Thus, some of the OMT techniques fail to reveal their true potential because they are utilized without knowledge of their exact physiologic, cellular, and molecular effects, preventing significant advancement in osteopathic medicine. Our research approach is innovative*, in our opinion, because it represents a substantive departure from the status quo by proposing the use of OMT techniques based on their known physiologic, cellular, and molecular effects. Specifically, we propose utilizing the documented ability of some OMT techniques to augment parasympathetic nervous system activity[2,6-9] and to activate the cholinergic anti-inflammatory pathway[1,3,10], that would “reprogram” immune cells in the spleen to a more anti-inflammatory phenotype (e.g., less IL-6 mRNA expression). We will then capitalize on the physiologic and cellular effects of the splenic pump technique to translocate these “reprogrammed” immune cells (e.g., monocytes) from the spleen to the systemic circulation and to the site of potential inflammatory insults as illustrated by our preliminary data shown in the “Significance” section of this application. *Our mechanistically driven approach will potentially open new horizons in osteopathic medicine, because using established OMT techniques based on their known physiologic, cellular and molecular effects will allow these OMT techniques to reveal their full potential in the treatment of chronic inflammatory diseases.*

Research Strategy

Specific Aim: Maximize translocation of immune cells in response to the splenic pump technique.

Introduction: The splenic pump is an OMT technique that applies rhythmic compressions to the area over the spleen to enhance a patient's immune response. The optimal pressure and rate of compressions that result in a maximal immune response when applying the splenic pump technique are currently unknown. This lack of knowledge constitutes an *important problem* because it prevents the splenic pump technique from revealing its maximal potential when treating chronic inflammatory diseases. *The objective of this aim* is to establish the optimal pressure and rate of compressions that result in a maximal immune response when applying the splenic pump technique. To attain this objective, we will test the *working hypothesis* that the immune response to the application of the splenic pump technique depends on the rate of compressions and the pressure applied. Furthermore, we hypothesize that an optimal combination of pressure and rate of compressions exists that results in a maximal immune response. Our *approach* to testing the working hypothesis will be to assess the immune responses to the splenic pump technique performed with differing pressures and rates of compressions. As surrogate measures for the immune response, we will assess the translocation of immune cells from the spleen to the systemic circulation by flow cytometry and the changes in circulatory cytokine levels by Bio-Plex assays. The *rationale* for this aim is that its successful completion is likely to contribute a fundamental element of our base of knowledge, without which the relationship between applied pressures and rates of compressions and the immune response to the splenic pump technique cannot be understood. The acquisition of such knowledge is essential for the optimal use of the splenic pump technique to treat chronic inflammatory diseases. Upon completion of this Aim, it is our *expectation* that we will have established the optimal pressure and rate of compressions that result in a maximal immune response to the splenic pump technique. This is important, because this knowledge will form the foundation of subsequent studies, in which the splenic pump technique will be applied to patients with chronic inflammatory diseases.

Study Participants: The study will be conducted in adult generally healthy subjects (n=7) of both genders, recruited from Las Cruces, NM and surrounding areas through advertisement on the Burrell College research website, e-mails to potential participants (e.g., within the Burrell College and New Mexico State University community), and local media. Based on the local demographics, it is expected that the study population will consist of 50-60% Hispanics/Latinos, 30-40% Caucasians, 3-4% mixed races, 2-3% African Americans, 1-2% Native Americans, and 1-2% Asians with an age distribution of 48% of the study population to be between 18 and 44 years, 35% between 44 and 65 years, and 17% above the age of 65 years. *Exclusion criteria include:* age under 18 years; pregnancy; contraindications for the splenic pump technique (e.g., nearby wounds or abscesses, fever); splenomegaly; severe obesity (BMI>35 kg/m^2); any medication or medical condition that affects the immune system or study outcomes; and current drug or alcohol abuse. Prior to enrollment potential participants will review the consent form with one of the investigators. Once written consent is obtained, the subjects are asked to fill out a questionnaire that is designed to verify that the subject qualifies for inclusion in the study (based on exclusion criteria) and to obtain contact (name, address, phone, e-mail) and demographic (age, gender, ethnicity) information.

Experimental Protocol (Fig. 5): Subjects (n=7) will participate in five study sessions (3 consecutive study days each) separated by at least 4 weeks. Before enrolling in the study, written informed consent is obtained. On the first study day of all study sessions, subjects take an online questionnaire to check for exclusion criteria, and body weight and height will be determined to assess body mass index. At the beginning of the first study day and at the end of the third study day, blood is collected (15 mL, venipuncture, antecubital vein) for flow cytometry and determination of plasma cytokine levels. On each study day, the splenic pump technique is applied for a duration of 10 minutes after a 10-minute rest. On each day of the five study sessions the splenic pump is performed using one of five combinations of pressures and rates of compressions. The order of the combinations will be randomly assigned to the five study sessions and the same pressure and rate of compressions will be used on each of the 3 study days within a given study session. Importantly, the protocol for each study session (10 minutes of splenic pump on 3 consecutive days) resamples the protocol of our currently ongoing study that demonstrated that the splenic pump technique increases the number of circulating monocytes (Fig. 2).

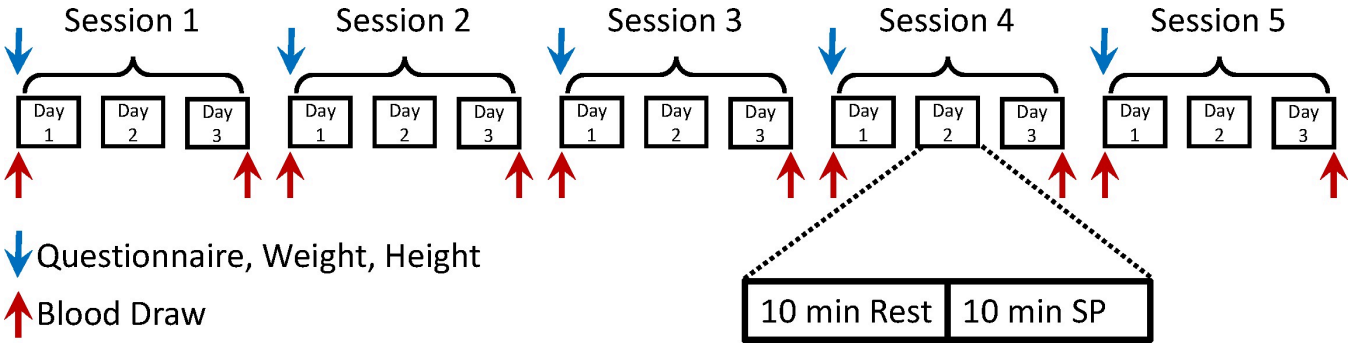

Fig. 5: Experimental Protocol.
Each session consists of 3 study days. Sessions are separated by a minimum of 4 weeks. SP: Splenic pump.

Controls: Each study participant serves as its own control, because data obtained at the beginning of a study session (on day 1, before the first splenic pump of the session) will be compared to data obtained at the end of a study session (on day 3, after the last splenic pump of the session).

Splenic Pump Technique: The splenic lymphatic pump technique, will be performed as a gentle alternating compression and relaxation over the spleen. With the patient supine, the osteopath places a posterior hand along the left lower ribcage, and a superior hand over the left costal arch, encompassing the spleen. The physician compresses and releases the spleen at a given rate of compressions and pressure for 10 min.[12] Five combinations of rates of compression and pressures will be randomly assigned to the five study sessions. While the splenic pump is performed, the rate of compressions will be timed using a metronome and the pressure will be monitored using a partially inflated blood pressure cuff placed under the left lower ribcage. The combinations of rates of compressions and pressures as illustrated in Fig 4 are:

Combination	Rate of Compressions	Pressure
1	10/min	2-4 mmHg
2	10/min	6-8 mmHg
3	30/min	2-4 mmHg
4	30/min	6-8 mmHg
5	120/min (vibration)	2-4 mmHg

Translocation of Immune Cells: Flow Cytometry will be used to determine the extent of translocation of immune cells (T-helper cells, cytotoxic T-cells, B-cells, monocytes, natural killer (NK) cells) from the spleen to the systemic circulation. Briefly, venous blood samples (EDTA) will be centrifuged, and plasma stored (-80°C) for determination of plasma cytokines. Red blood cells will be lysed (ACK lysis buffer) and leukocytes stained for flow cytometry. The following antibody panels will be used to determine the different cell types:

Fluorochromes	PerCP-Cy 5.5 (red)	PE (yellow)	FITC (green)
T-helper and cytotoxic T cells	CD3	CD4	CD8
B-lymphocytes	CD19	CD45	-
Monocytes	CD14	CD16	CD11b
Natural Killer (NK) cells	CD3	CD16	CD56

For compensation, we will stain cells with CD45 antibodies for the three fluorochromes. Appropriate isotype controls will be used to determine thresholds for positively stained cells for the 3 fluorochromes.

Interpretation and Anticipated Results: Translocation will be determined as the difference of the relative cell numbers (% of total number of leukocytes) determined at the end (day 3) and the beginning (day 1) of each study session. Based on our preliminary data (Fig. 2), we anticipate that the splenic pump technique will increase the relative numbers of circulating monocytes. We also anticipate that this increase in circulating monocytes depends on the combination of rates of compressions and pressures applied during the splenic pump intervention at the five study sessions.

