THESIS

EVALUATION OF ACUPUNCTURE AS AN ALTERNATIVE THERAPY IN A RODENT MODEL OF SPONTANEOUS OSTEOARTHRITIS

Submitted by

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ABSTRACT

EVALUATION OF ACUPUNCTURE AS AN ALTERNATIVE THERAPY IN A RODENT MODEL OF SPONTANEOUS OSTEOARTHRITIS

Osteoarthritis (OA) is a degenerative joint disease affecting nearly 250 million people globally. With clinical signs of severe and persistent joint pain, OA is a leading cause of physical disability throughout the world. When faced with the frustration of chronic discomfort and restricted mobility due to OA, many individuals have turned to acupuncture as an alternative therapy. Acupuncture is a traditional Chinese practice of medicine for pain alleviation that involves insertion of thin needles into the skin and underlying tissue. The needles may be manipulated via manual or electrical stimulation, referred to as manual acupuncture and electroacupuncture, respectively. However, the efficacy of acupuncture in managing OA pain is uncertain, as much of the evidence is of questionable quality. The overall goal of this project was to evaluate acupuncture in a rodent model of human OA such that unbiased conclusions regarding its effectiveness for symptom modification could be drawn. Unfortunately, the majority of laboratory models of OA are artificially induced via chemicals or surgery and may not adequately represent the spontaneous disease process that occurs in humans. In contrast, the Dunkin Hartley guinea pig is a natural disease model, with primary OA pathology that mirrors human disease.

As the major symptoms of OA are painful and decreased mobility, we were interested in evaluating the effect of acupuncture on a variety of mobility parameters using treadmill-based gait analysis and open-field enclosure monitoring. Additionally, as OA is an inflammatory

disorder, we were interested in evaluating the effect of acupuncture on systemic inflammation, as well as any potential effects on normal physiology. This is commonly done in veterinary species with minimally invasive blood tests, such as complete blood counts and serum biochemistries. Despite their widespread use in biomedical research, there are few published studies investigating normal reference ranges for these diagnostics in the Dunkin Hartley guinea pig. Therefore, the goal of the first study was to develop hematology and serum biochemistry reference intervals for this strain. Data from complete blood counts and serum biochemistries were compiled from control Dunkin Hartley guinea pigs from previous studies to establish reference ranges for this model. Data were stratified by sex to determine specific reference intervals for males and females, and significant differences in parameters were investigated based on age and sex. The results of this study provide a foundation for interpreting these common diagnostic and laboratory blood tests in the Dunkin Hartley strain.

The second and third studies evaluated electroacupuncture and manual acupuncture for the treatment of OA utilizing the Dunkin Hartley guinea pig model. Results of these studies demonstrated that electroacupuncture improved mobility, but not structural changes, in the knee joint. Conversely, manual acupuncture did not improve mobility parameters, but trended toward a decrease in knee joint histology scores compared to control groups. The results of these studies provide evidence that short-term treatment with electroacupuncture, but not manual acupuncture, is effective for symptom modification in this animal model of OA. Long-term studies are still needed to determine mechanisms for disease modification with these modalities.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I.

LITERATURE REVIEW

1. Osteoarthritis

Osteoarthritis (OA) is a devastating and debilitating joint disease affecting 242 million people globally.[1] OA can affect any joint in the body, but most commonly affects the knees, hips, and hands.[2] Pain, the hallmark symptom of OA, contributes to functional limitations and ultimately decreased quality of life.[3] According to the Centers for Disease Control and Prevention (CDC), approximately 43.5% of the 54.4 million US adults diagnosed with OA reported limitations in their daily activities.[4] Out of 291 conditions, the Global Burden of Disease 2010 study ranked OA as the 11th highest contributor to global disability and 38th highest in disability-adjusted life years.[5] Additionally, OA was the second most costly health condition treated at American hospitals in 2013 and accounted for $16.5 billion of health care expenses.[6] Adults with arthritis also averaged $4,040 less pay than adults without the disease.[7] With the aging population and increased rates of obesity, the OA burden is rapidly rising. Diagnoses in the United States are projected to increase to 78.4 million by 2040,[8] creating a major challenge for health care systems.

1.1 Risk factors

While a number of risk factors are involved in the development of OA, age is one of the most significant. This is likely due to exposure of various risk factors and age-related biological changes within the joint structures over time, such as cartilage thinning, loss of muscle strength, and oxidative damage.[9] According to the CDC, approximately 7.1% of young adults aged 18-44

years and 29.3% of middle-aged adults aged 45-64 years were reported to have doctor-diagnosed arthritis. The majority of adults diagnosed with arthritis were 65 years of age or older, with almost half (49.6%) reporting to have arthritis.[4]

After 50 years of age, women are more likely than men to develop OA.[10] It is estimated that 9.6% of men and 18.0% of women over 60 years of age have symptomatic OA.[11] In a meta-analysis of sex differences in OA prevalence, incidence, and severity, females were found to have an overall higher risk of OA and tended to have more severe knee OA, particularly after menopausal age.[12] The higher incidence of OA in women after menopause suggests estrogen deficiency may be involved in development of disease.[10] Cohort studies reported estrogen replacement therapy decreased risk of progressive knee OA and may have a protective effect for post-menopausal women with OA.[13,14]

OA prevalence also varies among races and ethnicities. In the Beijing Osteoarthritis Study, hip OA in Chinese individuals was 80-90% less frequent than in Americans from the Framingham (Massachusetts) Osteoarthritis Study.[15] However, Chinese women had a higher prevalence of both radiographic and symptomatic knee OA compared to American women of similar ages. Prevalence of knee OA in Chinese men was comparable to that of American men.[16] The Johnston County Osteoarthritis Project determined that African Americans had a slightly higher prevalence of both radiographic and symptomatic knee OA, and significantly higher prevalence of severe radiographic knee OA compared to Caucasians.[17] Interestingly, both African American and Caucasian women had similar prevalence of hip OA (23% and 22%, respectively), but African American men had a slightly higher prevalence of hip OA (21%) compared to Caucasian men (17%).[18] Additionally, in a longitudinal study, African American

males were found to have a higher risk of medial knee joint space loss over time compared to African American females and Caucasians.[19]

Joint injury, particularly of the knee, is one of the strongest risk factors for development of OA.[9,20] In a population-based study by Kellgren and Lawrence, 35% of males and 15% of females were found to have OA at the site of an injury, with the knees being the most common site in both sexes.[21] Similarly, the Framingham study found the rate of knee OA to be higher in those with a history of knee injury compared to those without. Additionally, follow-up studies of patients with meniscal tears requiring menisectomy and anterior cruciate ligament tears have shown a high prevalence of radiographic knee OA, pain, and functional limitations.[22–24]

Developmental abnormalities also contribute to the development of OA. Congenital subluxation,[25] Legg-Calvé- Perthes disease,[26] and slipped capital femoral epiphysis[27] have been associated with the development of hip OA. Additionally, hip-knee-ankle malalignment changes the load distribution at the knee joint, contributing to OA progression. A longitudinal cohort study found that compared to baseline, varus alignment was associated with a 4-fold increase in the odds of medial OA progression, whereas valgus alignment was associated with a 5-fold increase in the odds of lateral OA progression.[28]

For many years it was unknown if obesity was a risk factor for OA or was the result of decreased mobility from OA. The Framingham Study determined a strong association of pre-existing obesity with the later development of radiographic OA, particularly for women.[29] Additionally, women who lost approximately 5 kg of weight decreased their risk of developing knee OA by 50%.[30] Increased risk of OA in overweight persons is likely due to overloading of the weight-bearing joints, leading to cartilage breakdown.[10] Obesity has also been associated with hand OA,[31] suggesting a possible metabolic or inflammatory role.

Epidemiological studies have provided evidence that genetics is a strong determinant of OA.[32] Familial clustering of Heberden's nodes, enlargements of the terminal interphalangeal joints, was first studied in the early 1940s.[33] Familial clustering of hand and knee OA was later confirmed in epidemiological surveys in the United Kingdom.[34] Other familial studies have shown that offspring of individuals with a total knee replacement for OA had increased risk of worsening knee pain,[35] as well as greater worsening of radiographic OA (joint space narrowing and osteophytes) over time.[36] Additionally, twin studies have shown a heritability of 39 – 65% in radiographic hand and knee,[37] approximately 60% in hip,[38] and approximately 70% in spinal OA[39]. Genome-wide association studies have identified approximately 30 loci associated with OA;[40] however, little is known about their function in disease pathogenesis.[41]

1.2 Pathogenesis of osteoarthritis and joint pain

OA is generally classified as either primary or secondary. Primary OA is also referred to as spontaneous or naturally-occurring OA, and the underlying cause of disease is unknown. On the other hand, secondary OA occurs due to a preexisting joint abnormality, such as a trauma, congenital joint disorder, or other arthropathy.[42] In primary OA, the mechanisms leading to disease onset and joint pain are still poorly understood. OA was long-believed to be a non-inflammatory "wear and tear" of the articular cartilage due to abnormal mechanical loading or injury. It is now well-recognized that inflammation is involved in the pathogenesis of OA and contributes to its symptoms of joint pain, stiffness, and swelling.[43] Additionally, disease involves the entire joint organ, including the articular cartilage, subchondral bone, bone marrow, synovium, joint capsule, menisci, ligaments, and periarticular muscles.[44]

The pathological hallmark of disease is damage and remodeling of the articular cartilage.

Articular cartilage is composed of an extracellular matrix rich in collagen and proteoglycans. Normally, chondrocytes produce low levels of degradative and synthetic enzymes for matrix remodeling. In OA, chondrocytes fail to maintain homeostasis between synthesis and degradation and release more degradative enzymes. Breakdown products from collagen and proteoglycan cause the release of proinflammatory cytokines such as tumor necrosis factor-α (TNFα), interleukins, chemokines, nerve growth factor, prostaglandins, and matrix metalloproteinases (MMPs) from chondrocytes, osteoblasts, and synoviocytes. MMPs degrade articular cartilage, leading to fissuring of the cartilage surface, decreased cartilage thickness, and joint space narrowing. Over time, the cartilage becomes completely destroyed, exposing the underlying subchondral bone.[45] Inflammation also stimulates endothelial cells and fibroblasts to produce vascular endothelial growth factor (VEGF), leading to increased angiogenesis in the synovium and osteochondral junction.[43] In response, endochondral ossification occurs at the osteochondral junction, forming osteophytes and subchondral bone cysts.[46]

Mechanisms of OA-related joint pain likely involve joint nociception and the peripheral and central nervous systems.[47] The knee joint is highly innervated by sensory nerve fibers, including small myelinated Aδ (group III) fibers and unmyelinated C (group IV) fibers. These fibers are activated by noxious mechanical, thermal, or chemical stimuli, including inflammatory mediators.[48] Afferent fibers transmit pain inputs from the joint to the dorsal horn of the spinal cord to the brain. Descending pathways from the brain to the spinal cord modulate pain signals to increase or decrease pain perception.[46]

Although articular cartilage destruction is the hallmark of OA, cartilage is avascular and aneural and, therefore, not a direct cause of joint pain. In contrast, the subchondral bone, synovium, ligaments, and joint capsule all contain nerve endings that could be a source of

nociceptive pain in OA.[49] Several pro-inflammatory mediators, including nerve growth factor and prostaglandins, cause localized damage to tissues and activate peripheral nociceptors.[50] Structural changes, such as bone marrow lesions, subchondral bone remodeling, and osteophyte formation, have also been shown to be associated with pain. Additionally, neovascularization of the articular cartilage and menisci stimulates the development of sensory nerves in these tissues, further activating joint nociceptors.[47]

Normal joint tissues are insensitive to pain generation, as a low pain threshold would result in any movement being painful.[49] In chronic inflammatory states, the threshold for local nerve excitation and transmission may be lowered. This leads to increased responsiveness of the peripheral nociceptors, termed peripheral sensitization. Additionally, central nervous system pathways may be affected by persistent stimulation of dorsal root ganglia from inflammatory cytokines, leading to central sensitization. Sensitization may lead to hyperalgesia (increased pain sensitivity to a noxious stimulus) or allodynia (pain in response to a normally non-noxious stimulus). Sensitization may also explain why OA pain becomes more severe over time and why some individuals become resistant to analgesic treatment.[48]

1.3 Management

As the complex pathogenesis of OA is still not well understood, there are currently no treatments that can restore the normal cartilage structure. Treatment is typically focused on preserving quality of life by increasing the patient's functional capabilities and reducing pain with a combination of nonpharmacologic and pharmacologic modalities. When conservative treatment fails, joint replacement may be indicated.[51] The American Academy of Orthopaedic Surgeons (AAOS),[52] Osteoarthritis Research Society International (OARSI),[53] American College of Rheumatology (ACR),[54] European League Against Rheumatism (EULAR),[55] and National

Institute for Health and Care Excellence (NICE),[56] among others, have published guidelines for the management of knee OA. There is broad agreement on recommendations by these various organizations, with a few exceptions.[57]

For non-pharmacologic management, a combination of education, exercise, and weight loss is widely recommended as the first-line treatment.[58] Self-management programs are used to educate patients about the disease, treatments, and lifestyle changes to promote well-being and better manage symptoms.[2] These programs have been shown to be beneficial, although the effect size is small.[54] There is consistent evidence that low-impact aerobic exercise, such as walking, cycling, and aquatic exercise, and strength training of the lower limb reduces pain and improves physical function in the knee.[59,60] However, no recommendations have been established regarding specific exercise regiments, including frequency, duration, intensity, and specific muscle groups to be strengthened.[60] Weight loss is strongly recommended for overweight and obese individuals with knee OA, as it has been shown to decrease pain and improve function.[61] Research has also shown weight loss to have a positive effect on changes in OA biomarkers[62] and medial articular cartilage structure, including increased proteoglycan content and reduced cartilage thickness losses.[63] Other nonpharmacologic management recommendations may include the use of supportive walking devices, but there is disagreement in recommendations for knee braces and heel wedges. [57]

Paracetamol/acetaminophen has historically been recommended for first-line pharmacologic management of OA due to its perceived safety compared to non-steroidal anti-inflammatory drugs (NSAIDs) and opioids.[64] However, paracetamol has been found to be largely ineffective for pain control,[65] and NSAIDs are often substituted. Unfortunately, NSAIDs can be associated with serious gastrointestinal side effects, which may outweigh their benefits. Due to

safety concerns, NSAIDs should be taken at the lowest effective dose for the shortest duration with a gastroprotective agent, such as a proton-pump inhibitor.[2] Selective cyclooxygenase-2 (COX-2) inhibitors were developed to reduce gastrointestinal risk, but these have been associated with an increased risk for cardiovascular events. Alternatively, topical NSAIDs may be used as they have been found to be effective in reducing OA pain and may be safer than oral NSAIDs.[66] There is controversy regarding the use of glucosamine and/or chondroitin,[57] mostly due to concerns regarding publication bias and heterogeneity of results in randomized clinical trials.[67] Intra-articular corticosteroid injections are generally recommended and commonly used to treat knee OA.[68] Still, there is debate over their long-term therapeutic effects and safety of repeated injections.[69,70]

Opioids are also often considered for the treatment of severe knee OA pain. Most guidelines suggest limiting their use until after inadequate response to other nonpharmacologic and pharmacologic treatments or as a last resort therapy for those awaiting surgery.[71] While opioids have been found to significantly decrease pain, their benefits are limited by frequent side effects, such as nausea, vomiting, constipation, dizziness, and somnolence.[67] Additionally, concerns regarding addiction to opioids limits the prescription of these medications.[2]

Alternative and complementary treatments include lasers, ultrasound, transcutaneous electrical nerve stimulation, electrotherapy, Tai Chi, and acupuncture. Recommendations for these therapies remain controversial among organizations. Although acupuncture is one of the most heavily researched of all the complementary therapies for OA, insufficient evidence exists to provide a general recommendation for its use.[57] This is likely due to issues in study design, which is discussed in more detail below.