Plasma Cytokine Levels: Plasma will be analyzed for cytokine levels (GM-CSF, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α) using a Bio-Plex Immuno Assay kit ((#M 50000007A, Bio Rad, USA) according to

the manufacturer's instructions. Previously, we have successfully used the same Bio-Plex Immuno Assay kit for a different study. Thus, we have experience using this system and are confident that this technique will yield reliable data.

Interpretation and Anticipated Results: The effect of the splenic pump on plasma cytokine levels will be determined by the difference in cytokine levels in plasma obtained at the end (day 3) and the beginning (day 1) of each study session. Of particular interest is the IL-6 response to the splenic pump intervention, because IL-6 is a "signature cytokine" for monocytes.[13] Our preliminary data show a 60% increase in circulatory monocytes in response to the splenic pump (Fig. 2). However, spleen-derived monocytes express less IL-6 mRNA (Fig. 3). Thus, the effect of the larger number of circulating monocytes on plasma IL-6 levels may be offset by reduced IL-6 mRNA expression by spleen-derived monocytes. Therefore, it is reasonable to anticipate that IL-6 plasma levels may not change or may even decrease. This anticipated finding is significant, because monocytes have been demonstrated to increase pro-inflammatory IL-6 production in chronic inflammatory diseases, such as psoriasis.[14]

Power Analysis: In our ongoing study, the splenic pump increased circulating monocytes from 1.4±0.6% to 2.2±0.8% (mean±SD). Based on this data, *we will need 7 subjects to achieve an acceptable statistical power (type II or 1-β error) of 80%* in a paired t-test at a type I (α) error of 5%.

Statistical Analysis: The effect of the splenic pump technique on translocation of immune cells and plasma cytokine levels will be determined by the difference of relative cell numbers and plasma cytokine levels at the end and the beginning of each study session. To test if these effects are statistically significant ($P<0.05$), paired t-tests will be performed to compare the values obtained at the beginning and end of each study session. To identify the combination of rate of compressions and pressure applied during the splenic pump technique that results in the greatest translocation of immune cells and/or greatest effect on plasma cytokine levels, one-way ANOVAs for repeated measures (each subject undergoes all five study sessions) will be performed to compare the effects of the splenic pump during the five study sessions on translocation of immune cells and plasma cytokine levels.

Potential Problems and Alternative Strategies: This study is based on the premise that the splenic pump technique modulates the immune system. Our preliminary data solidly supports this premise by demonstrating an increase in the relative number of circulating monocytes following application of the splenic pump technique (Fig. 2). Our working hypothesis is that this effect of the splenic pump technique depends on the rate of compressions and the pressure applied. Although unlikely, there is a remote possibility that this working hypothesis will be invalidated when it is tested objectively. If this happens, the alternative hypothesis would be that all five tested combinations of rates of compression and pressures are equally effective in modulating the immune system. In this case we would compare the results of our preliminary data that were obtained using a rate of 20 compressions/min at a pressure of 2-4 mmHg with the results of the proposed study (that will test different rates of compression) to determine a combination of rate of compressions and pressure to be used for future studies.

Timeline: There will be a total of 35 study sessions (7 subjects and 5 study sessions each). We anticipate conducting one study session (3 study days) per week for a total of 35 experimental weeks. Thus, even when considering vacation and holiday times, we are confident that we can complete the 35 study sessions within the one-year funding period.

Future directions: Our long-term goal is to establish a novel approach to use OMT techniques, including the splenic pump technique, as alternative or adjuvant treatment modalities for chronic inflammatory diseases. At the conclusion of the proposed study, we anticipate having identified the optimal combination of rate of compressions and pressures that result in a maximal immune response to the application of the splenic pump technique in healthy study participants. The next step towards achieving our long-term goal will be to apply the splenic pump technique to patients with chronic inflammatory diseases using the optimal parameters identified in the proposed study. Specifically, we are considering applying the splenic pump technique to patients with psoriasis, because the clinical efficiency of the splenic pump could be easily assessed by measuring the size of skin lesions.

Procedures, situations, or materials that may be hazardous to personnel: We will collect blood samples that may potentially be infectious. To minimize the potential risks associated with blood-borne pathogens, blood draws will be done by trained and experienced phlebotomists and all blood work will be conducted in certified biological safety cabinets.

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Assessing the anti-inflammatory properties of cannabinoids by flow cytometry

Michael Woods, PhD

Hub Site: Las Cruces

Number of Students Accepted: 3

Assessing the anti-inflammatory properties of cannabinoids by flow cytometry

Significance

Neutrophil Extracellular Traps (NETs)—extracellular webs of chromatin containing proteases—play a role in the pathogenesis of numerous autoimmune and inflammatory diseases, including rheumatoid arthritis, multiple sclerosis and COVID-19.[1] Therefore, inhibition of NETosis may represent a novel method for treating or preventing the progression of some diseases. Cannabinoids derived from the hemp plant *Cannabis sativa* have demonstrated beneficial properties linked to the known medicinal effects of medical marijuana through modulation of the endocannabinoid system. Cannabidiol (CBD) in particular possesses anti-inflammatory and anti-oxidant properties through agonistic and antagonistic pathways.[2] In particular, CBD has been shown to interact with GPR55, which is expressed on human neutrophils and is responsible for regulating neutrophil degranulation and reactive oxygen species production.[3]

Limited data exist to speak directly to the ability of CBD to regulate NETosis. Fundamentally, our work hypothesizes that CBD negatively regulates NETosis by interfering in NADPH oxidase-driven ROS production, histone citrullination, or both. CBD modulates the effects of LPS in stimulating NETosis in neutrophils from psoriatic patients.[4] In our hands, CBD has shown potential as a regulator of NETosis; however, the results have been inconclusive, to date. Our initial studies in 2019 used immunofluorescent microscopy to demonstrate that CBD pre-treatment had a modest effect on inhibiting NETosis (**Figure 1)**. Follow-up studies in 2020 measured NETosis using an indirect assessment of soluble elastase activity and demonstrated a similar trend toward NET inhibition at higher doses of CBD (**Figure 2**). These observations have been hampered by a high rate of spurious NET formation, which is a widely reported phenomenon in NET-related research. Furthermore, we have yet to assess the effects of CBD in combination with other hemp-derived cannabinoids.

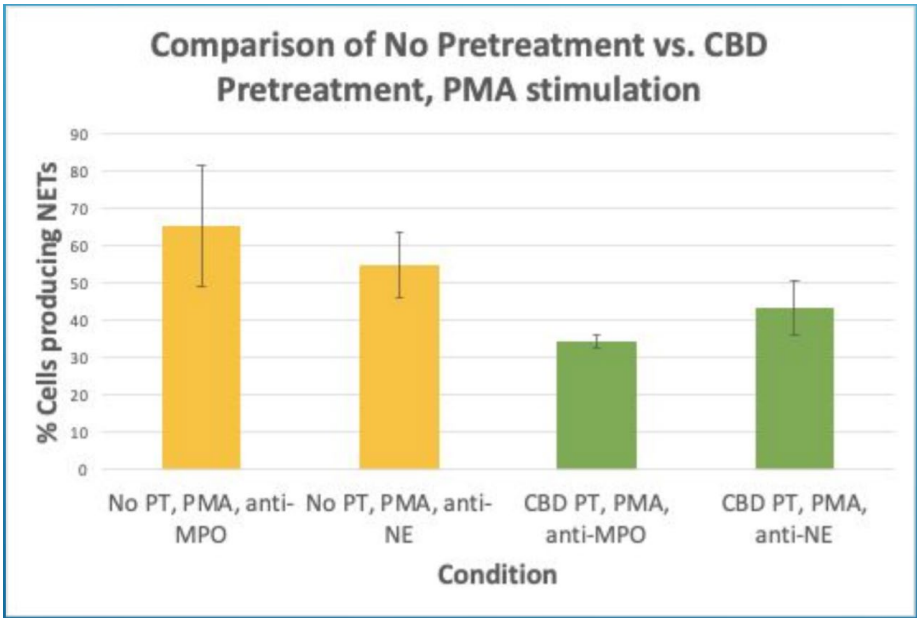

Figure 1. CBD pre-treatment inhibits PMA-induced NETosis as measured by immunofluorescent microscopy. Human neutrophils were pre-treated for 1 hour with 10 μM CBD and then stimulated with PMA. NETosis was assessed by counting the number of cells forming NETs.