2. Acupuncture

Acupuncture is a component of Traditional Chinese Medicine (TCM) that involves the insertion of needles into the skin to achieve a therapeutic effect. It is most commonly used for pain alleviation, though it has been used for a multitude of other conditions such as migraines, nausea, vomiting, infertility, obesity, and insomnia, among others.[72] The concept of "Qi" is central to the TCM theory of acupuncture. Qi is the "life force" that flows throughout the body via channels termed "meridians". Excesses or deficiencies of Qi are said to cause disease. Needles are inserted into acupuncture points along the meridians and stimulated to elicit a dull, aching sensation referred to as *de qi*. This is believed to restore the flow of energy throughout the body. Generally, the needles are manipulated by hand, which may be referred to as manual acupuncture. In contrast, electroacupuncture involves running small amounts of electrical current through the needles with enough intensity to make the muscles twitch.[73]

2.1 History

While acupuncture is commonly believed to have originated in China, there is evidence that it may have originated over 5000 years ago in Europe. Otzi the "Tyrolian Ice Man" was a hunter whose body was preserved in an Alpine glacier around 3300 BC and emerged in 1991. Otzi's body carried 15 groups of tattoos arranged primarily on the back and legs. Based on their simple linear shape and location on less visible parts of the body, the tattoos were likely not for decoration or ritual. Instead, the tattoos may have served a therapeutic purpose. Acupuncture experts determined that nine of the tattoo groups were located directly on or within 6 mm of traditional acupuncture points. Interestingly, these tattoo points are the same as traditional acupuncture points used to treat medical conditions that Otzi was found to have had at the time he died – lumbar arthrosis and abdominal disorders (intestinal parasites).[74]

Still, the idea that acupuncture originated in China is widely accepted. The first known documentation of acupuncture as a system of diagnosis and treatment is *Huang Di Nei Jing* (*The Yellow Emperor's Internal Classic*), which dates between 200-100 BC.[73] The text was likely a compilation of traditions passed down over the centuries, as the concept of meridians in which Qi flows were well-established by this time;[75] however, specific anatomical locations of acupuncture points were not developed until later.[76]

The practice continued to evolve over the years and became a routine practice in China along with massage, diet, and herbs.[75] During the Ming Dynasty (1368 – 1644), *The Great Compendium of Acupuncture and Moxibustion* was published, forming the basis of modern acupuncture. The text contained descriptions of 365 acupuncture points in which needles could be inserted to modify the flow of Qi.[76]

In the 17TH century, missionaries from Europe brought the Christian religion, as well as Western medicine, to China.[77] During this time, acupuncture had lost much of its appeal and became regarded as superstitious and irrational compared to Western medicine. In 1822, acupuncture was excluded from the Imperial Medical Institute. Acupuncture and other forms of traditional Chinese medicine were eventually outlawed by the Chinese authorities in 1929.[73] Although interest in acupuncture in China declined during this time, its popularity spread to other Eastern nations and the West. With the Chinese communist victory in the War of Liberation in 1949, Mao Tse Tung wanted to improve health services for the poor by ensuring more doctors become trained in traditional Chinese medicine. Teaching of both traditional Chinese medicine and Western medicine was instituted in medical colleges.[77]

Electroacupuncture was developed in China in the 1950s for surgical analgesia. With manual acupuncture, the anesthetists had to continuously manipulate the needles by hand

throughout the operation. Electroacupuncture was developed to alleviate them of this task.[73] During a visit to China in 1972, President Nixon and his entourage were introduced to this technique to suppress surgically-evoked pain.[77] Subsequently, teams of U.S. physicians traveled to China to assess this technique for themselves. While they determined that acupuncture was not acceptable as a sole analgesic, their reports stimulated further research investigations into the use of acupuncture for pain. Over time, acupuncture has become more widely accepted in the West due to positive clinical trials, as well as discoveries of mechanisms of action.[73]

2.2 Mechanisms of acupuncture

As the TCM mechanism of acupuncture is difficult to comprehend from a Western perspective, there have been strong efforts to determine the mechanisms of acupuncture analgesia in scientific terms. While these mechanisms are still not well understood, it is widely believed that acupuncture exerts its effects on the nervous system.[73] In the 1970s, topographic mapping revealed that most acupuncture points and meridians were located on or near peripheral nerve trunks and branches.[78] Additionally, the analgesic effect of acupuncture needles was abolished after injection of a local anesthetic into the surrounding tissue.[79] These studies suggested that the mechanism of acupuncture is associated with peripheral nerve fibers.

Other pioneering studies implicated a role of opioid peptides in acupuncture analgesia by determining that naloxone, an opioid antagonist, inhibits the effects of acupuncture analgesia in mice[80] and humans.[81] Additionally, mice genetically deficient in opioid receptors were found to have decreased acupuncture analgesia.[82] Further, by using different opioid receptor antagonists, it was demonstrated that varying stimulation frequencies of electroacupuncture caused different opioid receptors to be activated. Low frequency stimulation likely released β-endorphin, enkephalin, and endomorphin that act on μ and δ opioid receptors, and high-frequency

stimulation likely released dynorphin to act on κ opioid receptors.[83] In another landmark study, electroacupuncture increased concentrations of β-endorphin in the CSF of patients with pain, whereas β-endorphin levels did not rise in control patients.[84]

There is also evidence that the serotonin and noradrenaline descending pain inhibitory pathways may play a role in acupuncture analgesia. The 5-HTP serotonin precursor was found to enhance the effects of electroacupuncture analgesia, whereas serotonin receptor antagonists were found to reduce the analgesic effects in mice.[85] Moreover, numerous studies have demonstrated that lesions of the nucleus raphe magnus, where serotonin cells are primarily located, decrease analgesic effects of electroacupuncture.[86]

In addition to providing pain relief, acupuncture has been shown to have anti-inflammatory effects in inflammatory pain models. Using a complete Freund's adjuvant model of inflammatory pain, Lao et al. determined that electroacupuncture treatment resulted in significantly longer paw withdrawal latencies and decreased paw edema of the inflamed paw compared to control animals.[87] In the same model, they also determined that electroacupuncture increased plasma levels of corticosterone, and anti-inflammatory effects were partially blocked in adrenalectomized rats. These results suggest acupuncture may suppress inflammation by activating the hypothalamus-pituitary-adrenal axis.[88] In a carrageenan-induced paw inflammation in rats, electroacupuncture analgesia was completely blocked in a dose-dependent manner by intraplantar injection, but only partially blocked by an intraperitoneal injection of naloxone. This suggested that in addition to opioid receptors in the CNS, peripheral opioids released at the site of inflammation are also involved in the anti-inflammatory mechanism of electroacupuncture.[89]

Based on these initial studies, the mechanism of acupuncture analgesia has been hypothesized as follows: acupuncture stimulates small diameter nerves in the skin (Aδ) or

muscle (Groups II & III) which sends impulses to the spinal cord and the brain to cause analgesia. The spinal cord, midbrain, and hypothalamus/pituitary are activated to release neurotransmitters (endorphins and monoamines) that block pain by preventing the release of substance P. At the level of the spinal cord, stimulation of sensory afferents causes the release of enkephalin from interneurons in the dorsal horn. Enkephalins act segmentally within the substantia gelatinosa to block the transmission of pain. Sensory nerve fibers also synapse with nerve cells that travel via the spinothalamic tract to the periaqueductal grey (PAG) of the midbrain. This causes the release of enkephalin which activates the serotinergic-mediated pain inhibitory system that descends from the PAG to the dorsal horns. A synergistic effect of serotonin and norepinephrine inhibits spinal cord pain transmission. From the pituitary gland, β-endorphin is released into the blood and CSF, which also activates the descending pain inhibitory system in the PAG of the midbrain. The pituitary gland also releases adrenocorticotrophin hormone (ACTH) that acts at the adrenal cortex to release cortisol into the blood, which may explain some of acupuncture's anti-inflammatory effects.[86]

Despite advances in the understanding of pain-relieving and anti-inflammatory mechanisms of acupuncture, OA-specific mechanisms remain unclear. More recently, studies have been performed to assess acupuncture mechanisms for chronic pain relief and chondroprotective effects in induced models of joint degeneration. Using an intra-articular injection of collagenase in a rat model, low frequency electroacupuncture was suggested to attenuate pain through μ-opioid and δ-opioid, but not by κ-opioid receptors.[90] Electroacupuncture has also been shown to inhibit chronic pain in a mouse model of monosodium iodoacetate-induced knee joint degeneration by reducing expression of IL-1β through activation of the CB2 receptor.[91] Electroacupuncture was found to increase estrogen levels, inhibit expression of

MMP-13, decrease body weight, and prevent cartilage degeneration in rabbits with ovariectomy-induced OA.[92] In rats with anterior cruciate ligament transection-induced OA, electroacupuncture was determined to have prevented the degeneration of articular cartilage through regulation of MMP-13 and inhibition of p38, ERK1, and c-Jun mitogen-activated protein kinases (MAPKs). Using ovariectomized rats, the same group found that electroacupuncture inhibited subchondral bone deterioration by regulating RANK/RANKL/OPG signaling and protected articular cartilage by inhibiting MMP-13.[93]

In summary, the mechanism by which acupuncture exerts its therapeutic effects is still poorly understood and likely occurs through multiple pathways. Although acupuncture has been shown to be beneficial for knee OA pain, further research is still needed to fully elucidate its mechanisms.

2.3 Challenges in acupuncture research

Although acupuncture has become increasingly accepted as a valid therapy for the treatment of OA pain, many organizations have stated there is still not enough evidence to provide a general recommendation for its use.[52,54,56] A number of systematic reviews of randomized controlled trials have assessed the efficacy of acupuncture for knee OA pain.[94–99] While positive effects of acupuncture are reported, the evidence is often stated to be of poor quality, leading to uncertainty in its efficacy.[96] Additionally, although a large amount of acupuncture studies have already been performed in China, most are published in national journals that are not translated to Western languages.[100]

For acupuncture to become more readily incorporated into Western medical practices, clinical trials need to demonstrate its ability to be superior to that of a powerful placebo.[77]

Further, studies should be double-blinded to prevent bias. Since there is no true placebo control for an acupuncture needle, the term "sham" is used instead for any control procedure that is used to make the participant believe they have had treatment. There are two types of sham controls commonly used in acupuncture studies: non-penetrating and penetrating. Non-penetrating sham controls have included tapping of the skin with a guide tube, toothpick, or a blunted needle. To keep the patient blinded, these can only be applied convincingly on points out of the patient's line of vision, such as the back, or behind a barrier. Additionally, the patient could be blindfolded. With penetrating sham controls, real acupuncture needles are either placed in the wrong site, or in the right site but superficially and without stimulation. However, it is difficult to place needles in a wrong site, as patients expect to be needled somewhere near the site of pain. Additionally, needle insertion, even superficially, is likely to have some kind of physiological effect on the patient, and false negative results are likely. Further, while blinding of the patient is possible, it is inevitable that the acupuncturist is aware of the treatment being administered.[73]

Another problem is that acupuncture is an extremely complex therapeutic with many variables involved, such as the choice of acupuncture points, type of stimulation, and frequency and duration of treatment.[77] Acupuncture treatments are practitioner-dependent and not standardized for different conditions, making it difficult to compare study outcomes. Sample sizes are often small due to underfunding, leading to inadequate statistical power. Additionally, patients are often responsible for evaluating their own progress, leading to further bias.[86] These methodological limitations indicate that high quality clinical trials are still needed.

3. Overview of Animal Models of Osteoarthritis

As the course of disease is slow and unpredictable, studying OA in humans is challenging. Additionally, clinical symptoms of pain do not always coincide with molecular and

structural changes within the joint. For these reasons, a variety of laboratory animal species have been used as models of OA, each with their own advantages and disadvantages. Unfortunately, there is no single animal model that reflects all aspects of human disease.[101]

Common small animal models of OA include the mouse, rat, guinea pig, and rabbit, and large animal models include the dog, goat/sheep, horse, and nonhuman primates. Choice of model depends on several factors including the type of OA being studied, length of experiment, husbandry costs, ease of handling, and outcomes. Small animal models are generally cheaper, easier to handle, and have a faster course of disease compared to large animal models. However, treatments in these models may not be as equally translatable to humans due to differences in anatomy and physiology. On the other hand, the anatomy of large animal models is much more similar to that of humans. However, length of disease progression, high costs of care, as well as ethical issues may be obstacles to their widespread use.[102]

3.1 Classification of OA models

Models of OA are typically classified into spontaneous (naturally-occurring or genetically modified) or induced (surgically or chemically) models.[102] Surgical procedures most often involve the knee joint and produce joint instability and alter load bearing to induce OA.[103] These procedures include, but are not limited to, anterior cruciate ligament transection, meniscal tear, partial or total meniscectomy, or creation of articular grooves.[101,102] Surgical models have the advantage of reproducibility (depending on expertise) and rapid disease progression; however, there is little correlation to the natural disease process seen in primary OA of humans. Thus, these models are best-suited for studying secondary (post-traumatic) OA.

Chemically-induced models include an intra-articular injection of a toxic or inflammatory compound, such as papain, sodium monoiodoacetate, quinolone, collagenase, carrageenan, or Freund's adjuvant.[101,102] These models are easy to induce and highly reproducible. Additionally, there is minimal risk of infection as compared to surgically-induced models. However, chemical induction does not correlate to the disease pathogenesis seen in primary or secondary OA and is more commonly used to study other inflammatory arthritides or OA pain-related behaviors.[102,104]

Genetically-modified mouse strains have been designed to develop OA without intervention. These transgenic mice have frequently been used to study the effects of single genes in the pathogenesis of OA. Studies using these mice have helped establish the molecular basis of OA, including the role of pro-inflammatory cytokines.[102] However, as OA is a polygenic disease, knockout of a single gene may oversimply the disease process and limit its translatability.[101,102] Natural models of slowly-progressing OA most closely simulate the disease process of primary human OA. Yet, these models take longer for OA to develop, making it difficult to conduct short-term studies. As experimental time increases, the cost of housing these animals will also increase.[102]

The two most common rodent models of primary OA are the STR/ort mouse and the Dunkin Hartley guinea pig. The STR/ort mouse resulted from a piebald mutation in the STR/N1 strain, which exhibited spontaneous OA at a young age. The strain is now a well-recognized model of naturally-occurring OA in the knee and other joints, particularly in males. OA spontaneously develops early in life at 18 weeks of age and has similar characteristics to human disease. However, studies suggest that these mice have an inherent endochondral ossification defect as the major driver of their OA pathology, which may limit their translatability to human disease.[105] While mouse models are beneficial for their low cost and commercial availability of

reagents, their small size leads to dissimilarities in joint mechanics compared to humans and limits the amount of available tissue for biochemical analyses.[101]Additionally, mice exhibit differences in cartilage anatomy compared to humans including decreased thickness (50-fold thinner than humans), a thick layer of calcified cartilage, and lack of 3 distinct zones of chondrocytes.[101]

The Dunkin Hartley guinea pig develops age-related, spontaneous OA of the knee and other joints, with histologic changes that are nearly identical to primary OA of humans. Disease onset typically occurs by 3 months of age, with OA pathology becoming advanced by 16 months of age.[106] The guinea pig is large enough to provide adequate tissue samples for histological and biochemical analyses, but small enough to allow use of sufficient numbers of animals to acquire statistically significant data.[107] Guinea pigs also share a similar Vitamin C requirement to humans[108], which is particularly important for studies evaluating oxidative damage. However, guinea pigs have few available reagents and are more costly compared to mice. Despite these limitations, the Dunkin Hartley guinea pig is the most frequently used animal model to study spontaneous OA.[102]

3.2 Dunkin Hartley guinea pig model of OA

Naturally-occurring degenerative joint disease in guinea pigs was first reported by Silverstein and Sokoloff. In their laboratory, 20 retired breeders (aged 29 to 51 months) of strains 2 and 13 were found to have osteoarthritic changes of several joints with the knees the most severely affected. Males were found to be more frequently and severely affected compared to females. However, the report primarily describes bony changes, particularly osteophyte formation, with little regard to changes in the articular cartilage.[109]

Bendele was the next to note spontaneous OA changes in male and female Hartley guinea pigs while evaluating their use as a suitable animal model for surgically-induced cartilage degeneration. A study was performed to characterize sequential histopathologic OA changes in operated and non-operated femorotibial joints of partially meniscectomized male and female guinea pigs at 1, 2, 3, 6, and 12 weeks post-surgery. Cartilage degeneration was noted in the contralateral non-operated limb in some animals at 3 weeks post-surgery and in all animals 12 weeks post-surgery.[110] This study was followed by a light and electron microscopic assessment of spontaneous knee joint cartilage degeneration to determine the incidence, age of onset, and characterization in male Hartley guinea pigs aged 61 to 365 days old.[107,111] Early degenerative changes characterized by proteoglycan loss and cartilage surface fibrillation were first observed on the medial tibial plateau in 89 day old animals. Changes became progressively more severe with age and resembled those of humans with OA.[107]

The pathology of OA in Dunkin Hartley guinea pigs shares many characteristics with the disease in humans. Like humans, the guinea pig knee is a diarthrodial joint composed of cartilage, bone, synovium, and supporting structures, and the pathogenesis of OA involves all of these tissues. Disease occurs primarily in the medial compartment of the knee joint as the medial aspect of the knee is predominately loaded in guinea pigs. Generally, disease is bilaterally symmetrical and develops spontaneously in both male and female guinea pigs. However, males grow and gain weight faster, resulting in more consistent pathological alterations.[112] Also similar to humans, disease worsens with increased age and body weight.[113] Given this, Dunkin Hartley guinea pigs have become the most commonly used strain to model OA due to their strong histological resemblance to human primary OA.

Disease onset typically occurs when animals reach 3 months of age and 700 g of weight. Initial histologic lesions are observed on the medial tibial plateau and consist of focal chondrocyte death, proteoglycan loss, and articular cartilage fibrillation. At approximately 6 months of age and 900 g of weight, moderate lesions will be present in 90 – 100% of medial tibial plateaus. Lesions involve chondrocyte loss extending into the upper middle zone, articular cartilage fibrillation, and proteoglycan loss. Small osteophytes may be present. There is no evidence of subchondral bone, meniscal, or femoral cartilage changes at this stage. At 9 months of age, animals will have mild to moderate medial tibial cartilage degeneration, mild femoral condylar degeneration, and tibial osteophytes. Other potential changes include degenerative changes in the menisci, thickened synovial membranes, and subchondral bone sclerosis. By 1 year of age, disease becomes more severe. Degenerative changes in the cartilage involve the entire medial compartment of the knee. Proteoglycan loss with fibrillation and hypocellularity extend into the deep zone. Degenerative changes to the menisci are severe. Papillary proliferation of the synovium can be observed. There is also extensive subchondral bone sclerosis with bone cysts, and large osteophytes may be present. In 18 – 24 month old animals, there is dramatic recontouring of medial surfaces, and degenerative changes on the lateral sections of the joint.[112]

Primarily based on the 1971 Mankin scheme for humans,[114] numerous scoring systems have been developed for histological evaluation of knee OA in guinea pigs. In an effort at standardization, the OARSI has put forth specific criteria for assessing OA in the guinea pig, termed the OARSI-HISTOgp recommendations. These guidelines include grading of the articular cartilage structure, proteoglycan content, cellularity of chondrocytes, presence of osteophytes, and tidemark integrity. Two independent, blinded evaluators and scoring of one

intact central section are advised. Scores should be tabulated separately for each joint section (medial tibia, medial femur, lateral tibia, and lateral femur). With 0 as normal cartilage, the system scores articular cartilage structure 0 – 8, proteoglycan content 0 – 6, cellularity 0 – 3, tidemark integrity 0 or 1, with an optional osteophyte score 0 – 3. These scores can be summed for compartmental (medial and lateral) and total joint scores.[113]

3.3 Outcome measures of OA

Using animal models, the major goals of OA research are to study the pathology of disease or evaluate efficacy of treatment.[102] Histopathology has long been the gold standard for outcome assessment in animal models of OA. Still, there is a need for less invasive measures of monitoring disease progression and response to treatment.[101] These include imaging, local and systemic biomarker measurements, pain assessments, and gait outcomes.[102]

Radiographs are the standard for OA diagnosis and progression in humans as they allow for the evaluation of bony changes such as joint space width, osteophytes, subchondral bone sclerosis, and bone cysts. However, radiographs cannot be used to evaluate changes to the cartilage and menisci and are not sensitive enough to detect early OA changes.[102] Additionally, it is well-known that radiographic findings are weakly associated with symptoms of OA knee pain.[115] In contrast, magnetic resonance imaging (MRI) has the advantage of visualizing the cartilage, menisci, ligaments, and synovium and has shown a stronger correlation with OA pain in humans. Despite its high cost, MRI is being increasingly used and is anticipated to replace radiography in both clinical and research settings.[102]

Biomarkers can also be used to evaluate changes in disease progression, but there is debate on which ones should be measured for OA studies.[101] Urine and serum biomarkers may

be used to evaluate systemic inflammation but may be influenced by other diseases. Biomarkers in synovial fluid may be more useful as they represent the local environment of the joint. Yet, repeated joint aspirations may increase inflammation within the joint, and synovial fluid is limited in small animal models.[104] Biomarkers have been evaluated in many of the larger animal models, including the Dunkin Hartley guinea pig.[116] A combination of catabolic, anabolic, and inflammatory biomarkers are recommended to provide the most accurate disease characterization.[104]

Systemic inflammation can also be measured using a variety of hematologic and biochemical parameters. Complete blood count (CBC) and serum biochemistry are easy to use, noninvasive, and inexpensive tests commonly used in animal species to diagnose disease, monitor disease progression, and evaluate response to treatments. Dogs with experimental stifle OA were found to have significant differences in platelet counts and alkaline phosphatase compared to controls.[117] However, these assays have not yet been evaluated in the more commonly used rodent models of OA.

As the radiographic and histologic severity of OA is only weakly associated with clinical symptoms,[49] pain and gait assessments are critical outcome measures in studies utilizing animal models of OA. Unfortunately, studies investigating these outcomes with animal models are limited as the majority have focused on the pathophysiology of joint pathology or disease-modifying therapies.[118] Various pain assessments have included von Frey algesiometry, Hargreaves testing, ultrasonic vocalizations, pressure application measurement devices, spontaneous behavior assessment, and facial expressions.[118,119] Gait analysis can be used to evaluate pain, but it is difficult to interpret whether observed gait changes are due to OA pain or altered joint biomechanics.[118] Some published methods have included measurement of stride

length from inked paw prints[120] or an automated treadmill system.[121,122] Although mice, rats, and guinea pigs are the most commonly used species for investigating OA, these measures are challenging in rodents as they are prey species that mask their pain and lameness.[118]

CHAPTER II.

AGE- AND SEX-RELATED DIFFERENCES IN HEMATOLOGY AND SERUM BIOCHEMISTRY PARAMETERS OF THE DUNKIN HARTLEY GUINEA PIG[1]

1. Introduction

Due to their docile nature, small size, and biological similarities to humans, guinea pigs have been a mainstay of biomedical research for hundreds of years. They are most commonly used in allergy, immunology, infectious disease, nutritional, auditory, and osteoarthritis studies, among others.[123,124] The standard laboratory guinea pig for research is the Dunkin Hartley, an outbred, smooth-coated, albino strain. The strain was first developed by Dunkin and Hartley in 1926 and is commercially available from several laboratory breeders.[124]

Complete blood counts (CBCs) and serum biochemistries are blood tests routinely performed to screen health status in both animals and humans. Reference intervals, defined as the set of values comprising 95% of the healthy reference population,[125] are essential for laboratory diagnostic testing as well as clinical decision-making. The American Society for Veterinary Clinical Pathology (ASVCP) has set forth guidelines for determining reference intervals for veterinary species.[125] Despite their widespread use in research, there are few publications reporting normal hematology and clinical chemistry reference intervals in guinea pigs, particularly of the Dunkin Hartley strain. Waner et al. compared hematologic and clinical

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chemistry parameters of IAF hairless and normal Dunkin Hartley guinea pigs. However, the study was limited by a small number of animals of the same age and sex. Additionally, values were determined using mean and standard deviation, which are not the recommendations set forth in the current ASVCP guidelines.[125] Prior studies have determined reference intervals in Weiser-Maples[126] and strain 13[127] guinea pigs. However, there are likely differences in hematology and biochemistry parameters between strains of guinea pigs, similar to other laboratory rodents.[128–130] These studies in guinea pigs[126,127] and other laboratory rodents[129–133] have also demonstrated age and sex to be important factors impacting hematologic and biochemical parameters.

The purpose of this study was to develop CBC and serum biochemistry reference intervals for the Dunkin Hartley strain and determine age- and sex-related differences. To accomplish this, we accumulated historical CBC and serum biochemistry data from healthy control animals used in our laboratory's previous studies that represent both males and females of a large age range.

2. Materials and Methods

2.1 Animals

Retrospective CBC and serum biochemistry data from 91 male (age, 2 – 15 mo; 948.83 ± 173.30 g) and 52 female (age, 2 – 9 mo; weight, 783.41 ± 189.29 g) Dunkin Hartley guinea pigs were included in this study. Serum biochemistry data was unavailable from 8 females. All guinea pigs were purchased from Charles River Laboratories (Wilmington, MA). All animals were singly-housed in 30.80 cm x 59.37 cm x 22.86 cm isolator cages (Maxi-Miser Interchangeable IVC Caging, Thoren, Hazleton, PA) with 0.125-in. corn cob bedding (Harlan, Madison, WI) and

a red hut (BioServe, French Town, NJ). Caging was changed 2 – 3 times weekly. Teklad Global Guinea Pig Diet 2040 (Envigo, Madison, WI) and filter-sterilized water were provided *ad libitum*. Hay cubes (PMI Nutrition International LLC, Brentwood, MO) were provided daily. Animal rooms were maintained at a 12:12h light:dark cycle, 20 - 26° C temperature, and 30 – 70% humidity. All animals were free of Sendai virus, lymphocytic choriomeningitis virus, pneumonia virus of mice, guinea pig adenovirus, guinea pig reovirus, *Helicobacter* spp., *Mycoplasma pulmonis*, and ectoparasites. All activities were performed in accordance with *The Guide for the Care and Use of Laboratory Animals* and approved by the Colorado State University Institutional Animal Care and Use Committee.

2.2 Blood collection and analysis

All blood was collected from guinea pigs anesthetized with 3 – 5% isoflurane. Blood was collected ante-mortem from the cranial vena cava with a 25-gauge needle and 1-mL syringe or at study harvest via direct cardiac puncture with a 20-gauge butterfly catheter. Collected blood was placed into 0.5 mL EDTA microtubes and red top serum collection tubes. After clotting, red top tubes were placed into a centrifuge at 5000x*g* for 15 minutes for serum collection. EDTA microtubes and serum aliquots were submitted to the Colorado State University Clinical Pathology Laboratory. CBCs were performed using the Advia 120 hematology analyzer (Siemens, Munich, Germany). Automated parameters included: hemoglobin (Hgb), hematocrit (Hct), red blood cells (RBC) red blood cell distribution width (RDW), mean cell volume (MCV), mean corpuscular hemoglobin concentration (MCHC), cell hemoglobin concentration mean (CHCM), platelets, mean platelet volume (MPV), and white blood cells (WBC). Manual blood film differentials were performed to determine heterophil, lymphocyte, Foa-Kurloff, monocyte, eosinophil, and basophil counts. When blood films were unavailable, the automated WBC

differential counts were used for heterophils, lymphocytes, monocytes, eosinophils, and basophils. The Roche Cobas 6000 (Basel, Switzerland) was used to measure the following parameters in serum: glucose, blood urea nitrogen (BUN), creatinine, phosphorus, calcium, magnesium, total protein, albumin, globulin, albumin/globulin ratio (A/G), cholesterol, creatine kinase (CK), total bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), γ-glutamyl transferase (GGT), sodium, potassium, chloride, bicarbonate, anion gap, and iron.

2.3 Statistical analysis

Following guidelines provided by the ASVCP,[125] reference intervals were determined using Reference Value Advisor v2.1[134] The nonparametric method was performed to determine the 2.5th and 97.5th percentile of each parameter to serve as the lower and upper limits of the reference interval, respectively. The 90% confidence intervals of the lower and upper limits of the reference interval were then determined using the bootstrap method. Due to the low sample size for Foa-Kurloff cells in females (< 40), the nonparametric method could not be performed. Instead, the data was assessed for normality using the Anderson-Darling test. As the distribution was not Gaussian, data were transformed using the Box-Cox method and rechecked for distribution. The parametric method was performed to determine the reference interval with 90% confidence intervals of the reference limits.

Age- and sex-associated differences were analyzed using Prism (version 8.4.0, GraphPad Software, La Jolla, CA). Age correlation was determined using the Spearman coefficient. Normality was assessed using the D'Agostino-Pearson normality test. An unpaired t-test for normally-distributed data or a nonparametric Mann-Whitney test for non-normally distributed data was used to assess sex-associated differences. Results were considered statistically

significant with a P value < 0.05.

3. Results

3.1 Hematology

Reference intervals were established for hematology parameters of Hgb, Hct, RBC, RDW, MCV, MCHC, CHCM, MPV, platelets, WBC, heterophils, lymphocytes, monocytes, Foa-Kurloff cells, eosinophils, and basophils in 91 male Dunkin Hartley guinea pigs of 2 – 15 mo of age (Table 2.1) and 52 female Dunkin Hartley guinea pigs of 2 – 9 mo of age (Table 2.2). Manual WBC differential counts were unavailable from 8 males and 20 females, and automated WBC counts were unavailable from 4 females. Therefore, reference intervals for heterophils, lymphocytes, monocytes, eosinophils, and basophils were calculated from 83 males and 48 females. As automated counts could not be used for Foa-Kurloff cells, reference intervals were determined from 83 males and 24 females.

Age correlation of hematology parameters in male and female guinea pigs is shown in Table 2.3. In both sexes, MPV, heterophils, and basophils were correlated with age, with females more highly correlated than males. In males alone, HCT (r = 0.3013), MCHC (r = -0.2701), CHCM (r = -0.3705), platelets (r = -0.2944), and MPV (r = 0.3587) were correlated with age. In females alone, monocytes (r = 0.3129) and eosinophils (r = 0.3685) were correlated with age.

Sex differences in RBC (Figure 2.1), platelet (Figure 2.2), and WBC parameters (Figure 2.3) were also determined. Male guinea pigs had significantly higher levels of Hgb ($P < 0.0001$), Hct ($P < 0.0001$), RBC ($P < 0.0001$), MCHC ($P = 0.0019$), CHCM ($P < 0.012$), WBC ($P = 0.0472$), and heterophils ($P < 0.0001$) compared to females. Females had higher MCV ($P < 0.0001$), platelets, ($P < 0.0001$), MPV ($P < 0.0001$), and eosinophils ($P < 0.0001$) than males.