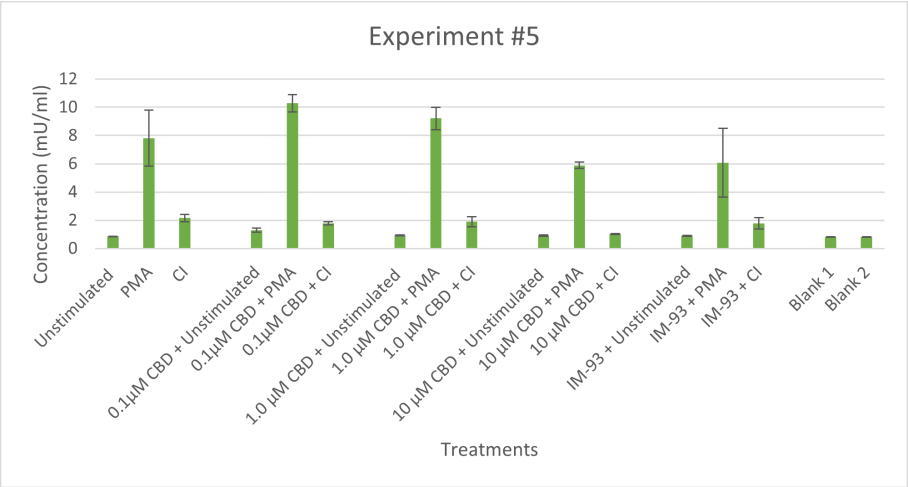

Figure 2. CBD may have a dose-dependent inhibitory effect on PMA-induced NETosis. Human neutrophils were pre-treated with varying doses of CBD and stimulated with PMA. NETosis was assessed by measuring soluble elastase activity. IM-93, a known NET inhibitor, was used as a positive control.

Specific Aims

1. **Optimize techniques for isolating pure neutrophil populations from human blood samples using negative selection and flowy cytometric characterization.**

2. **Determine the effect of CBD alone or in combination with other cannabinoids on the rate of PMA and LPS-induced NETosis by flow cytometry.**

3. **Determine the effect of CBD alone or in combination with other cannabinoids on ROS production in response to PMA or LPS stimulation.**

Innovation

According to one estimate, the CBD market will grow to $19.5 billion in 2025, pending FDA approval of CBD as a legal additive in 2022.[5] As cannabinoid use spreads, there will be greater need for understanding the potential health benefits of CBD and related compounds. This work seeks to better describe the effect of CBD has on mechanisms related to inflammation and disease.

Research Strategy

This proposal seeks to develop a new method for us to measure NETosis and associated responses using flow cytometry. Burrell currently operates a Guava EasyCyte Pro 5HT three-color, five channel flow cytometer. Most flow cytometric techniques described to date utilize complex, multi-laser or imaging flow cytometry systems to measure NETosis.[6] Our technique will be based on a recently-described protocol for measuring NETosis in mouse bone marrow-derived neutrophils,[7] which we will adapt for use with human peripheral blood derived neutrophils. Briefly, we will isolate neutrophils from whole blood collected from volunteer

research participants. We will isolate neutrophils by negative selection and characterize the cell purity by flow cytometry, as illustrated in **Figure 3**. This is important because previous methods used in our laboratory were highly sensitive to cell numbers, which could explain some of the experimental variability observed in our experiments. By analyzing cells by flow cytometry we 1) can assess the purity of our cell population after negative selection, and 2) reduce the effect of variable cell numbers because cells are interrogated in a cell-by-cell basis.

In the next phase of the experiment, we will stimulate NETosis and measure the number of NETs using SYTOX Green staining, as illustrated in **Figure 4**. SYTOX Green stains extracellular DNA, which is a component of NETs. Alternatively, we can stain some cells with fluorescent antibodies against myeloperoxidase or H3-Cit, which are alternative markers of NETosis; however, this limits our ability to characterize cell purity using the available single laser technology.

We will use a similar technique to assess reactive oxygen species generation is purified neutrophils underdoing NETosis. In this experiment, we will replace SYTOX Green with Total Reactive Oxygen Species (ROS) Assay Kit 520 nm, which contains the necessary reagent and buffer for identifying ROS in cells by flow cytometry in the FITC channel. ROS generation is important for the initiation of NETosis in response to PMA stimulation and this will allow us to begin to pick apart the pathways potentially influenced by CBD and related compounds.

There are several potential limitations to this technique and potential challenges to overcome. First and foremost, neutrophils are extremely delicate and will initiate NETosis in response to harsh pipetting or the generation of bubbles. We have observed this phenomenon in previous experiments, which has clouded our results to date. Based on our experience, this is highly dependent on experimenter technique and comfort with the protocol. Unnecessary or unexpected delays in processing of the samples also introduces variability as neutrophils are short-lived and do not respond well to prolonged storage. Therefore, we will attempt to streamline these experiments to minimize the amount of time from blood collection to analysis.

The biohazardous risks associated with this study involve handling human blood of unknown status. Student investigators will be required to complete CITI and in-person laboratory safety training and will work under the direct supervision of the principle investigator. Phlebotomy experience is preferred.

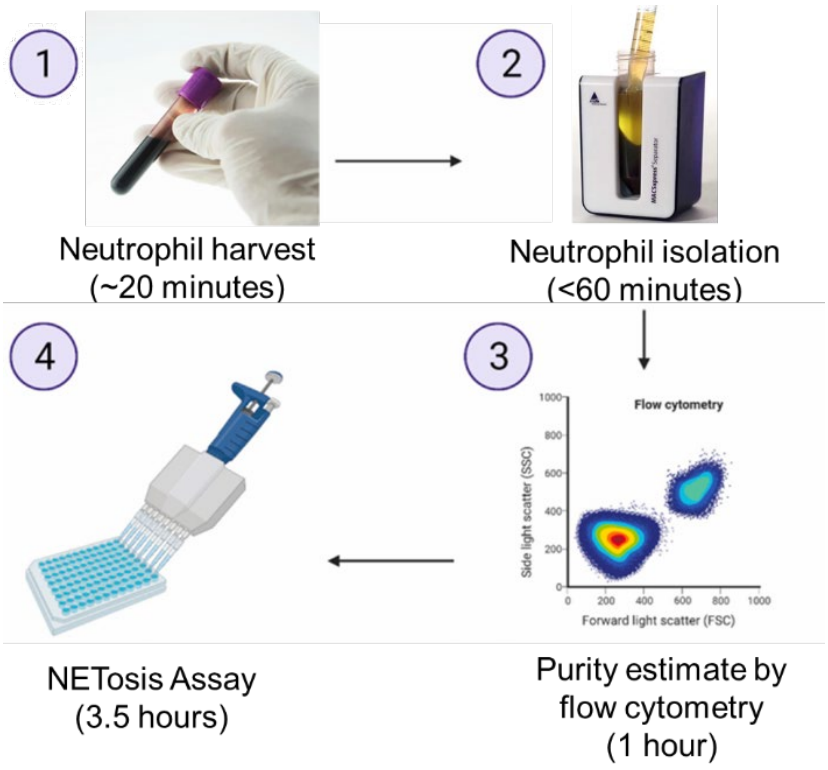

Figure 3. Process for harvesting whole blood, isolating neutrophils by negative selection, and determining purity of the cell population using flow cytometry.

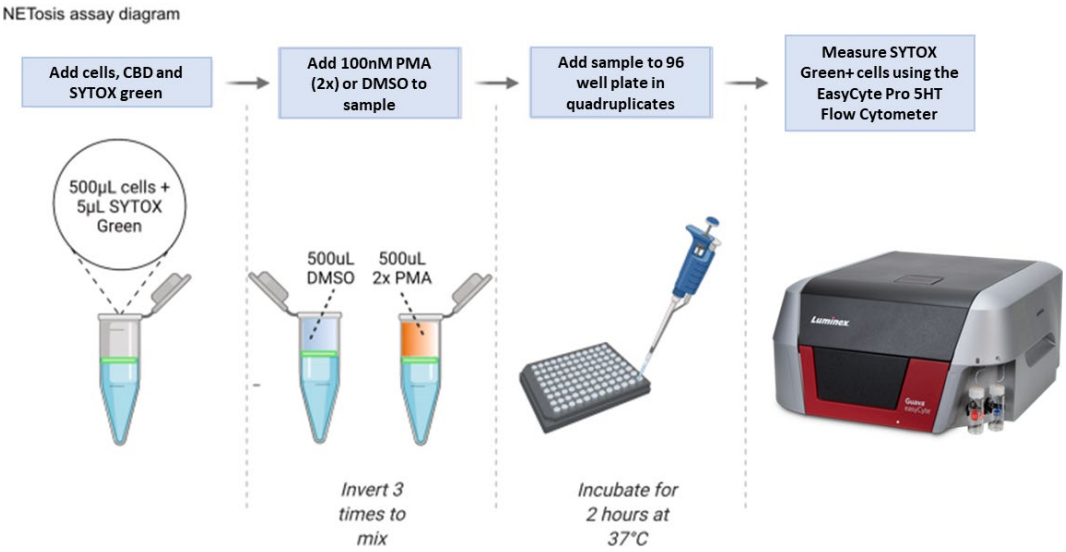

Figure 4. NETosis assay diagram illustrating the process by which neutrophils will be stained by SYTOX Green, pretreated with CBD and stimulated with NET agonists. SYTOX Green stains extracellular DNA, which is a marker of NETosis.

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Development of RT-LAMP Assay For detection of Arboviruses in the Context of homogenized Mosquito tissues

Debra Bramblett, PhD and Michael Woods, PhD

Hub Site: Las Cruces

Number of Students Accepted: 2

Mentor(s):
Debra Bramblett, PhD
Associate Professor
Biomedical Sciences

Michael Woods, PhD
Associate Professor
Physiology and Pathology

Project Title:
DEVELOPMENT OF RT-LAMP ASSAY FOR DETECTION OF ARBOVIRUSES IN THE CONTEXT OF HOMOGENIZED MOSQUITO TISSUES.

Project Abstract:
The long-term goal of this proposal is to establish an institutional capability to conduct arbovirus surveillance in New Mexico and elsewhere, and to position BCOM to play a central role in responding to emerging infectious diseases that may one day impact the southwestern US. The explosive emergence of Zika, Dengue and Chikungunya into Central America and the Caribbean in recent years has raised concerns that these diseases may one day establish themselves in the United States just as West Nile virus did following its initial introduction into the US in 1999. Furthermore, a warming climate has expanded the range of the primary mosquito vector for these viruses, Aedes aegypti, to include most of the southern United States, including New Mexico. The US is no less immune to emerging infectious diseases than any other place in the world, and therefore it is essential that we prepare for the inevitability that these "foreign" diseases will one day be our diseases.

To accomplish our goal, we propose to develop an isothermal nucleic acid amplification technique capable of detecting multiple emerging arboviruses in a rapid, "low-tech" format that can be performed in the field or at the bedside without the need for complex or expensive equipment. This assay will be based on a multiplexed reverse transcription-loop-mediated isothermal amplification (RT-LAMP) reaction targeting West Nile virus (WNV), Zika virus, Dengue virus (DENV) and Chikungunya virus (CHIKV). About four years ago we started by developing the protocoll for the West Nile Virus based on the work of Parida et al. Three years ago, we developed novel RT-LAMP primers specific for ZIKA virus. Last summer we successfully optimized the RT-LAMP assays specific to West Nile sequences and Zika virus sequences in the context of human body fluids (Urine, whole blood and plasma) using several different readout mechanisms including fluorescence, colorimetric (pH based or hydroxynapthol blue dye), and lateral flow assay.

During the 2022 summer research program, we plan to test the Zika and WNV assay in the context of homogenized Mosquito. For this purpose, we will need to develop a RT-LAMP assay for an endogenous target, most likely mosquito Actin and/or cytochrome oxidase c subunit I (COI) using online bioinformatics software for LAMP primer designed called Primer explorer. CO1 has been used to differentiate between mosquito species by several researchers (Chan et al 2014, Morlais 2014) and the invertebrate actin-1 sequence has been used as an internal control to test the quality of RNA from mosquitoes collected in vector surveillance studies by

Staley et al. Also, we plan to develop RT-LAMP primers for one or more of the other Arboviruses as we intend to develop a multiplex assay. We are in the process of developing one tube a multiplex LT-LAMP assay using a method called QUASR (Ball 2016) that utilizes a fluorescence read out and should there be time, we hope to optimize this assay as well. The specificity our assays and the presence of specific targets in homogenized mosquitoes will be confirmed by nucleic acid sequencing.

In the long term, we hope to develop a collaboration with NMSU and/or UTEP faculty to develop a microfluidics chip platform for these the RT-LAMP assays optimized to be used for regional surveillance and/or bedside assays. The hope is to develop a chip that can be used to screen for multiple viruses in one assay. We will need to optimize each LT-LAMP assay for detection of viral sub-types that are either present in our region (i.e the El Paso WNV strain) now or have the potential to spread to this region based on present of the natural vector. Once the LT-LAMP assays have been thoroughly validated we will then proceed to developed the microfluidics chip platform for regional surveillance and or bedside assay.

Specific Aims for the 2022 summer student project:

• Specific Aim (Student) #1: Optimize RT- LAMP reaction conditions for the Westnile and Zika virus specific primer sets in the context of homogenized mosquito, spiked with synthetic or genomic viral RNA.

• Specific Aim (Student) #2 Use bioinformatics tools to develop/design Reverse-Transcription Loop-Mediated Isothermal Amplification (RT-LAMP) primers specific to Mosquitos *CO1* and invertebrate *actin-1* to be used for internal controls

• Specific Aim (Student) #3 Screen wild caught frozen mosquitoes provided by a collaborator for the presence of viral RNA using the optimized LT-LAMP assays.

BCOM Medical students will be intimately involved in all aspects of the larger project. Due to the limited experience and time available to most medical students, we plan to establish student projects that will be meaningful and fruitful in a short period of time.

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The SHERLOCK assay, a specific high-sensitivity enzymatic unlocking assay, for virus detection

Debra Bramblett, PhD

Hub Site: Las Cruces

Number of Students Accepted: 2

As seen in the Zika (2015) and Coronavirus SARS-COV2 (2019) outbreaks, rapid diagnosis and response time are key to minimizing the devastating impact of a virus. Our long-term goal is to develop cheap and fast diagnostic techniques to prevent uncontrolled viral outbreaks. The *Flaviviridae* are a virus family with high morbidity and mortality, responsible for neuroinvasive diseases with potentially long-term cognitive and neurologic effects such as meningitis and encephalitis. Many Flaviviruses are mosquito-transmitted, and rising global temperatures promote the survival of Flavivirus' mosquito vectors across larger areas, resulting in the rapid spread of Flaviviruses.

The purpose of this research project is to create a SHERLOCK assay, specific high-sensitivity enzymatic unlocking assay, for virus detection for the detection of certain *Flaviviridae*. SHERLOCK, is a CRISPR-based isothermal reaction that offers quick and highly specific viral detection potentially without the need for expensive lab equipment. However, SHERLOCK is a novel molecular technique that few labs have tested and which is not commercially available as a kit. It is however being used for the detection of the SARS-CoV2 virus and has been validated clinically (Patchsung, 2020). The overall goal of our study is to optimize and standardize a protocol for the use of SHERLOCK in detecting synthetic Zika and West Nile RNA. No live virus will be used for the development of the SHERLOCK assay rather we will be using synthetic viral RNA purchased from ATCC and genomic viral RNA provided by collaborators.

Our interest in the SHERLOCK assay started about two years ago when a Burrell student learned of the technique though a literature review (Kellner et al). Development of the assay required the expression of Caspase 13a (CAS13a) as this protein is not commercially available. The Cas13a gene, derived from *L. wadeii* and cloned into the pET vector by Wang et al and made available to researchers globally. Large bacteria cultures and purification of the CAS13a protein was performed in the laboratory of Dr. Eric Yukl in the Biochemistry department at NMSU. During the 2021 summer research program, we were able to use the CAS13a protein isolated by Dr. Yukl to get SHERLOCK working for the detection of Zika virus, but it requires further optimization.

SHERLOCK reactions involve two stages: an isothermal Reverse Transcriptase Recombinase Polymerase Amplification (RT-RPA) and a Cas (CRISPR) reaction for detection and fluorescence reporting. The reporter is a short, quenched, ribo-oligonucleotide (Rnase Alert v2 by Invitrogen) that is cleaved by activated Cas13a, causing fluorescence.

Experimental design

The SHERLOCK assay protocol has 2 basic steps. Step1. Amplification of the viral RNA by recombinase polymerase amplification (RPA) reaction followed by transcription (c) occurs in a 1.5 m Eppendorf or PCR tube. The purpose of the amplification is to increase the sensitivity of the overall SHERLOCK reaction. We will be running the RPA assay in the context of homogenized mosquito tissue "spiked" with known viral RNA obtained from outside sources. No live virus will be used or processed in the Burrell Laboratory. Whole, frozen, mosquitos will

be homogenized in buffers from a MegaMax RNA isolation kit (Applied Biosystems) purchased from thermoFisher (AM1939), which will serve to inactivate any pathogens (viral or protozoan) potentially carried by the wild caught mosquitoes. We will also test the SHERLOCK assay in the context of certified clean blood and urine purchase from Fisher Scientific. Such body fluidics will be "spiked" with viral RNA isolated by outside resources. No live virus will be used or processed in the Burrell Laboratory. Step 2. Cas13a will be loaded with a virus specific crRNA template (b). The crRNA and a reporter oligonucleotide will be designed using bioinformatic tools freely available online. The target viral RNA will be detected by a fluorescent signal generated by the CRISPR collateral detection reaction. The CAS13a is "trained" to be specific for a particular virus using a virus specific guide RNA crRNA. The crRNA bousd by CAS13a hybridizes to the specific viral target RNA and upon hybridization the collateral RNAse activity of CAS13a become active. Activated CAS13a then cleaves a non-specific RNA reporter that has covalently attached fluorophore (Green) and a quencher molecules (Black). Cleavage of the commercially available reporter (RNAase Alert v2) separates quencher from fluorophore allowing for fluorescent detection using CFX96 real time thermocycler.

Specific Aims

1. Optimization of the existing fluorescent SHERLOCK assay for Zika virus detection in the context of mosquito homogenous.
2. Development of a new fluorescent SHERLOCK detection assay for West Nile virus by testing a series of target specific RPA primer and crRNA sets.
3. Development of an instrument free method for SHERLOCK detection of viruses by incorporating LFA technology.