Table 2.1. Descriptive statistics and reference intervals for hematology parameters of male Dunkin Hartley guinea pigs (age, 2 – 15 mo).

Parameter	Mean	Median	SD	Min	Max	Reference Interval	90% CI for lower limit	90% CI for upper limit
HGB (g/dL)	15.58	15.6	0.66	13.5	17.3	14.2 - 16.94	13.50 - 14.52	16.50 - 17.30
HCT (%)	47.5	48	2.2	42	53	42.6 - 52.7	42 - 44	51 - 53
RBC (10^6/µL)	5.79	5.81	0.26	5.06	6.42	5.25 - 6.24	5.06 - 5.37	6.18 - 6.42
MCV (fl)	82.3	82	2.2	77	89	78 - 87	77 - 78.3	85 - 89
RDW (%)	13.02	12.7	1.29	11	16.3	11.13 - 15.61	11 - 11.4	15.3 - 16.3
MCHC (g/dL)	32.8	33	0.9	31	35	31 - 35	31 - 32	34 - 35
CHCM (g/dL)	32.79	33	1.05	31	37	31 - 35	31 - 31	34.7 - 37
PLT (10^3/µL)	483.6	488	95.5	112	697	206.2 - 686.8	112 - 363.8	648 - 697
MPV (fl)	8.17	8.3	0.63	6.6	9.2	6.83 - 9.17	6.6 - 7.03	9 - 9.2
WBC (10^3/µL)	5.59	5.5	1.68	2	9.8	2.86 - 9.51	2 - 3.1	8.95 - 9.8
Heterophils (#)	2.58	2.52	1.01	0.98	5.49	1.1 - 5.24	0.98 - 1.32	4.81 - 5.49
Lymphocytes (#)	2.48	2.4	0.82	0.56	4.82	1.08 - 4.51	0.56 - 1.19	3.74 - 4.82
Foa-Kurloff cells (#)	0.22	0.19	0.17	0	0.76	0 - 0.71	0 - 0	0.50 - 0.76
Monocytes (#)	0.22	0.18	0.18	0	0.99	0 - 0.73	0 - 0.03	0.54 - 0.99
Eosinophils (#)	0.09	0.07	0.12	0	0.91	0 - 0.39	0 - 0	0.21 - 0.91
Basophils (#)	0.02	0	0.03	0	0.11	0 - 0.1	0 - 0	0.1 - 0.11

Table 2.2. Descriptive statistics and reference intervals for hematology parameters of female Dunkin Hartley guinea pigs (age, 2 – 9 mo).

Parameter	Mean	Median	SD	Min	Max	Reference Interval	90% CI for lower limit	90% CI for upper limit
HGB (g/dL)	14.77	15	0.88	11.9	16.3	12.36 - 16.24	11.9 - 13.43	15.9 - 16.3
HCT (%)	45.7	46	2.8	40	52	40.3 - 51.4	40 - 41	50 - 52
RBC (10^6/µL)	5.39	5.47	0.35	4.16	6.17	4.36 - 6.08	4.16 - 4.84	5.85 - 6.17
MCV (fl)	84.9	84.5	3	79	95	79.3 - 94.7	79 - 81	89 - 95
RDW (%)	13.36	13.3	1.3	11.2	17.2	11.2 - 16.84	11.2 - 11.26	15.42 - 17.2
MCHC (g/dL)	32.3	32	1	30	34	30.3 - 34	30 - 31	34 - 34
CHCM (g/dL)	32.3	32	1	30	34	30 - 34	30 - 31	34 - 34
PLT (10^3/µL)	597.7	561.5	156.5	333	1025	337.6 - 998.4	333 - 361.4	849.9 - 1025.0
MPV (fl)	8.62	8.7	0.43	7.5	9.3	7.57 - 9.3	7.5 - 7.8	9.2 - 9.3
WBC (10^3/µL)	5.03	5.2	1.47	1.9	9.1	2 - 8.94	1.9 - 2.86	7.1 - 9.1
Heterophils (#)	1.8	1.75	0.81	0.4	4.7	0.41 - 4.59	0.40 - 0.93	3.57 - 4.7
Lymphocytes (#)	2.63	2.66	0.85	0.48	4.29	0.61 - 4.20	0.48 - 1.56	3.9 - 4.29
Foa-Kurloff cells (#)	0.17	0.14	0.16	0	0.58	0 - 0.57	0 - 0	0.42 - 0.74
Monocytes (#)	0.19	0.13	0.28	0	1.91	0 - 1.61	0 - 0.03	0.34 - 1.91
Eosinophils (#)	0.2	0.18	0.17	0	0.8	0 - 0.78	0 - 0.03	0.45 - 0.8
Basophils (#)	0.04	0	0.06	0	0.29	0 - 0.25	0 - 0	0.10 - 0.29

Table 2.3. Spearman correlation (r) of hematology parameters with age in male and female Dunkin Hartley guinea pigs.*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

	Males	Females
HGB (g/dL)	0.1769	-0.1057
HCT (%)	0.3013**	-0.07527
RBC (10^6/µL)	0.2053	-0.1649
MCV (fl)	0.1968	0.2611
RDW (%)	0.05095	-0.0182
MCHC (g/dL)	-0.2701**	-0.1397
CHCM (g/dL)	-0.3705***	-0.1196
PLT (10^3/µL)	-0.2944**	0.198
MPV (fl)	0.3587***	0.7228****
WBC (10^3/µL)	0.1193	0.2616
Heterophils (#)	0.2448*	0.4214**
Lymphocytes (#)	-0.08401	-0.05978
Foa-Kurloff cells (#)	0.1011	-0.3536
Monocytes (#)	-0.1578	0.3129*
Eosinophils (#)	0.124	0.3685**
Basophils (#)	0.2262*	0.3225*

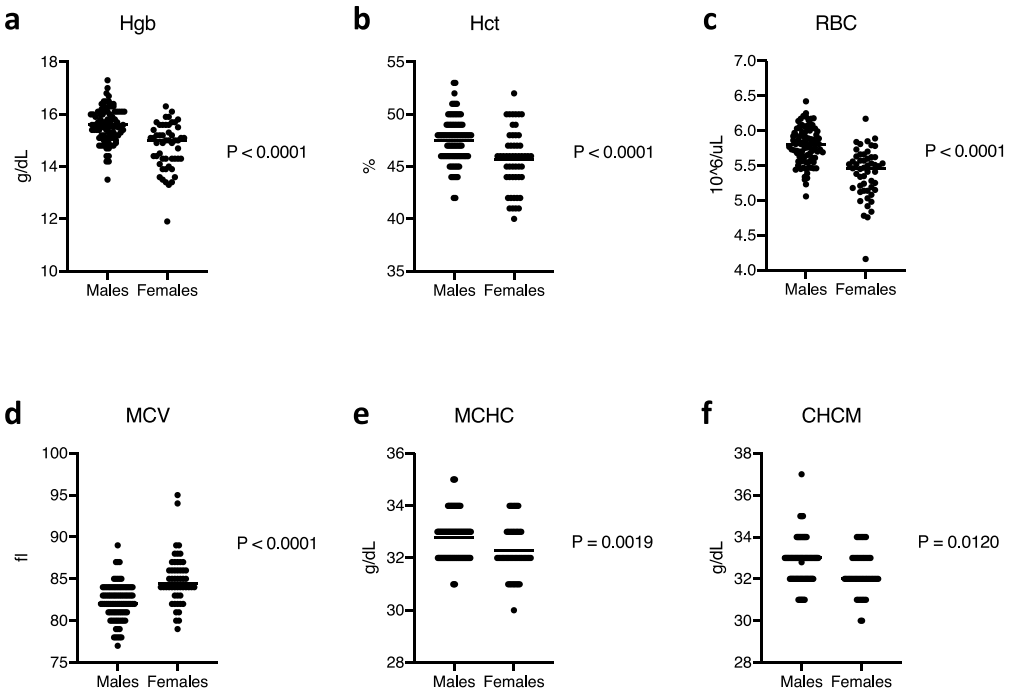

Figure 2.1 Sex-related differences in RBC parameters Hgb (a), Hct (b), RBC (c) MCV (d), MCHC (e), and CHCM (f). Black lines represent mean values for normally distributed data (Hct, MCHC) and median values for non-normally distributed data (Hgb, RBC, MCV, CHCM). P-values were determined by unpaired t-tests for normally distributed data and non-parametric Mann-Whitney tests for non-normally distributed data.

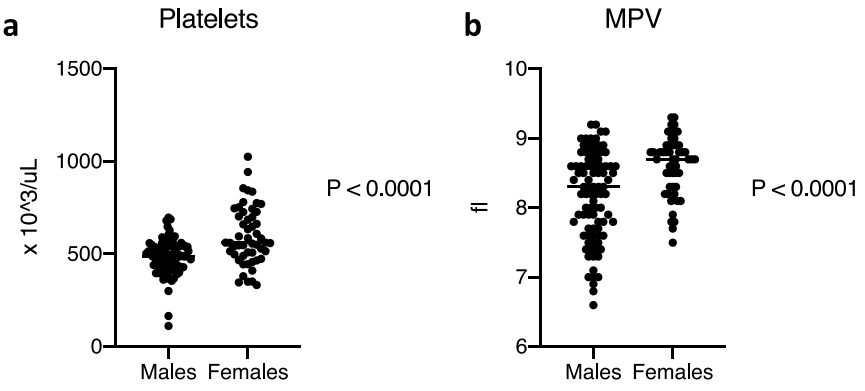

Figure 2.2. Sex-related differences in platelets (a) and MPV (b). Black lines represent median values. P-values were determined by non-parametric Mann-Whitney tests.

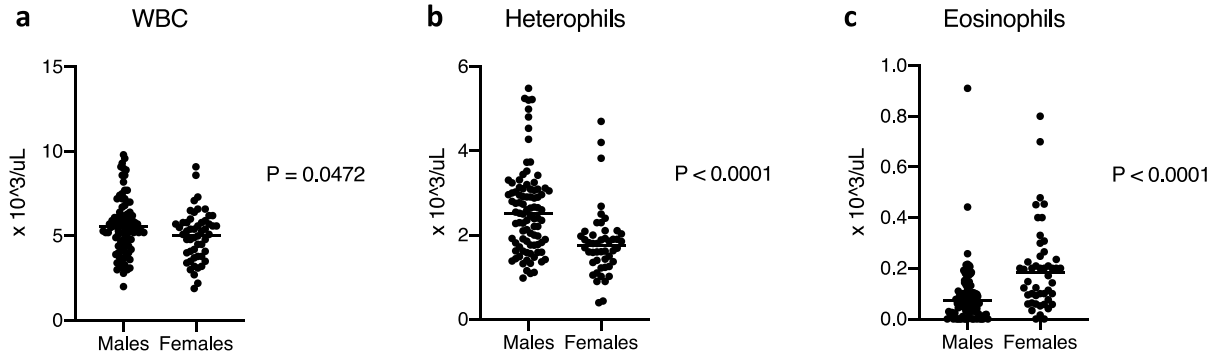

Figure 2.3. Sex-related differences in WBC (a), heterophils (b), and eosinophils (c). Black lines represent mean values for normally distributed data (WBC) and median values for non-normally distributed data (heterophils, eosinophils). P-values were determined by unpaired t-tests for normally distributed data and non-parametric Mann-Whitney tests for non-normally distributed data.

3.2 Serum biochemistry

Reference intervals were also established for the following serum biochemistry analytes: glucose, BUN, creatinine, phosphorus, calcium, magnesium, total protein, albumin, globulin, A/G, cholesterol, CK, total bilirubin, ALP, ALT, AST, GGT, sodium, potassium, chloride,

bicarbonate, anion gap, and iron in 91 male Dunkin Hartley guinea pigs of 2 – 15 mo of age (Table 2.4) and 44 female Dunkin Hartley guinea pigs of 3 – 9 mo of age (Table 2.5).

Age correlation of serum biochemistry parameters in males and females is presented in Table 2.6. In both sexes, BUN, A/G, ALP, ALT, AST, and GGT were correlated with age. Of these analytes, males were more strongly correlated with A/G (r = -0.702) and ALP (r = -0.5961), and females were more strongly correlated with BUN (r = 0.5394), AST (r = 0.3812), and GGT (r = 0.4669). ALT was positively correlated with age in males (r = 0.2503) but negatively correlated with age in females (r = -0.3282). Many more analytes were correlated with age in males than females, including glucose (r = 0.4744), creatinine (r = 0.4848), phosphorus (r = -0.4881), calcium (r = 0.3563), total protein (r = 0.6631), albumin (r = 0.2171), globulin (r = 0.7739), bicarbonate (r = 0.3979), and anion gap (r = -0.3471). Cholesterol (r = 0.5284) and iron (r = 0.3081) were correlated with age in females, but not males.

Sex-associated differences in serum biochemistry parameters are shown in Figure 2.4. Male guinea pigs had higher levels of ALT ($P = 0.0005$), sodium ($P = 0.005$), and bicarbonate ($P = 0.0231$) compared to females. Females had higher levels of total protein ($P = 0.0175$), albumin ($P = 0.0348$), and cholesterol ($P < 0.0001$) compared to males.

Table 2.4. Descriptive statistics and reference intervals for serum biochemistry parameters of male Dunkin Hartley guinea pigs.

Parameter	Mean	Median	SD	Min	Max	Reference Interval	90% CI for lower limit	90% CI for upper limit
Glucose (mg/dL)	237.1	227	68	125	558	139.5 - 466.8	125 - 156	374 - 558
BUN (mg/dL)	20.7	20	4	11	43	16 - 32.1	11.0 - 16.0	26.8 - 43
Creatinine (mg/dL)	0.32	0.3	0.07	0.2	0.5	0.2 - 0.4	0.2 - 0.2	0.4 - 0.5
Phosphorus (mg/dL)	5.47	5.4	1.03	3.5	8.6	3.63 - 7.98	3.5 - 3.95	7.63 - 8.6
Calcium (mg/dL)	11.09	11.1	0.57	8.9	12.5	9.82 - 12.18	8.9 - 10.39	11.98 - 12.5
Magnesium (mg/dL)	3.61	3.6	0.55	2.6	5.1	2.79 - 5.1	2.6 - 2.8	4.53 - 5.1
Total Protein (g/dL)	5.13	5.1	0.33	4.4	5.8	4.43 - 5.78	4.4 - 4.63	5.7 - 5.8
Albumin (g/dL)	3.07	3.1	0.15	2.8	3.4	2.8 - 3.4	2.8 - 2.8	3.3 - 3.4
Globulin (g/dL)	2.06	2.1	0.25	1.4	2.5	1.45 - 2.5	1.4 - 1.63	2.48 - 2.5
A/G Ratio	1.51	1.5	0.19	1.16	2.1	1.27 - 2.08	1.16 - 1.3	1.9 - 2.1
Cholesterol (mg/dL)	31.9	31	9.1	17	72	17.5 - 52	17 - 20.2	45.8 - 72
CK (IU/L)	625.1	405	817.6	141	6907	141.9 - 2891.5	141 - 153	1676.2 - 6852.8
Total Bilirubin (mg/dL)	0	0	0.02	0	0.2	0 - 0	0 - 0	0 - 0.2
ALP (IU/L)	109.2	91.5	52.4	29	280	34.3 - 254.4	29 - 48.8	223 - 280
ALT (IU/L)	40.8	37.5	14.5	24	110	24.3 - 97.4	24 - 26	64.9 - 110
AST (IU/L)	65.4	53	39.2	22	279	26.2 - 190.7	22 - 30	123.4 - 279
GGT (IU/L)	17.4	17	7.7	6	37	6.0 - 35.9	6.0 - 6.0	31 - 37
Sodium (mEQ/L)	136.8	137	2.6	127	144	128.2 - 142.1	127 - 133	139.7 - 144
Potassium (mEQ/L)	5.162	4.97	1.04	3.83	9.96	3.87 - 8.22	3.83 - 3.99	6.88 - 9.96
Chloride (mEQ/L)	100.85	101.2	3.04	88.5	107.3	92.43 - 106.84	88.5 - 96.2	105.48 - 107.3
Bicarbonate (mEQ/L)	24.07	25	3.75	14.8	32.7	16 - 30.9	14.8 - 16.8	29 - 32.7
Anion Gap (mmol/L)	17.1	16	3.2	11	26	12.3 - 25.8	11.0 - 13.0	22.8 - 26
Iron (µg/dL)	289.5	288.5	25.8	230	350	230.4 - 349.6	230.0 - 250.1	331.6 - 350

Table 2.5. Descriptive statistics and reference intervals for serum biochemistry parameters of female Dunkin Hartley guinea pigs.