Now that we have validated successful detection of synthetic Zika RNA using the primers and crRNA sequences as described by Kellner et al., we would now like to increase the fluorescence output and sensitivity and develop an instrument free detection system. Previously, we made efforts to optimize SHERLOCK for the Zika virus target by modifying the RT-RPA protocol provided by *TwistDx™* and the Cas13a reaction protocol published by Kellner et al . We have varied incubation temperature, incubation time, primer concentration, and magnesium acetate concentration in the RT-RPA reaction. In addition, we have added a crRNA-CAS13a incubation step and a mid-incubation vortex step to improve sensitivity. However, these modifications need to be repeated to verify their impact on sensitivity and enhancing fluorescence output. Further work must be done to develop a novel West Nile virus SHERLOCK protocol using modifications that we have incorporated into the original SHERLOCK and using West Nile specific RPA primers and crRNA.

Also, we have found that Lateral flow (LFA) detection to be a simple and easy readout method for RT-LAMP, a different nucleic acid amplification method that we are also using in the lab for viral detection. Other researchers have used LFA to generate an instrument free method for SHERLOCK detection of viruses (Myhrold et al 2018).

This summer, we plan to determine the best West Nile RT-RPA primers and crRNA sequences using the recommended primer and guide characteristics and using free software algorithm available online (xxx) to identify a series of potential RPA primer and crRNA guide sets specific for West Nile Virus. A series of 5 to 10 primer and guide sets will be ordered from ID and each set will used in a SHERLOCK assay to determine which primer/guide set is optimal for West Nile virus detection.

SHERLOCK is known for being sensitive to the single nucleotide level and could be used for a variety of applications other than viral detection such as identification of single nucleotide polymorphism associated with human disease states. In the future, we hope to demonstrate this level of sensitivity in using viral subtypes. We plan to investigate the specificity of SHERLOCK by gradually introducing single-nucleotide changes into the established crRNA, potentially allowing us to distinguish between different strains of WNV. We are also interested in developing a multiplex Caspase detection assay in the long term (Gootenberg et al, 2018).

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Effect of FIO2 on BP responses during and immediately after exercise

Pedro Del Corral, PhD

Hub Site: Las Cruces

Number of Students Accepted: 4

Note: Participation in this project will require meeting with Dr. Del Corral before January 14, 2022, because this project will already start on Saturday January 29th.

Mentor(s):
Pedro Del Corral, MD, PhD
Physiology and Pathology

Project Title:
Effects of FIO2 on Blood Pressure During and Immediately After Exercise

This project will require meeting with faculty before Jan 14th 2022 to determine a match, project will start Saturday January 29th.

Project Abstract:
Significance: During incremental exercise, cardiac output increases to meet the increase in oxygen delivery to contracting skeletal muscle. This is accompanied by gradual increases in systolic blood pressure (SBP) favoring contracting muscle perfusion pressure and ultimately skeletal muscle oxygenation. There are healthy individuals that show an abnormal increase in SBP during exercise, known as "exaggerated exercise blood pressure response" (EEBP). The EEBP response is a predictor of "masked" and future hypertension, thus represents an early warning of future cardiovascular disease. The etiology of the EEBP is not well understood, part of it is thought to be link to endothelial dysfunction. Hyperoxia has been associated to vasodilation or vasoconstriction depending on the anatomic location of the vasculature, and the FIO2 used, with most of the vasculature showing vasoconstriction at FIO2 of 1.0. Little is known about the global effects of moderate hyperoxia on the SBP during exercise. This is important to investigate because the wide spread use of commercial hyperoxic and hypoxic systems available to the general population.

Specific Aims: The aim of the present study is to characterize the effect of breathing low and moderately high FIO2 mixture on the SBP response during incremental and sabmaximal exercise. Specifically, the present study will examine how low (FIO2= 0.13) and moderately elevated (FIO2= 0.50) affect the SBP during exercise, and immediately after exercise. This intervention might shred some light on factors affecting EEBP.

Innovation: the findings of this study will describe any detrimental or ergogenic effects of opposing FIO2's on SBP during and after exercise with potential implications for clinical populations. For instance, pulse oximetry is a non-invasive way to monitor arterial hemoglobin oxygen saturation usually, at rest is approximately 97-99%, and during intense exercise often drops 95%. However, a subset of individuals shows a deeper drop reaching values below 90%, known as "exercise-induced hemoglobin oxygen desaturation" (EIHD), leading to reduced diffusion of oxygen from the capillaries to contracting skeletal muscle cells. The EIHD is also frequently encountered in patients with COPD and pulmonary hypertension. These patients show a significant improvement in HbO2 saturation when treated with hyperoxia (FIO2 0.4-1.0, depending on severity), and similar observations have been reported in healthy individuals (regardless of training status).

Research Strategy: Eight to nine subjects will be recruited via word of mouth, flyers posted in the community, and through e-mails (e.g., to Burrell or NMSU students or to standardized patients at Burrell College). Once a potential subject expresses interest in participating in the study, the consent form and other relevant information will be conveyed through e-mail and telephone (where

applicable). All participants will be free of any medical disease/condition that prevents them from participating in vigorous exercise that could have altered their cardiovascular responses to exercise. Inclusion criteria: ages 20-40yrs, BMI ≤ 30, with resting BP <140/90 mmHg. Exclusion criteria: resting BP ≥140/90, cardiovascular/pulmonary disease, or any medical disease/condition that prevents them from participating in vigorous exercise.

Study design: Each subject will report to the laboratory for four to six separate visits, all at the same times (+/- 1h). During Visit-1 and Visit-2, volunteers will undergo exercise tests to maximal and submaximal effort on a cycler ergometer, respectively, under normoxic air (FIO2 of 0.209, ambient air). Subjects will then be assigned (balanced) to complete Visit-3 & Visit-4 for the same exercise tests while breathing moderate hyperoxic air (FIO2 of 0.5) or moderate hypoxic air (FIO2=0.13). The subject assignment will be balance to achieved 8-9 subjects to complete Visit-3 and Visit-4 under hyperoxia, and 8-9 subjects under hypoxia. Subjects from either group may opt to complete two additional visits (ie., Visit-5 & Visit-6) if they elect to complete both hyperoxia and hypoxia studies, if so, sample size will be 8-9 subjects. In all exercise tests, we will measure blood pressure, heart rate, rate of perceived exertion, and blood lactic acid. The maximal exercise tests (Visit-1 and Visit-3, and optional Visit-5) will be design to last 12-15 min. During the post-exercise periods of Visit-2 & Visit-4 (and optional Visit-6) the subjects blood pressure and heart rate will be measured at 10 minute intervals for 1-hour.

Resting measurements: Subjects will be asked to avoid caffeine and vigorous exercise for 24 hours prior to measurements. Subjects will be seated in a silent room with their feet flat on the ground for at least 5 minutes before measurements begin. An automated heart rate monitor and an appropriately sized cuff, based on arm length and circumference will be used to ensure accurate BP measurements. To obtain the BP measurement the subject's arm will be supported and the cuff positioned at the level of the right atrium. Cardiovascular measurements will be reassessed 5 minutes later, with the two values averaged. If the BP values differ by +/- 10 mmHg, researchers will conduct a third measurement will be obtained. Following BP measurements, a baseline capillary blood sample (100µl) will be obtained from a finger prick and analyzed for lactate and endothelin-1.

Exercise Studies: All exercise studies will occur between 8am to 11am under overnight fasted conditions. Height, weight, %-body fat (Biolectrical impedance, Tanita scale), and seat adjust for the cycle ergometer will be determined. During Visit-1 & Visit-3, (and optional Visit-5) a cycle ergometer (Lode Corvival) incremental exercise test will be conducted in 2 to 3 minute stages. Starting resistance will be based on the subjects fitness and weight aimed at completing the test in 12-15 minutes. Heart rate will be monitored (Polar Heart rate monitor) throughout the test and recorded during the last fifteen seconds of each stage. Blood pressures will be obtained by a validated automated system (Tango Plus, SunTech Medical, Morrisville, NC) in the last 45 seconds of each stage. At the end of each exercise stage, subjects will be asked to rate their exercise level of perceived exertion (RPE) using an RPE 6-20 scale. While on the cycle ergometer, the subject will have a fitted mask connected to a tub to breath in air from a 1,100 liter gas reservoir containing ambient air (FIO2= 0.209), moderate hyperoxia (FIO2= 0.50) or hypoxic air (FIO2= 0.14) by using EWOT Energy Plus System (Portland, Oregon). During Visit-2 (FIO2: 0.2093) and Visit-4 (and optional Visit-6) (FIO2: 0.50/0.14) the subjects will

exercise for 30 minutes at 65% of their peak workload achieved in Visit-1 and Visit-3 (and optional Visit-5) (respectively) under similar conditions and measuring the same variables. The HR, BP, and RPE will be measured at 5, 10, 20, and 30 minutes after the onset of exercise. A 10 µl capillary blood sample will be obtained at 5 minutes and 30 minutes of exercise to measure blood lactate, and additional sample (100 µl) will be obtained at 30 minutes post-exercise. For Visit-1 and Visit-3 (and Optional Visit-5), there will be resting and 3 min post-exercise samples, immediately after collecting the blood sample, subjects will then be moved to a seated chair where final blood pressure & HR measurements will be taken at 10 min post-exercise. Thereafter, the subject will be given a snack ~50 g of carbohydrate and protein bar/drink. For Visits-2 & Visit-4 (and optional Visit-6), once subects finished the exercise study, the subjects will be transferred to a chair and remained seated, there will be serial blood pressure & heart rate measurements at 10 minutes intervals to 60 min post-exercise. Again, at the end of the experiment subjects will be given a snack ~50 g of carbohydrate and protein bar/drink.