Parameter	Mean	Median	SD	Min	Max	Reference Interval	90% CI for lower limit	90% CI for upper limit
Glucose (mg/dL)	267.9	232.5	96.8	159	526	160.8 - 522.6	159 - 177	465 - 526
BUN (mg/dL)	20.1	19.5	3.8	13	32	13.1 - 31.6	13 - 14.4	25 - 32
Creatinine (mg/dL)	0.32	0.3	0.05	0.2	0.4	0.2 - 0.4	0.2 - 0.21	0.4 - 0.4
Phosphorus (mg/dL)	5.53	5.55	0.99	3.6	7.7	3.66 - 7.65	3.6 - 4.21	7.16 - 7.7
Calcium (mg/dL)	11.11	11.1	0.49	10	12.3	10.04 - 12.3	10 - 10.41	11.96 - 12.3
Magnesium (mg/dL)	3.43	3.5	0.47	2.2	4.2	2.26 - 4.19	2.2 - 2.71	4.09 - 4.2
Total Protein (g/dL)	5.28	5.3	0.34	4.5	6	4.53 - 5.99	4.5 - 4.71	5.7 - 6
Albumin (g/dL)	3.14	3.2	0.22	2.7	3.6	2.7 - 3.59	2.7 - 2.8	3.49 - 3.6
Globulin (g/dL)	2.14	2.1	0.18	1.8	2.6	1.81 - 2.58	1.8 - 1.9	2.4 - 2.6
A/G Ratio	1.48	1.5	0.11	1.2	1.65	1.2 - 1.65	1.2 - 1.3	1.6 - 1.65
Cholesterol (mg/dL)	47.7	37.5	25.4	12	119	13.3 - 118.1	12 - 26.1	107.4 - 119
CK (IU/L)	532.8	381	489.4	125	2382	127.4 - 2367.9	125 - 191.3	1247.5 - 2382
Total Bilirubin (mg/dL)	0	0	0	0	0	0 - 0	0 - 0	0 - 0
ALP (IU/L)	118.1	105	50.6	51	253	51.9 - 249.5	51 - 60	216.9 - 253.0
ALT (IU/L)	33.9	31.5	14.2	15	105	15.1 - 99.3	15 - 18.4	48.3 - 105
AST (IU/L)	68.8	54	42.9	27	215	27 - 209.8	27 - 28.6	151.4 - 215
GGT (IU/L)	16	16	6.4	0	30	0.9 - 30	0 - 7.1	26.6 - 30
Sodium (mEQ/L)	135.5	136	2.9	127	142	127.4 - 141.8	127 - 131	139.8 - 142
Potassium (mEQ/L)	5.24	4.88	1.09	3.89	9	3.90 - 8.75	3.89 - 4.03	6.9 - 9
Chloride (mEQ/L)	100.49	101.3	3.31	90.5	105.6	90.64 - 105.49	90.5 - 93.2	104.6 - 105.6
Bicarbonate (mEQ/L)	22.92	23.55	3.82	2.5	28.4	4.5 - 28.31	2.5 - 19.61	26.5 - 28.4
Anion Gap (mmol/L)	16.8	17	2.1	13	22	13 - 21.9	13 - 14	20 - 22
Iron (µg/dL)	297.1	300.5	39.9	186	369	190.9 - 368.6	186 - 233	356 - 369

Table 2.6. Spearman correlation (r) of serum biochemistry parameters with age in male and female Dunkin Hartley guinea pigs. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001

	Males	Females
Glucose (mg/dL)	0.4744****	0.1055
BUN (mg/dL)	0.3247**	0.5394***
Creatinine (mg/dL)	0.4848****	0.001359
Phosphorus (mg/dL)	-0.4881****	-0.1233
Calcium (mg/dL)	0.3563***	-0.02177
Magnesium (mg/dL)	0.179	-0.05542
Total Protein (g/dL)	0.6631****	0.07104
Albumin (g/dL)	0.2171*	-0.1304
Globulin (g/dL)	0.7739****	0.2849
A/G Ratio	-0.702****	-0.3542*
Cholesterol (mg/dL)	0.1184	0.5284***
CK (IU/L)	-0.03027	0.09774
Total Bilirubin (mg/dL)	0.08037	-
ALP (IU/L)	-0.5961****	-0.4756**
ALT (IU/L)	0.2503*	-0.3282*
AST (IU/L)	0.2215*	0.3812*
GGT (IU/L)	0.2656*	0.4669**
Sodium (mEQ/L)	-0.007717	0.01635
Potassium (mEQ/L)	-0.1218	0.1874
Chloride (mEQ/L)	-0.1349	0.03105
Bicarbonate (mEQ/L)	0.3979***	-0.2446
Anion Gap (mmol/L)	-0.3471***	0.2147
Iron (µg/dL)	-0.06317	0.3081*

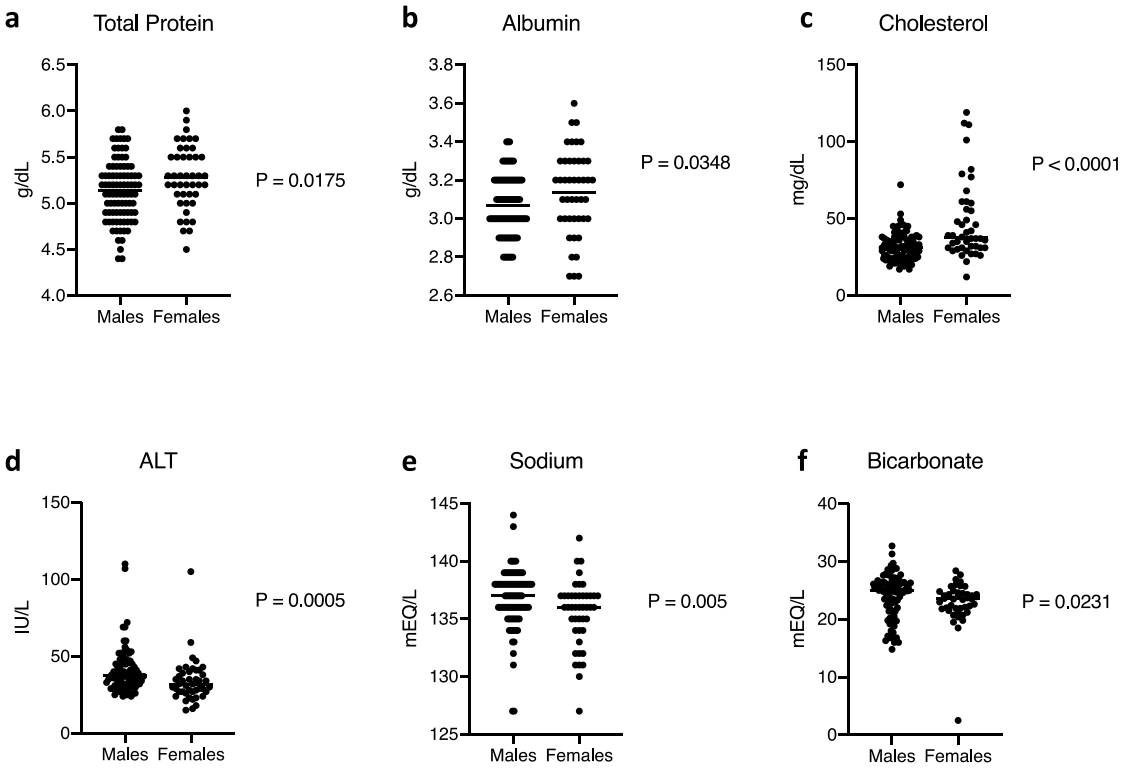

Figure 2.4. Sex-related differences in serum biochemistry parameters total protein (a), albumin (b), cholesterol (c), ALT (d), sodium (e), and bicarbonate (f). Black lines represent mean values for normally distributed data (total protein, albumin) and median values for non-normally distributed data (cholesterol, ALT, sodium, bicarbonate). P-values were determined by unpaired t-tests for normally distributed data and non-parametric Mann-Whitney tests for non-normally distributed data.

4. Discussion

Comprehensive reference intervals for hematology and serum biochemistry have not previously been developed for the Dunkin Hartley strain. This study established reference intervals for hematologic and biochemical parameters of male and female Dunkin Hartley guinea pigs according to the ASVCP guidelines and determined age- and sex-associated differences. These results provide the foundation for interpreting hematology and serum biochemistry results of the Dunkin Hartley guinea pig strain.

Hematologic and biochemical parameters in this study appeared to be similar to those previously reported in Dunkin Hartley guinea pigs,[135] with the exception of ALP. As the prior study only utilized 2-month-old guinea pigs, it is not surprising that the reported reference interval for ALP was much higher than in our study. In rodents and other species, bone-associated isoforms of ALP are elevated in younger animals due to bone growth.[136]

There were several age- and sex-related changes in hematology parameters in the Dunkin Hartley strain. In particular, a strong sex difference was observed in the erythrocyte indices. Males had significantly higher Hgb, Hct, and RBC compared to females. Similar findings have been reported in numerous animal species, including other rodents[127,131,132] as well as humans.[137] This may be due to the varying effects of estrogen and testosterone on erythropoietin production.[137] Hct was positively correlated with age, and MCHC and CHCM were negatively correlated with age in males. Platelets and MPV were both higher in females compared to males. While MPV was strongly correlated with age in both males and females, the number of platelets decreased with age in males. In contrast, platelets were positively correlated with males in Weiser-Maples guinea pigs.[126] Other RBC and platelet parameters were not correlated with age in either strain 13[127] or Weiser-Maples guinea pigs.[126]

Males had significantly higher WBC counts, due to higher numbers of heterophils, compared to females. Females had more eosinophils compared to males, similar to female strain 13 guinea pigs.[127] In other strains of guinea pigs, heterophils have been shown to increase with age, while lymphocytes decrease with age.[126,127] We found that while heterophils did increase, lymphocytes did not decrease with age in Dunkin Hartley guinea pigs. Additionally, basophils were significantly increased with age in both sexes of Dunkin Hartley guinea pigs, and

monocytes and eosinophils were significantly increased with age in females. However, basophils were negatively correlated with age in female Weiser-Maples guinea pigs.[126]

In the current study, Foa-Kurloff cells were not correlated with age, and there was no significant difference in the number of cells between males and females. Foa-Kurloff cells are a type of estradiol-dependent white blood cell unique to guinea pigs that contain a large granular intracytoplasmic inclusion. Although the function of these cells is unknown, they are thought to have natural killer cell activity and protect the fetus during pregnancy. Foa-Kurloff cells are commonly associated with pregnancy in older females and are reported to rarely be seen in young animals or males.[136] However, higher numbers of Foa-Kurloff cells were reported in male strain 13 guinea pigs than females.[127] Other studies did not report numbers of Foa-Kurloff cells.[126,135] As this cell type is not recognized in automated hematology analyzers, manual differential leukocyte counts are particularly important for accurate white blood cell counts in guinea pigs. At this time, our white blood cell counts may be influenced by the lack of manual blood film evaluations in several guinea pigs. WBC differential counts will be completed for these guinea pigs at a later date.

Numerous age- and sex-related differences were also found in serum biochemistry parameters of Dunkin Hartley guinea pigs. Glucose was positively correlated in male Dunkin Hartley guinea pigs, but negatively correlated in strain 13 males. Additionally, our reference intervals for glucose were much higher than those of strain 13 and Weiser-Maples guinea pigs,[126,127] which may be due to variations in diet or fasting status. Animals were not fasted prior to blood collection in the current study. As fasting has been shown to affect numerous clinical pathology parameters in rats,[138] it may also influence similar parameters in guinea pigs.

Similar to other strains, creatinine levels were higher in males, and BUN, creatinine, and calcium were positively correlated with age.[126,127] An increase in these parameters may be associated with the development of renal disease. Spontaneous renal lesions, such as nephrosclerosis, are a common incidental finding in guinea pigs that may result in renal insufficiency. Total protein was also significantly increased with age in males, particularly due to an increase in globulin. However, females had higher total protein levels overall due to increased albumin. Similar age-related increases in total protein were observed in Weiser-Maples guinea pigs,[126] but not strain 13 guinea pigs.[127]

ALP and phosphorous decreased with age, likely due to the decline in bone growth as animals reached skeletal maturity. In the guinea pig, bone growth ceases by 4 months of age.[113] While ALT, AST, and GGT were correlated with age in Dunkin Hartley guinea pigs, this was not observed in other strains.[126,127] Interestingly, ALT was positively correlated with age in male Dunkin Hartleys, but negatively correlated with age in females. This resulted in significantly higher levels of ALT in males compared to females. Male strain 13 guinea pigs were also noted to have higher ALT than females. Increased levels of ALT, AST, and GGT may be indicative of hepatocellular disease.

Female Dunkin Hartleys had an age-related increase in cholesterol, which resulted in significantly higher levels compared to males. In Weiser-Maples guinea pigs, cholesterol was increased with age in males rather than females.[126] Dunkin Hartley females also had an age-associated increase in iron, but there was no significant difference in total levels between males and females. Males had significantly increased sodium and bicarbonate levels compared to females. Additionally, bicarbonate was positively correlated with age in males, which led to a negative age correlation in anion gap. This differs from Weiser-Maples guinea pigs where

sodium was positively correlated with age in females but not males.[126] Bicarbonate and iron were not evaluated in other guinea pig studies.

In addition to strain, age, and sex, blood parameters may be affected by other factors that should be considered when applying these reference intervals to other animals. For example, all guinea pigs included in this study were anesthetized with isoflurane for blood collection. As blood collection is challenging in this species, anesthesia is often necessary to collect large blood volumes to minimize trauma and stressful handling. Isoflurane has been shown to increase white blood cells and liver enzymes and decrease red blood cells and plasma proteins in Dunkin Hartley guinea pigs. Additionally, this study included data from blood collected from the cranial vena cava, as well as directly from the heart. In rodents, hematologic and biochemical parameters can vary when blood is collected from different anatomical locations.[140–145] It should also be noted that all guinea pigs in this study were housed at an elevation of approximately 5,000 ft, which may impact RBC parameters. Humans at high altitude have been found to have increased erythropoietin and hemoglobin levels, as well as increased red blood cell volume.[146]

In conclusion, this study found several important differences in hematologic and biochemical parameters of Dunkin Hartley guinea pigs based on age and sex. Additionally, our results showed many differences between Dunkin Hartley and other strains of guinea pigs, which further emphasizes the need for strain-specific reference intervals. Establishing these differences provides valuable insight into their physiology to better evaluate diagnostics and experimental results.