Serum Analysis: Blood will be collected via a finger prick collection system (100µl tube, SARSTEDT). The first finger prick will be to the subject's non-dominate index finger after it has been cleaned with alcohol. This collection will be for 100µl of whole blood which will be processed to obtain serum samples for storage -20°C to determine endothelin-1 concentration at a later date. Informed Consent and Verification of Inclusion/Exclusion Criteria: Data collection will take place at the Human Physiology Laboratory at 9035 Advancement Avenue, Las Cruces, NM 88007. The Consent Form will be reviewed with the potential study participant before written informed consent is obtained. A health history questionnaire will be filled by the subject through to verify inclusion/exclusion criteria and to obtain contact data (name, address, phone, e-mail) and age, birth assigned gender. Subjects who do not provide blood lipid information (or glucose) in the last 12 months will undergo finger prick blood collection (40 microliters) for analysis in the laboratory during the screening process. For subjects who do not qualify based on the answers provided in the health history questionnaire the participation in the study ends at this point and no further data will be collected from such individuals.

Effects of exercise and transcutaneous auricular vagus nerve stimulation (taVNS) on parasympathetic tone

Pedro Del Corral, MD, PhD and Harald M. Stauss, MD, PhD

Hub Site: Las Cruces

Number of Students Accepted: 4

Effects of exercise and transcutaneous auricular vagus nerve stimulation (taVNS) on parasympathetic tone

Significance

The health benefits of physical activity were first documented in the landmark study by Paffenbarger and co-workers published in 1971 (1). This study demonstrated that San Francisco longshoremen with energy expenditures of over 8,500 kcal/week have significantly lower death rates from coronary heart disease compared to a control group with lower weekly energy expenditures. Even though the beneficial effects of physical activity and exercise are known for over 50 years, the Surgeon General Report on Physical Activity and Health published by the Centers for Disease Control and Prevention states that 25% of the US adult population is not active at all and only 25% of the adult population engages in the minimal recommended level of physical activity. One proposed mechanism by which physical activity or exercise improves health is through an increase in resting parasympathetic nervous system activity and activation of the cholinergic anti-inflammatory pathway (2). This proposed mechanism suggests that health benefits – like those induced by physical activity - may also be obtained by other interventions that increase resting parasympathetic nervous system activity. One such non-exercise-based interventions is non-invasive transcutaneous auricular vagus nerve stimulation (taVNS). *Our long-term goal* is to establish interventions other than exercise or physical activity that result in similar activation of resting parasympathetic tone and, therefore, health benefits just as regular exercise. *This long-term goal is significant* because 75% of the adult US population does not engage in the recommended intensity of physical activity. This huge population may benefit from non-exercise-based interventions that may potentially elicit the same health benefits as regular exercise. The *objective of this study* is to test the hypothesis that pupillometry can be used to efficiently and reliably assess parasympathetic tone in a large cohort-based future study, investigating the health benefits of non-exercise-based interventions that may potentially improve health through an increase in parasympathetic tone.

Specific Aims

Specific Aim 1: In this aim we will test the *hypothesis* that acute changes in parasympathetic tone in response to non-invasive transcutaneous auricular vagus nerve stimulation (taVNS) can be efficiently and reliably quantified by pupillometry. We will validate pupillometry against other established methods of assessing parasympathetic tone, including heart rate variability, Valsalva maneuver, and the cold face test (diving reflex). The *significance* of this Aim is that taVNS – just like physical activity - may increase parasympathetic tone and therefore may induce health benefits like that of endurance training.

Specific Aim 2: In this aim we will test the *hypothesis* that the decrease in parasympathetic tone during submaximal exercise and the subsequent increase in parasympathetic tone upon cessation of exercise can be quantified efficiently and reliably by pupillometry. Pupillometry will be validated against the heart rate and heart rate recovery responses before, during and after submaximal exercise. For additional validation of pupillometry we will also assess heart rate variability, the response to the Valsalva maneuver and to the cold face test before and after exercise. The *significance* of this Aim is that

it will establish the use of pupillometry as a reliable technique to assess changes in parasympathetic tone during exercise.

Innovation

The status quo in assessing parasympathetic tone in research studies is to apply heart rate variability analysis. This technique requires recording of an EKG under stationary conditions for a minimum of 5-10 minutes. During exercise, stationary conditions cannot always be achieved. In addition, the effects of exercise on respiration makes interpretation of high frequency heart rate variability (at the respiratory rate) very difficult if not impossible. This problem is important, because parasympathetic tone is assessed through the high frequency component of heart rate variability. This study is potentially innovative, because we will deviate from the status quo by replacing heart rate variability analysis with pupillometry to assess parasympathetic tone. Pupillometry can be done in less than one minute, does not require stationary conditions for a prolonged period of time, and is not confounded by the effects of exercise on respiration. Another advantage of pupillometry over heart rate variability analysis is that it is feasible in large scale population studies because the procedure is very quick (less than one minute) and does not require time-consuming computer analyses.

Research Strategy

Specific Aim 1

To test the hypothesis that acute changes in parasympathetic tone in response to non-invasive transcutaneous auricular vagus nerve stimulation (taVNS) can be efficiently and reliably quantified by pupillometry.

Experimental Protocol: Before, during and after 15 minutes of taVNS application, parasympathetic tone will be assessed by pupillometry, heart rate variability analysis (based on EKG recordings), phase 4 of the response to the Valsalva maneuver, and the cold face test. Correlation analysis will be used to validate pupillometry against the other measures of parasympathetic tone.

Specific Aim 2

To test the hypothesis that the decrease in parasympathetic tone during submaximal exercise and the subsequent increase in parasympathetic tone upon cessation of exercise can be quantified efficiently and reliably by pupillometry.

Experimental protocol: Subjects will be instrumented with EKG electrodes for continuous heart rate monitoring and subsequent heart rate variability analysis. Parasympathetic tone will be assessed under resting conditions (before the start of exercise) by pupillometry, the Valsalva maneuver, and the cold face test. Then subjects will start submaximal exercise on a cycle ergometer at a moderate exercise intensity for 30 minutes. At 10 minutes and 20 minutes into the exercise, pupillometry will be conducted. At 5 minutes and 25 minutes into the exercise, blood lactate will be determined. Upon termination of exercise, heart rate recovery will be determined for 5 minutes through EKG recording, immediately followed by pupillometry, the Valsalva maneuver, and the cold face test. These tests will then be repeated at 20, 40, and 60 minutes into the recovery from submaximal exercise.

Experimental Procedures

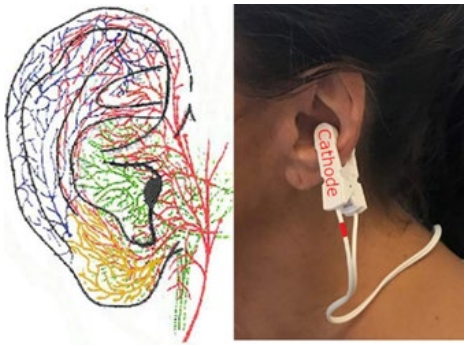

Fig. 1: taVNS

Transcutaneous auricular vagus nerve stimulation (taVNS): The auricular branch of the vagus nerve innervates the cymba conchae (3) as shown in Fig. 1, left. The clip electrode (Fig. 1, right) is connected to a transcutaneous electrical nerve stimulator (EMS 7500, Current Solutions, LLC, Austin, TX). This device is operated from a single 9V battery and not connected to any power outlets. The stimulation parameters will be 10Hz stimulation frequency and 300 µs pulse width. The stimulation current will be determined individually for each subject by slowly increasing the stimulation current until the subjects just barely feel a mild tingling sensation at the site of the electrode. This current will then be used for taVNS. In our previous studies (4, 5) this current was in the range of 20-30 mA. For this study, we will apply taVNS for 15 minutes.

Pupillometry: Pupillometry (6, 7) will be conducted using the PLR-3000 pupillometer (Neuroptics, Laguna Hills, CA). This device allows to elicit the pupillary light reflex while continuously recording the pupil diameter. The device automatically derives various parameters that all depend on parasympathetic tone, including the maximal (initial) and minimal (end) pupil diameter, the latency of constriction, the constriction velocity and the maximum constriction velocity. The test takes less than one minute.

Heart rate variability analysis: We will use time domain parameters of heart rate variability (SDNN and RMSSD) as well as frequency domain parameters of heart rate variability (low frequency (LF) and high frequency (HF) spectral power) to assess autonomic tone (8). RMSSD and HF spectral power are established measures of parasympathetic modulation of sinus node function.

Valsalva maneuver: Subjects will be instrumented with EKG electrodes for assessment of heart rate. For the Valsalva maneuver, subjects will perform a forced exhalation into a mouthpiece connected to a manometer for visual feedback. Subjects are instructed to generate a pressure of 40 mmHg for 15 s, while conducting the Valsalva maneuver (9). For assessment of parasympathetic tone, the heart rate response during phase 4 of the maneuver will be used.