CHAPTER III.

EVALUATION OF ELECTROACUPUNCTURE FOR SYMPTOM MODIFICATION IN A RODENT MODEL OF SPONTANEOUS OSTEOARTHRITIS[2]

1. Introduction

Osteoarthritis (OA) is the most common form of arthritis, affecting nearly 242 million people throughout the world.[1] OA, particularly of the hip and knee, is a leading contributor of chronic pain and disability, with many individuals reporting symptoms that limit their daily activities.[2,8] Additionally, the estimated total cost among American adults with arthritis in 2013 was $303.5 billion due to medical expenditures and lost earnings.[7] For those suffering with chronic discomfort, decreased mobility, high medical costs, and lost wages, OA can effectively diminish individuals' quality of life.[2]

Despite decades of research, there are no known treatments that prevent, slow, or reverse the progression of OA.[147] Treatment is focused on symptom modification with exercise, weight loss, and pain-relieving pharmacologic agents.[148] Acetaminophen and non-steroidal anti-inflammatory drugs are typically recommended as first-line treatments for OA management, with opioids recommended for refractory pain.[57] However, these drugs have limited efficacy in some individuals and are associated with numerous side effects that prohibit their use, particularly in

[2] A version of this manuscript will be submitted to Acupuncture in Medicine: **Personett AP, Afzali MF, Martinez RB, Culver LA, Leavell SE, Timkovich A, Sanford JL, Story MR, Santangelo KS.** 2020. Evaluation of Electroacupuncture for Symptom Modification in a Rodent Model of Spontaneous Osteoarthritis. Acupunct Med.

the elderly and patients with comorbidities.[149] With the projected increase of OA in an aging population,[8] there is a critical need for more efficacious treatments.

When faced with the frustration of chronic pain and limited mobility due to OA, many individuals have turned to acupuncture as a complementary therapy. Acupuncture is the traditional Chinese practice of needle insertion into the skin and underlying tissue with the goal of alleviating pain, tension, or stress.[72] Although acupuncture first started in primitive China, it is becoming increasingly accepted in Western culture as an alternative therapy for pain treatment.[73]

Electroacupuncture is a modern modification of traditional acupuncture that combines needling with electrical stimulation. In clinical trials, electroacupuncture has been reported to effectively alleviate clinical signs of patients with OA.[150–152] In a meta-analysis review, electroacupuncture was determined to be more effective than pharmacological treatment and manual acupuncture in reducing pain intensity and improving physical function in patients with OA. Additionally, there were no serious side effects with electroacupuncture, whereas nausea, abdominal distension, and constipation were reported with other pharmacological treatments.[98] Unfortunately, the efficacy of electroacupuncture in managing OA pain still remains uncertain, as much of the clinical trial evidence would benefit from improved study design.[96] Therefore, comprehensive and well-controlled studies in animal models are necessary to validate the use of electroacupuncture in clinical patients. Evidence for its efficacy would allow the medical community to provide better therapeutic recommendations for individuals suffering from this disease.

Electroacupuncture has been shown to have analgesic and anti-inflammatory effects in various laboratory models of induced arthritis. Rodent models of complete Freund's adjuvant-induced inflammation found that electroacupuncture decreased hyperalgesia,[153] arthritis index

scores, paw volume, and inflammatory mediators within the joint.[154,155] Additionally, electroacupuncture provided significant analgesic effects[156] and decreased knee joint histology scores in rat models of induced arthritis.[93,156,157] However, these models may not be applicable to primary OA that is seen in humans, and there are no reported studies to date investigating the efficacy of electroacupuncture in naturally-occurring models of OA.

The Dunkin Hartley guinea pig is an established, natural disease model of OA with primary knee pathology similar to humans.[106] The joint pathology of the guinea pig and human is both age-related, bilaterally symmetric, and susceptible to similar risk factors such as weight gain.[113] The purpose of this study was to evaluate electroacupuncture in the guinea pig model of spontaneous OA such that objective conclusions regarding its efficacy for symptom modification could be drawn. We hypothesized that guinea pigs treated with electroacupuncture would have improved gait and increased mobility compared to control guinea pigs. If found to be effective, electroacupuncture could be a low-risk, cost-effective therapy to greatly improve quality of life for individuals with OA.

2. Materials and Methods

2.1 Animals

Ten 12-month-old, male Dunkin Hartley guinea pigs (Charles River Laboratories, Wilmington, MA) were used in this study. All guinea pigs were singly-housed in solid bottom cages with corn cob bedding. Red huts and hay cubes were provided for enrichment. Animals were housed at a 12-12h light-dark cycle, 20 – 26° C temperature, and 30 – 70% humidity. Standard laboratory guinea pig chow and filter-sterilized water were provided *ad libitum*. All procedures were conducted in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the university's Institutional Animal Care and Use Committee.

2.2 Sample size calculation

Group size and power were determined using the statistical software at www.stat.uiowa.edu/~rlenth/Power. Based upon previous work,[122] stride length collected via treadmill-based gait analysis was selected as the principle outcome. Using a within group error of 0.5 and a detectable contrast of 0.5 in a linear regression model, power associated with Tukey/HSD post-test (alpha=0.5) was calculated as 0.87 with a sample size of 5 per experimental group.

2.3 Strategy

Dunkin Hartley guinea pigs, which characteristically have moderate OA at this age,[113] were randomly assigned to receive electroacupuncture (n = 5) or anesthesia only (n = 5). Animals were anesthetized with isoflurane, and treatments were performed three times weekly for three weeks. Gait and overhead enclosure monitoring were performed weekly. Animals were harvested two weeks after the final treatment session. Serum was collected for inflammatory biomarker testing, and whole knee joints were collected for histology and gene expression.

2.4 Electroacupuncture protocol

Each animal was anesthetized using 2 – 3% isoflurane with an oxygen flow rate of 2.0 L/min. Local acupuncture points at the level of, as well as proximal and distal to the stifle were chosen for pain modulation. Points were also chosen for autonomic nervous system support. Animals in the treatment group were placed in either left or right lateral recumbency, and acupuncture needles (SEIRIN J-type: No.1(0.16) x 30mm for electrical stimulation and SEIRIN J-15 No.01(0.14) x15mm for manual therapy) were placed at chosen points. Electroacupuncture was performed between Bladder (BL) 26-30, Gall Bladder (GB) 27-29 and Large Intestine (LI)

16-11. Electroacupuncture was set at low frequency with an amplitude just to a visible gentle twitch, for 5 minutes per side. In addition to electroacupuncture, manual acupuncture was also performed at the same time on LI 10, BL 23, 40, and 54, GB 30 and 34, and Stomach (ST) 36. The procedure was performed 3 times weekly for 3 weeks. There were no adverse reactions, and all animals recovered from anesthetic events without complication.

2.5 Open-field enclosure monitoring

Symptom modification was assessed using behavioral tracking software (ANY-maze, Stoelting Co., Wood Dale, IL) in a rectangular open-field apparatus. The apparatus consisted of a plastic black bin that provided contrast and measured 40" in length, 25" in width and 6" in height. Guinea pigs were randomly selected and placed in the center of the apparatus. They were allowed to explore the apparatus without interruption by the handlers (RBM, LAC, SEL, AT, & JLS). Each recording was performed for 10 minutes. All test trials were video–recorded, tracked, and analyzed with ANY-maze software (version 6.18). Guinea pigs were acclimated to the apparatus 3 weeks prior to baseline data collection, followed by weekly data collection after treatment. Acclimation and data collection were performed during the same time of day (10AM to 1PM) and by the same handlers. The final time point was collected 1 week after the last treatment.

2.6 Treadmill-based gait analysis

Gait analysis was performed using DigiGait™ (Mouse Specifics, Inc., Framingham, MA), a digital imaging treadmill system. Animals were acclimated to the system over 3 weeks. Training and data collection were performed during the same time period (10AM to 1PM) and involved the same handlers (RBM, LAC, SEL, AT, & JLS). All procedures were executed in the

dark (except for light emitted from the treadmill and computer screen), as due to their albinism, this is the environment in which the animals were most willing to run on the treadmill system. The order of which animals were utilized for gait analysis at each time point was randomly selected. Baseline gait analysis was performed the day immediately prior to the first electroacupuncture treatment. Subsequent data were collected 3 days after each treatment, with the final timepoint assessed one week following the last treatment.

2.7 Tissue harvest

Animals were harvested 2 weeks after the final electroacupuncture treatment. Animals were anesthetized with 3 – 5% isoflurane with an oxygen flow rate of 2 L/min. The thoracic cavity was opened, and blood was collected using a 20-gauge butterfly catheter via cardiac puncture. Collected blood was placed in a red top serum collection tube. After blood collection, the anesthetized animal was placed in a carbon dioxide chamber for euthanasia. Hind limbs were removed at the coxofemoral joint. The left limb was placed in 10% neutral buffered formalin for 48 hours and then transferred to PBS for storage. Prior to decalcification, right femur lengths were measured using calipers. Limbs were then transferred to a 12.5% solution of ethylenediaminetetraacetic acid (EDTA) at pH 7 for decalcification. EDTA was replaced twice weekly for 6 weeks.

2.8 Serum enzyme-linked immunosorbent assay for complement protein C3 and prostaglandin E2

Serum was collected at the time of euthanasia and stored at -80 °C until analysis (within 9 months of harvest). Serum protein analysis and quantification were conducted via a guinea pig-specific ELISA using C3 (Abcam, Cambridge, MA) and PGE_2 (ABclonal Science, Woburn,

MA) analysis kits in accordance with the manufacturers' instructions. The assays were conducted in technical triplicate.

2.9 Histologic grading of OA

After decalcification, coronal sections of the knee were paraffin embedded, and sections (5 μm) were taken from the center of the medial tibial plateau and stained with toluidine blue. Two blinded, independent evaluators (KSS and MFA) performed histological grading of four serial coronal sections using the OARSI-HISTOgp recommendations.[113] This semiquantitative histologic grading scheme is based on articular cartilage structure, proteoglycan content, cellularity, and tidemark integrity. Scores from each of the four anatomic locations were summed to obtain a total knee joint OA score for each guinea pig.

2.10 Quantitation of toluidine blue staining

The saturation intensity (calculated as lum) of proteoglycan stained positive with toluidine blue was determined in articular cartilage (superficial, middle, and deep zones) from the medial tibial plateaus using Image-Pro® (Media Cybernetics, Rockville, MD). All calculations were performed using identical thresholds across all photographs.

2.11 Gene expression of cartilage using Nanostring technology

Total RNA was extracted from cartilage and meniscus samples using a RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was quantified spectrophotometrically with a NanoDrop™ 2000 (ThermoFisher Scientific, Waltham, MA). A total of 250 ng of RNA, at a concentration of 20 ng/ml, was sent to the University of Arizona Genetics Core for analysis. Custom, guinea pig-specific probes were designed and synthesized by NanoString Technologies. Data analysis was

performed using nSolver™ software provided by NanoString Technologies. Results, reported as absolute transcript counts, were normalized to positive controls and housekeeping genes.

2.12 Statistical analyses

All data were subjected to normality testing via the Shapiro-Wilk test. For stride length, left and right hind paws were first evaluated individually. A paired t-test was performed to determine significant differences between limbs. No significant differences were observed, and the hind limbs were averaged together for each animal. For gait and enclosure monitoring data, the baseline measurements were subtracted from the final measurements to determine the change in parameter over time. Statistical differences were calculated by an unpaired t-test for normally distributed data or a Mann-Whitney test for non-normally distributed data. Statistical significance was set at $P < 0.05$. All statistical analyses were performed with Prism (version 8.0; GraphPad Software, La Jolla, CA).

3. Results

3.1 General description of study animals

All guinea pigs remained clinically healthy throughout the study. There was no significant difference in body weight between groups ($P = 0.3$). Mean total body weight was 1008.6 g (95% CI 891.9 – 1125 g) in the anesthesia group and 1085.2 g (95% CI 924.9 – 1245 g) in the electroacupuncture group. To ensure that differences in gait were not attributable to changes in skeletal properties, right femur lengths from all animals were measured. Femur lengths between the anesthesia (mean of 43.46 mm; 95% CI 41.34 – 45.58 mm) and electroacupuncture (mean of 43.83 mm; 95% CI 39.96 – 47.69 mm) groups were not significantly different ($P = 0.8$).

3.2 Overhead enclosure monitoring

Overhead enclosure monitoring was performed to measure changes in activity levels. Maximum (P = 0.03) and average (P = 0.005) speeds were significantly increased over time in the electroacupuncture group compared to the control group (Figure 3.1a and 3.1b). Electroacupuncture-treated guinea pigs had a significant increase in total distance traveled (P = 0.007) over time compared to control guinea pigs (Figure 3.1c). An example of a track plot displaying a guinea pig's path in the enclosure is shown in Figure 3.1d.

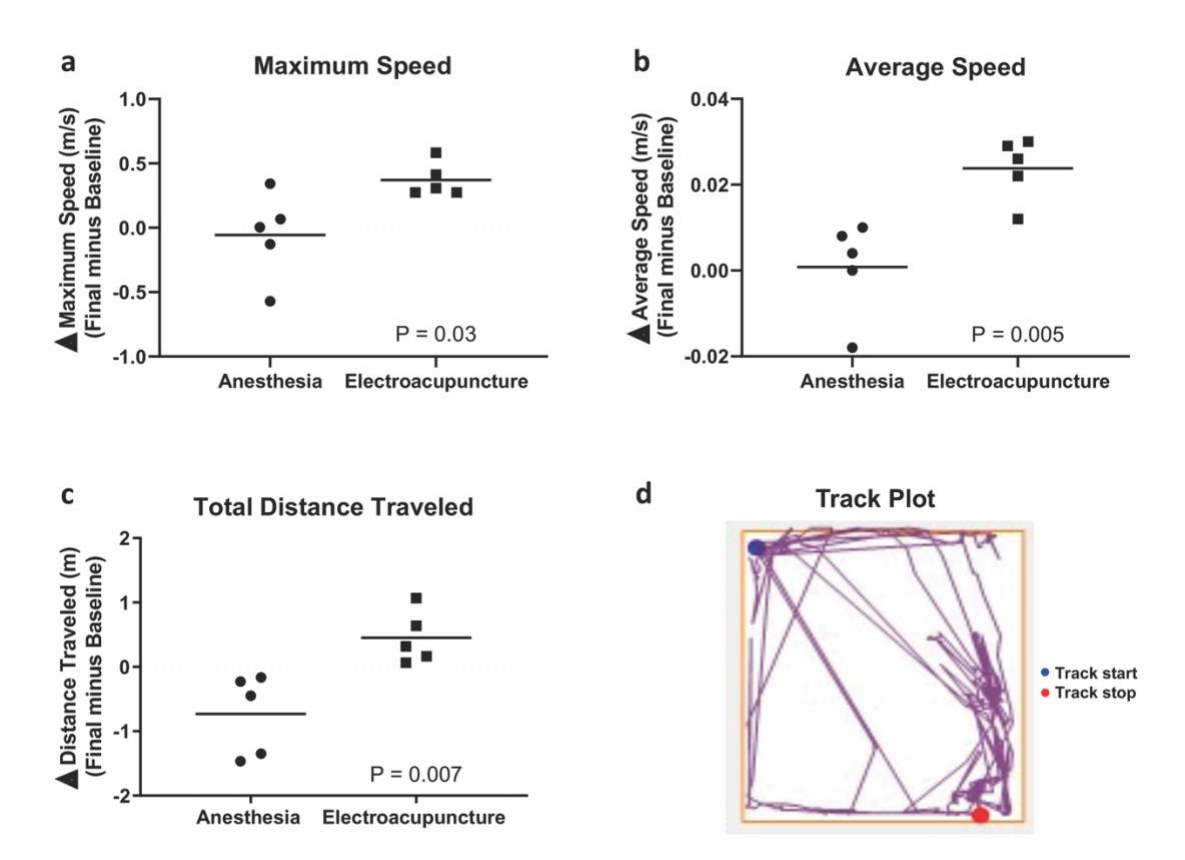

Figure 3.1. Enclosure monitoring results. Final minus baseline measurements of maximum speed (a), average speed (b), and total distance traveled (c) in anesthesia and electroacupuncture groups. Black lines represent mean values. P-values were determined by unpaired t-tests. A track plot is shown to demonstrate an electroacupuncture-treated guinea pig's route in the enclosure (d).