Cold face test: This test is based on the diving reflex which elicits a co-activation of the sympathetic and parasympathetic nervous system (10). Subjects will be instrumented with EKG electrodes to measure heart rate. For the cold face test ice pallets wrapped in a towel will be placed on the face of the participants for 2 min. Subjects will be instructed in advance to continue normal breathing and abstain from moving or talking during the cold face test. Parasympathetic tone will be assessed by the maximal decrease in heart rate induced by the cold face test.

Exercise protocol: Study participants will perform a moderate intensity exercise bout on a cycle ergometer at approximately 75% of age predicted maximal heart rate which corresponds to a rate of perceived exertion of approximately 13 (Borg scale 6-20) for 30 minutes. During the exercise protocol, EKG recordings and blood pressure (Tango Plus, Suntech Medical, Morrisville, NC) measurements will be obtained. At the 10^{th} and 20^{th} minutes the participants will temporarily (<1 min) stop pedaling and pupillometry will be performed. Subjects will then continue to pedal until the 30th minute. Additionally, blood lactate will be measured via finger prick at the 5^{th} and 25^{th} minute of the exercise protocol.

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What's growing in there?

Jon Jackson, PhD

Hub Site: Las Cruces

Number of Students Accepted: 3

RESEARCH PROPOSAL — WHAT'S GROWING IN THERE?

Significance (Maximal length 1/2 page)

1. Explain the importance of the problem or critical barrier to progress in the field that the proposed project addresses.

Notwithstanding the sobering fact that throughout history, cadavers have posed dangers to those working with them[1,2,3], human donor bodies are used in the anatomy instruction of many if not most medical schools today. Despite the advent of fixation, preservation, and disinfection techniques that allow an individual donor cadaver to be used for a long period of time, the fact is that a cadaver in an anatomy also represents a rich carbon source for microbial species introduced by happenstance or through general usage of the cadaver. It is a rare anatomy lab that has not at some point had to deal with unwanted or uninvited growth in and on the cadaver specimens under study. But what IS that stuff? The identities of flora and fauna one finds on and in a "typical" anatomy lab cadaver has not been the object of serious study to date. This project seeks to address this lack of attention to a common concern of anatomists.

2. Explain how the proposed project will improve scientific knowledge, technical capability, and/or clinical practice in one or more broad fields.

The problem of the transmission of disease by unfixed (unembalmed) bodies was indirectly solved by Lord Lister[4], who realized that infectious microorganisms could be kept at bay through the use of dilute phenol, thus ushering in antiseptic surgery. Soon after the wide-spread adoption of antisepsis as a strategy in surgery, this was carried over into the dissection lab, including the cleaning of hands and instruments before and after contact with the human body. Nonetheless, at some point, microbes overcome the forces of antisepsis, and can colonize the cadaver undergoing dissection. Under normal circumstances, these microbes are believed to arise from DISSECTOR contamination of the cadaver, and not from preexisting populations of microbes. We aim to see for ourselves on the bodies we are simultaneously dissecting.

3. Describe how the concepts, methods, technologies, treatments, services, or preventative interventions that drive this field will be changed if the proposed aims are achieved.

It is possible that the post-embalming regimen for handling human donor cadavers could be altered based on what is described through this research. It is also possible that what we discover will do nothing more than reinforce the importance of following universal precautions in work with human donor bodies in dissection.

Specific Aims (Maximal length 1 page)

1. State concisely the goals of the proposed research and summarize the expected outcomes, including the impact the results of the proposed research will exert on the field involved.

The goal of this project is to create a "microbial census" of what lives on and in a fixed human cadaver undergoing dissection in a typical gross anatomy lab setting.

2. List succinctly the specific aims of the research proposed.

The specific aims are to:

1) Sample select external and internal surfaces of human donor cadavers prior to and during the dissection process, and grow out the microbes from these swab samples in culture dishes.

2) Selection of colonies from the initial swab explants and expansion into pure colonies that will allow for characterization.

3) Inoculation of a pure culture into a commercially available kit that allows identification of the culture through read-out of chemical reactions in the growth media of the kit.[5]
4) Reporting of the findings of these inquiries and comparing our findings to those that have used unembalmed donor bodies in controlled decay situations (*i.e.*, "body farms")[6].

Innovation (Maximal length 1/2 page)

1. Explain how the application challenges and seeks to shift current research or clinical practice paradigms.

There is nothing particularly innovative about this proposed work, except in that it eschews reliance on the received wisdom that nothing survives the rigorous embalming process. Rather, this project proposes to determine firsthand whether or not the received wisdom is true in the case of the human donor bodies we utilize in the gross anatomy lab.

2. Describe any novel theoretical concepts, approaches or methodologies, instrumentation or interventions to be developed or used, and any advantage over existing methodologies, instrumentation, or interventions.

The novel concept behind this work is that a large amount of information could be gleaned through the straightforward use of established protocols[5] for microbial surveillance and identification in occupational and environmental settings, such as clinics, food service areas and hospitals. The techniques are not technically advanced or difficult beyond the level of what our team can collectively get our brains around and troubleshoot.

3. Explain any refinements, improvements, or new applications of theoretical concepts, approaches or methodologies, instrumentation, or interventions.

The noteworthy refinement to the existing protocols for identifying unknown microbes we propose to use is in the source material. And, just as it was once exciting to report what grew out of a swab explant sample from the surface of a student cell phone, the various locales we propose to sample will provide the students on this project with the opportunity to be among the first researchers to ever describe these kinds of findings from this setting. The only previous work of this nature on this problem of which microbial species were growing on a cadaver, grew out of infections that happened during transit between the donation program and the school where the dissection was to occur[7].

Research Strategy (Maximal length 4 pages)

1. Describe the overall strategy, methodology, and analyses to be used to accomplish the specific aims of the project. Include how the data will be collected, analyzed, and interpreted, and any resource sharing plans as appropriate.

We intend to follow a typical protocol for the identification of unknown microbial samples used in a typical undergraduate microbiology[5]. By photographing both the explant cultures and the source sites from the donor cadavers, our data should allow the generation of a site map wherein unique microbes might be found, as well as characterizing the flora common to multiple sites of the body's surfaces.

2. Discuss potential problems, alternative strategies, and benchmarks for success anticipated to achieve the aims.

Since this work has not been done before, it is anticipated that there may be trial and error with different growth and/or selective media that will aid in the identification of the microbes that will be encountered. Since the surveillance strategies for identifying microbial unknowns are reasonably standardized, by utilizing this fairly straightforward approach we will have lots of prior work from other settings to help inform our efforts.

3. If the project is in the early stages of development, describe any strategy to establish feasibility, and address the management of any high-risk aspects of the proposed work.

N/A. See below.

4. Point out any procedures, situations, or materials that may be hazardous to personnel and precautions to be exercised.

By definition, the project intends is to generate and expand cultures of unknown microbes from donor human cadavers. The word ***unknown*** is both a benefit and a danger in the ongoing work of the project — again, while there are ample exclusion criteria for donating a body at the time of death[8], it is never possible to rule out the possibility of culturing microbes from the external and internal surfaces of cadavers in the anatomy lab that could pose a danger to human health if handled with less than appropriate care. The project has undergone review by the Institutional Biosafety Committee, and requires clerical updating before it can receive final approval.

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RESEARCH PLAN

Significance (Maximal length 1/2 page)

1. Explain the importance of the problem or critical barrier to progress in the field that the proposed project addresses.
2. Explain how the proposed project will improve scientific knowledge, technical capability, and/or clinical practice in one or more broad fields.
3. Describe how the concepts, methods, technologies, treatments, services, or preventative interventions that drive this field will be changed if the proposed aims are achieved.

Specific Aims (Maximal length 1 page)

1. State concisely the goals of the proposed research and summarize the expected outcomes, including the impact the results of the proposed research will exert on the field involved.
2. List succinctly the specific aims of the research proposed (e.g., to test a stated hypothesis, create a novel design, solve a specific problem, challenge an existing paradigm or clinical practice, address a critical barrier to progress in the field, or develop new technology).

Innovation (Maximal length 1/2 page)

1. Explain how the application challenges and seeks to shift current research or clinical practice paradigms.
2. Describe any novel theoretical concepts, approaches or methodologies, instrumentation or interventions to be developed or used, and any advantage over existing methodologies, instrumentation, or interventions.
3. Explain any refinements, improvements, or new applications of theoretical concepts, approaches or methodologies, instrumentation, or interventions.

Research Strategy (Maximal length 4 pages)

1. Describe the overall strategy, methodology, and analyses to be used to accomplish the specific aims of the project. Include how the data will be collected, analyzed, and interpreted, and any resource sharing plans as appropriate.
2. Discuss potential problems, alternative strategies, and benchmarks for success anticipated to achieve the aims. You also may wish to include a discussion of future directions for your research, as well as a project timeline.
3. If the project is in the early stages of development, describe any strategy to establish feasibility, and address the management of any high-risk aspects of the proposed work.
4. Point out any procedures, situations, or materials that may be hazardous to personnel and precautions to be exercised.

References Cited

Attitudes about cadaver anatomy

Jon Jackson, PhD

Hub Site: Las Cruces

Number of Students Accepted: 2

RESEARCH PROPOSAL — ATTITUDES ABOUT GROSS ANATOMY

Significance (Maximal length 1/2 page)

1. Explain the importance of the problem or critical barrier to progress in the field that the proposed project addresses.

Human donor bodies are used in the anatomy instruction of many if not most medical schools today, especially and including the Burrell COM. At the time the decision was made to pursue the use of cadaveric material for anatomy instruction, two whole classes of students had matriculated and begun their studies at the school. Thus, the addition of cadaver anatomy "after the fact" provided a unique situation to examine the feelings of students, faculty and staff as to what kind of educational value and results might result from the addition of an Anatomical Gifts Program.

2. Explain how the proposed project will improve scientific knowledge, technical capability, and/or clinical practice in one or more broad fields.

This study, once completed and published, will provide valuable insight into how students, staff, and faculty reacted to the addition of cadavers to the curriculum at a medical school that had delivered basic science anatomical education without cadavers to two cohorts of first year medical students.

3. Describe how the concepts, methods, technologies, treatments, services, or preventative interventions that drive this field will be changed if the proposed aims are achieved.

Not likely to transform anything. However, the findings will be of interest owing to the unique nature of how the initial BCOM curriculum was organized.

Specific Aims (Maximal length 1 page)

1. State concisely the goals of the proposed research and summarize the expected outcomes, including the impact the results of the proposed research will exert on the field involved.

The goal of this project is to complete the analysis of survey response data, especially the open-ended responses, and characterize these responses so as to finalize this largely descriptive study.

2. List succinctly the specific aims of the research proposed.

The specific aims are to:

1) Using qualitative analysis software AND thematic analysis, collate the themes that emerge from the open-ended responses to the 2019 survey.
2) Using the themes in #1 above, place the results into the broader context of anatomy education in the 21st century.
3) Prepare a manuscript for publication in one of the several anatomy journals.
4) Prepare a poster for presentation of these findings at an appropriate regional or national meeting;

Innovation (Maximal length 1/2 page)

1. Explain how the application challenges and seeks to shift current research or clinical practice paradigms.

Polling medical faculty and students about the use of cadavers isn't particularly innovative. What ***is*** unique about this project is the situation where two consecutive classes of students had been admitted and undergone anatomy instruction ***without*** any cadaveric anatomy or donor bodies whatsoever. How these students report their "missing" of this chance (or not) is a story no one has told before, simply because no medical school started their anatomy instruction this way and switched so suddenly.

2. Describe any novel theoretical concepts, approaches or methodologies, instrumentation or interventions to be developed or used, and any advantage over existing methodologies, instrumentation, or interventions.

N/A.

3. Explain any refinements, improvements, or new applications of theoretical concepts, approaches or methodologies, instrumentation, or interventions.

N/A

Research Strategy (Maximal length 4 pages)

1. Describe the overall strategy, methodology, and analyses to be used to accomplish the specific aims of the project. Include how the data will be collected, analyzed, and interpreted, and any resource sharing plans as appropriate.

This is very straightforward. The student(s) working with this data will have to immerse themselves in the numbers and responses that comprise this anonymized data set, and look for themes in the open-ended responses and any correlation (however tenuous) between the various categories of responses (*i.e.*, identifiable groups, or linkages between responses to different attitudinal questions).

2. Discuss potential problems, alternative strategies, and benchmarks for success anticipated to achieve the aims.

When the work was begun, the school had some of the necessary software to do the "semi-automated" analysis. This may or may not still be the case, so students may have to try out some other software options to augment their own analysis of themes in the data.

3. If the project is in the early stages of development, describe any strategy to establish feasibility, and address the management of any high-risk aspects of the proposed work.

N/A.

4. Point out any procedures, situations, or materials that may be hazardous to personnel and precautions to be exercised.

N/A

References Cited

None at this time — part of the work will be constructing an appropriate listing of meaningful resources that place this study in the larger context of anatomy education in the mid-21st century.

Fascial attachments of Gluteus minimus: Implications for hip pain of endopelvic origin

Nancy Minugh-Purvis, PhD

Hub Site: Las Cruces

Number of Students Accepted: 2

This project is offered through the Distinction in Anatomy program.

It is no part of the Summer Research Experience.

Fascial attachments of Gluteus minimus: Implications for hip pain of endopelvic origin

Note: this project is offered through the Distinction in Anatomy program, not the Summer Research Experience.

Significance

Gluteus minimus, an abductor of the thigh at the hip orginating from the external ilium is universally described in anatomy texts as inserting into the greater trochanter of the femur (Williams, et al., 1995 and many others). As is true of all muscles, variations present themselves and g. minimus occasionally inserts into the hip joint capsule (Flack, et al., 2012) or sends fascicles continuous with piriformis, the gemelli, obturator internus and vastus lateralis (Flack, et al., 2012; Williams, et al., 1995).

In an examination of embalmed cadaveric material in the gross anatomy laboratory at Burrell College of Osteopathic Medicine during the summer of 2021, the PI observed >50% of g. minimus specimens passed through the greater sciatic foramen just superior to the ischial spine. Palpation confirmed tissue continuity into the pelvis. Consistent with our observations, Beck, et al. (2000) mention the most posterior origin of g. minimus as being inside the pelvis at the sciatic notch but do not further elaborate on the extent or specific location of this attachment site.

The greater sciatic notch opens into the pelvic cavity at the junction of the piriformis, coccygeus, levator ani, obturator internus and numerous pelvic fascial sheets. It is also in close proximity to the lowest extent of peritoneal membranes. Attachment to any or all of these structures by g. minimus has, to our knowledge, not been previously described. This is the initial phase of a study to examine whether endopelvic attachments of g. minimus could, in part, explain the chronic hip pain reported by some patients with pathologies of pelvic viscera (Rana, et al., 2001).

Specific Aims

This study will test the following hypothesis: Attachments of g. minimus include endopelvic fascias. In addition, this research will attempt to more precisely document the precise posterior attachments – bony as well as soft tissue – of g. minimus than what is currently provided in the foundational literature on human gross anatomy.

Innovation

Two aspects of this project present opportunities for innovative work, and depending upon initial outcomes there is also potential for additional innovative clinical applications. First, the endopelvic attachments of g. minimus have not been previously described, and as such this investigation would provide novel information regarding human pelvic anatomy.

A second innovation surrounds the development of techniques for the identification of pelvic fascias and their attachments. This is a difficult undertaking in embalmed cadavers. We hypothesize it can be facilitated by the utilization of injectable dyes into fascial planes. This will be tested on the same cadaveric specimens, utilizing regions other than the pelvis until sufficiently perfected for application to the pelvic region. The objectives will be to obtain a

visualization of the extent of fascial planes and to elucidate collagen fiber direction within the fascial sheets enveloping g. minimus in order to more clearly demonstrate continuity with other tissues. Some tissues might perhaps be harvested and subjected to bright field microscopy for documentation of fascial continuity. This will be done collaboratively between the PI, co-PI, and participating students.

Finally, the results of this study could have important implications for developing treatments for chronic pelvic pain through the application of osteopathic manipulative treatment to g. minimus. In patients with extensive scarring and adhesions due to pathology of pelvic viscera or surgical sequelae, targeted osteopathic manipulation of g. minimus could, via its fascial attachments, provide an easily accessible means for indirect treatment of endopelvic structures. Of additional note is the possibility that g. minimus manipulation could provide an alternative or supplemental means for treatment of the pelvic floor pain and dysfunction if the fascia of g. minimus proves, as expected, to have continuity with those comprising the pelvic floor such as obturator internus, coccygeus, and levator ani.

Research Strategy

Utilizing an estimated 5-8 cadavers (exact number to be determined, based upon specimen condition), available during the summer Distinction in Anatomy Program at Burrell College of Osteopathic Medicine, unilateral dissections of g. minimus will be conducted. During this process and upon completion, detailed notes will be made regarding the location and extent of all muscle attachments. Detailed photography will also record these findings.

As the muscle is initially revealed, care will be taken to preserve the enveloping deep fascia, and dye introduced into the fascial plane between it and the muscle fibers in order to visualize continuity between the g. minimus fascia and that of endopelvic fascias and other structural elements. This will also be described, and recorded photographically.

Possible problems which might be encountered with this project include the discovery of unusable specimens upon dissection. Substantial pelvic pathology could obstruct dissection of delicate tissues and thus reduce the number of specimens upon which our study is based.

References

Beck, M, JB Sledge, E Gautier, CF Dora, R Ganz 2000 The anatomy and function of the gluteus minimus muscle. J Bone and Joint Surg, 82-B (3).

Flack, NAMS, HD Nicholson, SJ Woodley. 2012. A review of the anatomy of the hip abductor muscles, Gluteus Medius, Gluteus Minimus, and Tensor Fascia Lata. Clinical Anatomy, 25:697-708.

Rana, S., RC Stanhope, T Gaffey, BF Morrey, DA Dumesic 2001 Retroperitoneal endometriosis causing unilateral hip pain. Obstet Gynecol, 98(5 Pt 2): 970-972.

Willliams, PL, et al., 1995. Gray's Anatomy (38th British Edition). NY: Churchill Livingston.