3.3 Treadmill-based gait analyses

Gait analysis was performed to evaluate movement-related knee joint nociception. Electroacupuncture-treated guinea pigs demonstrated a statistically (P = 0.01) longer stride length over time compared to control guinea pigs (Figure 3.2a). While not statistically significant (P = 0.1), the maximum speed reached trended toward an increase over time in electroacupuncture-treated guinea pigs compared to control guinea pigs (Figure 3.2b).

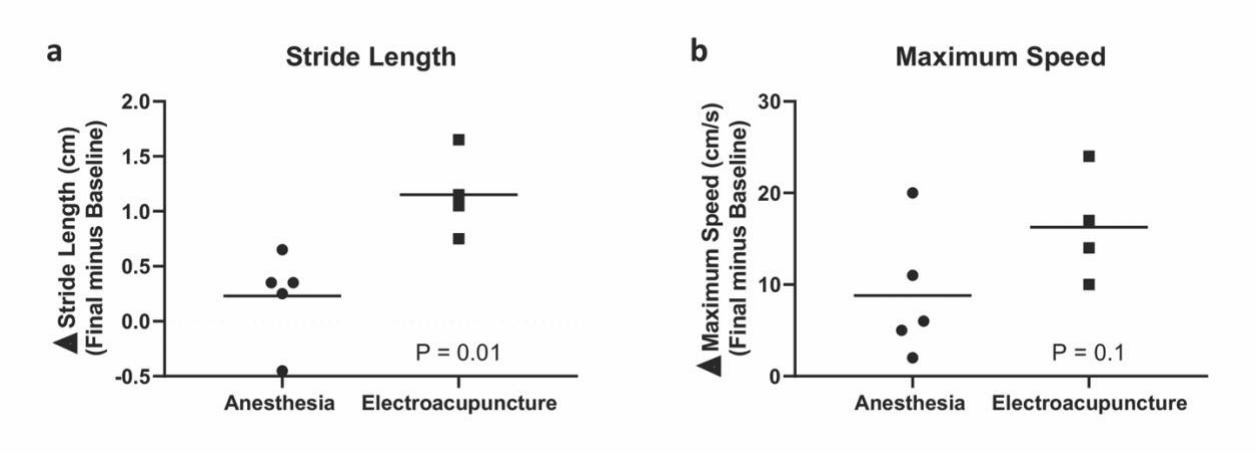

Figure 3.2. Gait analysis results. Final minus baseline measurements of stride length (a) and maximum speed (b) in anesthesia and electroacupuncture groups. Black lines represent mean values. P-values were determined by unpaired t-tests.

3.4 Serum inflammatory markers

C3 and PGE2 protein concentrations were evaluated to assess systemic inflammation. There were no significant differences between groups for either parameter. However, C3 trended toward a decrease in the electroacupuncture-treated group (P = 0.1) (Figure 3.3).

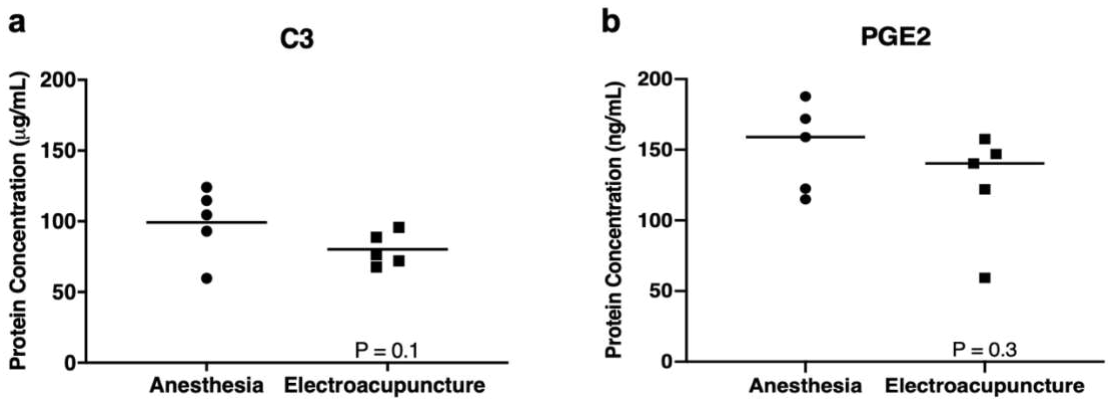

Figure 3.3 Serum protein concentrations of C3 (a) and PGE2 (b) in anesthesia and electroacupuncture groups. Black lines represent mean values. P-values were determined by unpaired t-tests.

3.5 Histologic assessment of OA

To evaluate articular cartilage abnormalities, histological assessment of joints was performed using the OARSI grading scheme for guinea pigs.[113] Representative lesions from each treatment group are shown in Figure 3.4a and 3.4b. There was no significant difference (P = 0.8) in OARSI scores between treatment groups (Figure 3.4c). Proteoglycan loss with hypocellularity and regions of chondrocyte clustering were noted in all animals, regardless of treatment group. These lesions were expected in Dunkin Hartley guinea pigs of this age.[113]

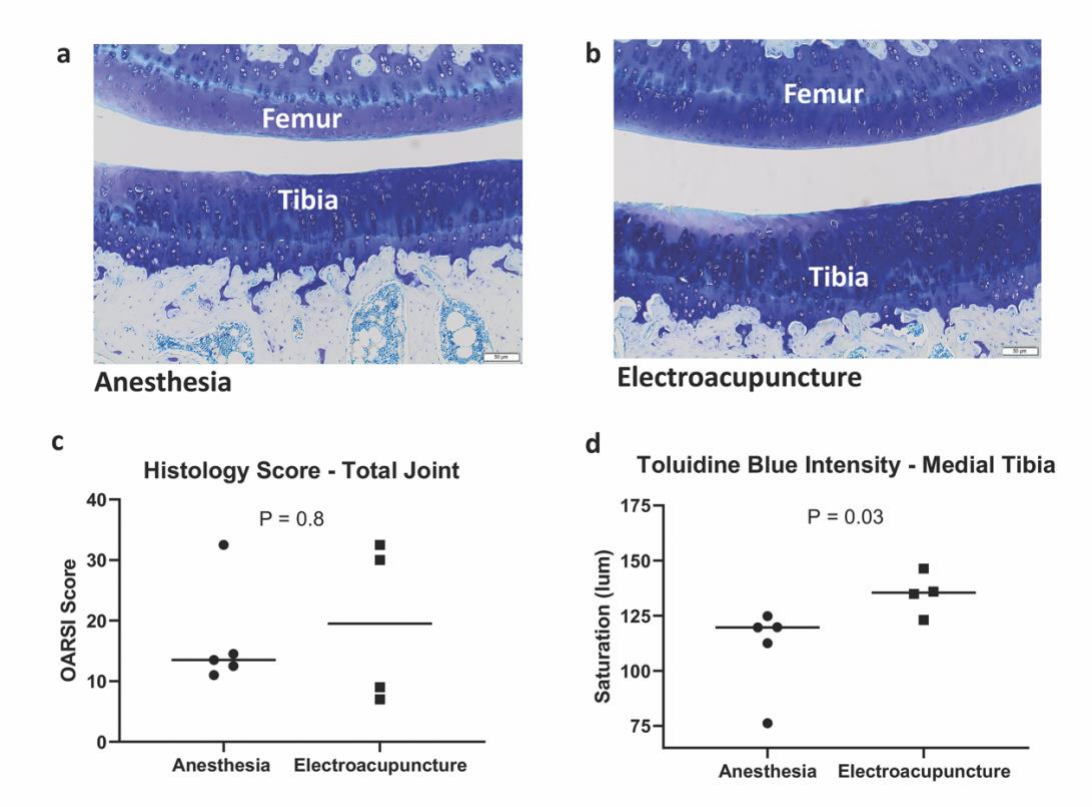

Figure 3.4. Histology results. Representative photomicrographs of toluidine blue-stained sections from medial compartments of knee joints of anesthesia- (a) and electroacupuncture-treated guinea pigs (b). Comparison of total joint histology scores (c) and toluidine blue saturation intensity (d) between anesthesia and electroacupuncture groups. Black lines represent median values. P-values were determined by non-parametric Mann-Whitney tests.

3.6 *Quantitative toluidine blue staining*

During grading of knee joints, dark proteoglycan staining was noted in the electroacupuncture group (Figure 3.4), which was confirmed by significantly higher saturation (lum). The electroacupuncture group had significantly (P = 0.03) increased saturation of toluidine blue compared to the anesthesia group (Figure 3.4d).

3.7 Cartilage gene expression

As the mechanism of action of electroacupuncture is still relatively unknown, we were interested to see if there were changes in gene expression within the knee cartilage with electroacupuncture treatment. Numerous genes involved in cartilage structure were upregulated in the electroacupuncture group (Figure 3.5). Compared to the control group, the electroacupuncture treated animals had higher expression of collagen type II alpha 1 chain (COL2A1) (P = 0.01), fibroblast growth factor 18 (FGF18) (P = 0.03), tissue inhibitor of metalloproteinases 1 (TIMP1) (P = 0.04), transforming growth factor β 1 (TGFβ1) (P = 0.01), and inducible nitric oxide synthase (iNOS) (P = 0.02). Additionally, the electroacupuncture-treated group had higher expression of antioxidant genes, including nuclear factor erythroid-2-related factor 2 (NRF2) (P = 0.05), peroxiredoxin 1 (PRDX1) (P = 0.06), and superoxide dismutase 2 (SOD2) (P = 0.02).

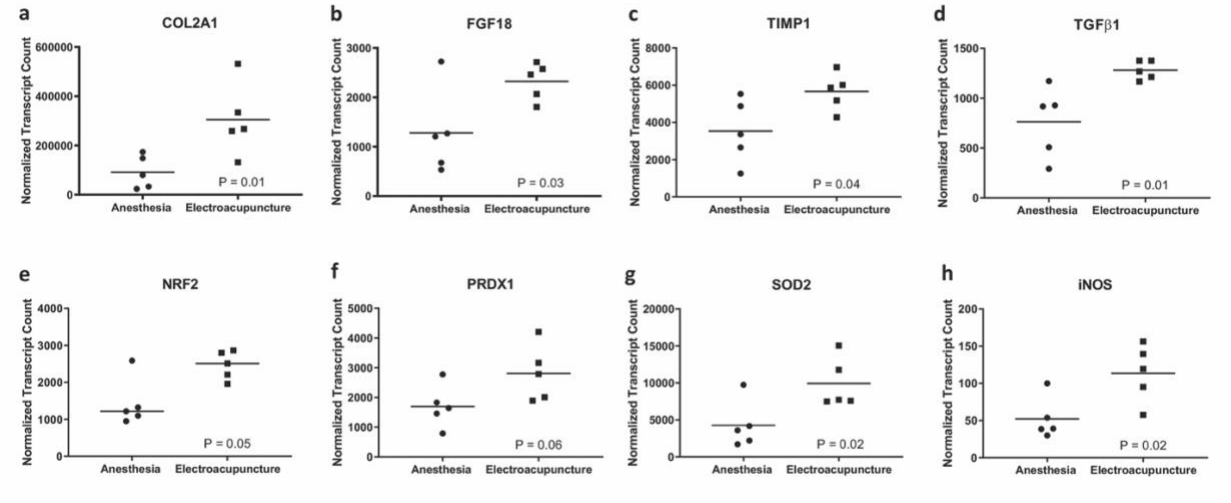

Figure 3.5. Gene expression results. Normalized mRNA counts for COL2A1 (a), FGF18 (b), TGFβ1 (c), TIMP1 (d), NRF2 (e), PRDX1 (f), and SOD2 (g) proteins in articular cartilage of anesthesia and electroacupuncture groups. Black lines represent mean values for normally distributed data (COL2A1, FGF18, TGFβ1, TIMP1, PRDX1, SOD2) and median values for non-normally distributed data (NRF2). P-values were determined by unpaired t-tests for normally distributed data and non-parametric Mann-Whitney tests for non-normally distributed data.

4. Discussion

The goal of the present study was to evaluate the efficacy of electroacupuncture for symptom modification using the Dunkin Hartley guinea pig model of spontaneous OA. The results of our study provide convincing evidence that electroacupuncture relieves movement-related nociception in this rodent model. In open-field tests, electroacupuncture-treated guinea pigs demonstrated an increase in total distance traveled, average speed, and maximum speed over time compared to control guinea pigs. Additionally, gait analysis showed that electroacupuncture-treated guinea pigs exhibited increased stride length and maximum speed over time compared to control guinea pigs.

Despite improvements in gait and mobility, electroacupuncture did not appear to improve the OA pathology of the knee joint, as total joint OA scores did not significantly differ between groups. This differs from other studies in rodent models where knee cartilage structure was improved with electroacupuncture compared to control groups.[93,156,157] A study using an anterior cruciate ligament transection model in rats found significantly decreased Mankin scores in the electroacupuncture-treated group compared to the control group when treatment was started 1 week post-surgery.[157] In a rat monosodium iodoacetate-induced model, electroacupuncture was only effective at decreasing joint scores when performed immediately post-injection. Delayed electroacupuncture treatments, starting at 7 or 14 days post-injection, did not improve joint scores.[156] A study inducing OA in rats via ovariectomy also saw differences in the treatment group when electroacupuncture was performed starting the day of surgery.[93] These studies provide evidence that electroacupuncture can mitigate OA if initiated at its onset, or shortly thereafter. In our current study, guinea pigs received electroacupuncture treatment at 12 months of age, a time when moderate OA has already developed in this model.[106] Additionally, these

studies utilized induced rat models of OA which may not adequately recapitulate effects on the natural OA disease process seen in guinea pigs and humans. Lastly, electroacupuncture was performed 5 – 6 days per week for as long as 12 weeks in these studies compared to 3 times weekly for 3 weeks in the current study. More frequent electroacupuncture treatments over a longer duration may be needed in the guinea pig model to see improvement in the joint structure.

Although there was no difference in joint scores between groups, our changes in systemic inflammatory mediators and cartilage gene expression still suggest a potential effect of electroacupuncture on OA disease modification. C3, an acute phase protein and biomarker of systemic inflammation in guinea pigs,[158] trended toward a decrease in the electroacupuncture group. Human studies have shown increases in serum C-reactive protein to be present in knee osteoarthritis and predictive of disease progression.[159] PGE2 is believed to mediate much of the inflammatory pain response,[160] and electroacupuncture has been shown to decrease pain behaviors and PGE2 in various rat models of pain.[161,162] However, there was no difference in levels of serum PGE2 between electroacupuncture-treated and control guinea pigs. In previous studies, tissues for PGE2 analysis were collected within 1 day of electroacupuncture treatment, compared to 2 weeks after treatment in the current study. Therefore, electroacupuncture may only have a short-term effect on systemic PGE2 levels.

Additionally, the knee cartilage gene expression profiles between electroacupuncture-treated and control guinea pigs differed. In general, expression of structural and antioxidant genes was increased in the electroacupuncture group. Since osteoarthritis is characterized by articular cartilage degeneration, upregulation of structural genes may be an indicator of cartilage tissue repair. Transcripts for type II collagen, a major constituent of the extracellular matrix of articular cartilage,[163] were significantly increased in the electroacupuncture group. Gene

transcripts of TIMP1 were also upregulated by electroacupuncture. TIMP1 is a protein that specifically inhibits matrix metalloproteinase 13 (MMP13), a protease associated with cartilage destruction and OA development.[164] Additionally, TGFβ1 and FGF18 transcripts were increased in the electroacupuncture group. TGFβ1 and FGF18 are proteins that play an important role in regulating cell proliferation, differentiation, and apoptosis in various tissues.[165,166] TGFβ1 signaling promotes chondrocytes to synthesize type II collagen and proteoglycan to form cartilage tissue. Inhibition of this signaling pathway leads to chondrocyte terminal differentiation and the development of OA.[166] FGF18 is an important regulator of chondrogenesis and osteogenesis.[167] A study found an intra-articular injection of FGF18 attenuated cartilage degradation, increased type II collagen deposition, and suppressed MMP13 expression in a rat post-traumatic osteoarthritis model.[168]

Antioxidant gene transcripts were also significantly increased (SOD2) or trended toward an increase (NRF2, PRDX1) in the electroacupuncture group compared to the control group. It has been established that cellular oxidative stress is a key contributor to the pathogenesis of OA.[169] Oxidative stress leads to accumulation of reactive oxygen species, causing abnormalities in the cartilage and bone metabolism and repair mechanisms.[169] NRF2 is a transcription factor that serves as a master regulator of cellular redox reactions[169] and has been demonstrated to be protective to cartilage and other joint tissues in post-traumatic OA.[170] SOD2 and PRDX1 are also important players in the reduction and detoxification of reactive oxygen species.[169,171] SOD2 has previously been found to be downregulated in OA cartilage of both humans[172] and guinea pigs.[173]

Interestingly, iNOS gene transcripts were also increased by electroacupuncture. iNOS is known to be upregulated in OA chondrocytes, leading to excess nitric oxide (NO) production.[174]

NO is long-believed to be catabolic and responsible for perpetuating the OA disease process; however, more recent evidence has suggested an anti-inflammatory role of NO in chondrocytes.[175] Additionally, NO is thought to have both positive and negative effects on pain perception.[176] Therefore, the role of NO in OA is not straightforward and requires further investigation.

Another intriguing finding of the study was that joints of electroacupuncture-treated guinea pigs had significantly increased intensity of toluidine blue staining compared to the control group. Toluidine blue is a cationic dye that is frequently used to stain proteoglycans and glycosaminoglycans due to its high affinity for sulfate groups.[177] Therefore, it is plausible that electroacupuncture increased the proteoglycan content within the joints. Aggrecan is the primary proteoglycan present in cartilage;[178] however, our gene expression data determined that aggrecan was not significantly increased in the electroacupuncture group (data not shown). Electroacupuncture may have influenced the production of smaller proteoglycans such as biglycan, decorin, and/or fibromodulin that are also important in maintaining the structure of cartilage. These small proteoglycans have been found to be degraded in OA cartilage and are glycosylated differently from those in normal cartilage.[179]

A general limitation of acupuncture research is the lack of standardization among treatment protocols. Point selection, needling depth, needle manipulation, and duration and frequency of treatments are all factors that widely vary in clinical practice and research studies. Thus, more comparative studies are needed to determine the optimal protocol for certain conditions. In the current study, electroacupuncture was performed 3 times weekly for 3 weeks. Perhaps if electroacupuncture treatments were performed daily and/or over a longer time period, we would have seen additional changes in molecular analyses and improvements in knee joint

structures. Another potential variable of the current study was our use of anesthesia. Anesthesia was necessary for proper needle placement in guinea pigs, but it is not typically used for electroacupuncture treatment of humans. Although unknown at this time, there may be potential effects of anesthesia on the mechanism of action of electroacupuncture.

Our results from this short-term study provide evidence that electroacupuncture had a positive effect on the modification of clinical signs, but not structural changes, in the Dunkin Hartley guinea pig model of OA. Based on our gene expression data, the upregulation of structural and antioxidant proteins may provide insight into the effect of electroacupuncture on OA repair mechanisms that could be seen at a later time point. Further investigations into mechanistic pathways of long-term disease modification are still needed to explain the efficacy of electroacupuncture in this rodent model.

CHAPTER IV.

EVALUATION OF MANUAL ACUPUNCTURE FOR SYMPTOM MODIFICATION IN A RODENT MODEL OF SPONTANEOUS OSTEOARTHRITIS[3]

1. Introduction

Osteoarthritis (OA) is a degenerative joint disease affecting nearly 250 million people worldwide, including 50 million adults in the United States.[8] With an aging population, the prevalence of OA is projected to increase to nearly 80 million US adults by 2040.[8] Due to the primary symptom of pain, OA is a major cause of physical disability and decreased quality of life throughout the world.[3,180]

Unfortunately, there are no approved drugs that prevent, halt, or reverse the progression of OA.[147] With no known cure, current treatment strategies are focused on reducing symptoms of joint pain and returning to normal functional capabilities. Common first-line treatments include exercise, weight loss, and pharmacologic pain control with paracetamol and NSAIDs.[2] However, these drugs are not effective for all individuals and have serious safety concerns, particularly in regard to gastrointestinal and cardiovascular side effects.[181] Therefore, safe and effective treatments for the management of OA pain are still needed.

[3] A version of this manuscript will be submitted to Acupuncture in Medicine: **Personett AP, Afzali MF, Martinez RB, Bork SB, Culver LA, Leavell SE, Sanford JL, Seebart CA, Timkovich A, Story MR, Santangelo KS.** 2020. Evaluation of Manual Acupuncture for Symptom Modification in a Rodent Model of Spontaneous Osteoarthritis. Acupunct Med.

Many patients are seeking alternative non-pharmacological treatments, such as acupuncture, for pain relief.[96] Acupuncture is a traditional Chinese practice of medicine that involves the insertion of thin needles into the skin to stimulate specific points on the body. The needles may be stimulated by hand (manual acupuncture) or small amounts of electrical current (electroacupuncture). Numerous studies have reported acupuncture to be beneficial for treating OA pain, although methodological flaws are often noted.[96,182,183] Because of these study limitations, there still remains insufficient evidence to provide a general recommendation for acupuncture.[57,96] Acupuncture was recommended by Osteoarthritis Research Society International,[53] but only conditionally recommended by the American College of Rheumatology.[54] Additionally, the American Academy of Orthopaedic Surgeons strongly recommended against acupuncture due to "lack of efficacy".[52] Therefore, further laboratory investigations using animal models are needed to justify the effectiveness of acupuncture for use in clinical patients with OA.

The Dunkin Hartley guinea pig develops age-related, spontaneous knee OA with histological changes similar to idiopathic human OA. The majority of laboratory studies evaluating acupuncture for OA have utilized animal models of acute inflammation[156] or surgically-induced OA,[92,93,157] which may not adequately represent the natural, idiopathic form of OA most commonly diagnosed in humans. Further, although manual acupuncture is commonly used in practice, most acupuncture research evaluates electroacupuncture due to its quantifiable frequency, intensity, and duration of needle stimulation.[184] While our previous study found electroacupuncture to improve symptoms in the guinea pig model of OA, manual acupuncture may produce different effects. Therefore, the purpose of this study was to evaluate manual acupuncture as a therapy for knee OA using the Dunkin Hartley guinea pig model of

spontaneous OA. We hypothesized that guinea pigs treated with manual acupuncture would have improved gait and increased mobility compared to sham-treated and untreated control guinea pigs.

2. Methods

2.1 Animals

This study was approached in two phases: one to evaluate efficacy of manual acupuncture using a treadmill-based gait analysis system; and the second to determine whether acupuncture resulted in improvement in voluntary movement parameters. First, 23 male Dunkin Hartley guinea pigs (age, 9 – 15 months) purchased from Charles River Laboratories (Wilmington, MA) were used in this aspect of this study. After completion, a second study was performed using 18 male Dunkin Hartley guinea pigs (age, 11 months). All guinea pigs were singly-housed and provided a red hut and hay cubes for enrichment. Standard laboratory guinea pig chow and filter-sterilized water were provided ad libitum. Animals were housed with a 12-12h light-dark cycle, 20 – 26° C temperature, and 30 – 70% humidity. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the university's Institutional Animal Care and Use Committee.

2.2 Sample size calculation

Group size and power were determined using the statistical software available at the following website: www.stat.uiowa.edu/~rlenth/Power. Based upon previous work,[122] stride length from treadmill-based gait analysis was selected as the representative outcome measure. Using a within group error of 0.5 and a detectable contrast of 0.5 in a linear regression model,

power associated with Tukey/HSD post-test (alpha=0.5) was calculated as 0.87 with a sample size of 5 per experimental group.

2.3 Strategy

In the initial study, animals were originally assigned to the following treatment groups: anesthesia, only; sheath tapping; off-point acupuncture; or acupuncture for knee OA. As there were no significant differences between the anesthesia and sheath tapping groups, these groups were combined into one control group for statistical analysis at the end of the study. Final groups were as follows: anesthesia (n = 9); off-point acupuncture (n = 5); and acupuncture for knee OA (n = 9).

All animals were anesthetized with isoflurane, and acupuncture or control treatments were performed once weekly for 3 weeks. Gait analysis was performed 3 times weekly (24 h, 72 h, and 1 week post-treatment). Animals were harvested 1 week after the final treatment session. At harvest, whole knee joints were collected for histology.

After review of the gait analysis results, a second study was performed to further evaluate symptom modification using open-field enclosure monitoring. Guinea pigs were divided into three groups: anesthesia (n = 6); off-point acupuncture (n = 6); or acupuncture for knee OA (n = 6). Open-field enclosure monitoring was performed weekly. All animals were harvested 1 week after their final treatment. At harvest, blood was collected for complete blood count (CBC), serum biochemistry, and serum protein analyses, and whole knee joints were collected for histology.

2.4 Acupuncture protocol

Each animal was anesthetized using 2 – 3% isoflurane with an oxygen flow rate of 2.0 L/min. Local acupuncture points at the level of, as well as proximal and distal to the stifle were chosen for pain modulation. Points were also chosen for autonomic nervous system support. Animals in the treatment group were placed in either left or right lateral recumbency, and acupuncture needles (SEIRIN J-15 No.01(0.14) x15mm) were placed at chosen points. Manual acupuncture was performed on Bladder (BL) 11, 23, 40, and 54, Large Intestine (LI) 11, Gall Bladder (GB) 29, 30, 34, and 39, and Stomach (ST) 36 for 10 minutes. The animals were then placed on their opposite side, and manual acupuncture was performed on BL 11 and 40, GB34, and ST36 for 5 minutes. A needle sheath was tapped on these same acupuncture points in the sheath tapping control group. For those in the off-point acupuncture group, 2 needles were placed along the spine of the scapula, where there are no known acupuncture points, for 10 minutes. They were then placed on their opposite side, and the procedure was repeated for 5 minutes.

2.5 Treadmill-based gait analysis

In the first study, gait analysis was performed using the DigiGait™ (Mouse Specifics, Inc., Framingham, MA) treadmill system. Animals were acclimated to the treadmill over a period of 3 weeks. Baseline gait analysis was performed the day prior to acupuncture or control treatment. Further data collections were performed 24 h, 72 h, and 1 week after each treatment. The order in which animals were selected for each gait analysis was randomly selected. All training and data collections were performed during the same time period (10AM – 1PM) by the same handlers (RBM, LAC, AT, & JLS). Gait analysis was performed in the dark (except for light emitted from the treadmill and computer screen), as this is the environment in which the animals were most willing to run.

2.6 Open-field enclosure monitoring

For the second study, open-field enclosure monitoring was assessed using ANY-maze behavioral tracking software (version 6.18, Stoelting Co., Wood Dale, IL). The apparatus consisted of a circular plastic blue bin that measured 45" in diameter and 6" in height. A red hut was placed along the edge of the apparatus in the same location for every recording. The guinea pigs were randomly selected for each recording. They were placed in the center of the apparatus and allowed to move freely for 10 minutes. Animals were acclimated to the apparatus for 1 week prior to baseline data collection. Baseline recordings were performed the day prior to the first acupuncture or control treatment. Subsequent recordings were performed the day prior to and after each treatment. All recordings were performed within the same time period (10AM – 3PM) by the same handlers (ARP, SBB, CAS). For optimal video-recording, all recordings occurred in the dark (with the exception of light from the laptop and a wall-mounted LED light bar).

2.7 *Tissue harvest*

Guinea pigs were harvested 1 week after their final treatment session. All guinea pigs were anesthetized with 3 – 5% isoflurane and euthanized via exsanguination. The thoracic cavity was opened to expose the heart, and blood was collected via intracardiac puncture using a 20-gauge butterfly catheter. Both hind limbs were removed at the coxofemoral joint. The left hind limb was placed in 10% neutral buffered formalin for 48 hours. After fixation, the left femur length was measured using calipers. The limbs were then transferred to a 12.5% solution of ethylenediaminetetraacetic acid (EDTA) at pH 7 for decalcification, and EDTA was replaced 2 – 3 times weekly for 8 weeks.

2.8 Complete blood count, serum biochemistry, and serum protein measurement

In the second study, blood collected at harvest was placed in 0.5 mL EDTA microtubes for CBCs. CBCs were performed at the Colorado State University Clinical Pathology Laboratory using the Advia 120 hematology analyzer (Siemens, Munich, Germany). White blood cell differentials were performed manually on blood films. Additional blood was collected into red top serum collection tubes. After clotting, red top tubes were placed in a centrifuge at 5000x*g* for 15 minutes for serum collection. One serum aliquot was submitted to the Colorado State University Clinical Pathology Laboratory for serum biochemistry using the Roche Cobas 6000 (Basel, Switzerland). Remaining serum was aliquoted and stored at -80 °C for C3 and PGE_2 analyses. Serum protein quantification was performed using C3 (Abcam, Cambridge, MA) and PGE_2 (ABclonal Science, Woburn, MA) guinea pig-specific ELISA kits. All assays were conducted in technical triplicate and performed as indicated by the manufacturers' protocols.

2.9 Histologic grading of OA

After decalcification, coronal sections of the knee joint at the level of the medial tibial plateau were paraffin embedded and stained with toluidine blue. Two blinded evaluators (ARP and KSS) performed grading of the coronal sections according to OARSI-HISTOgp recommendations.[113] The medial and lateral tibial plateaus, as well as the medial and lateral femoral condyles, were each scored individually based on articular cartilage structure, proteoglycan content, cellularity, and tidemark integrity. Scores from each of the anatomic locations were summed to obtain a total knee joint OA score for each animal.

2.10 Statistical analyses

Normality was evaluated for all data using the Shapiro-Wilk test. Longitudinal data from gait analyses and enclosure monitoring were assessed by ordinary two-way ANOVA with

Tukey's multiple comparisons tests. All other statistical analyses were evaluated by ordinary one-way ANOVA followed by Tukey's multiple comparisons tests for normally distributed data or Kruskal-Wallis tests followed by Dunn's multiple comparisons tests for non-normally distributed data. A P-value of < 0.05 was determined to be significant. All statistics were performed using Prism (version 8.4.0; GraphPad Software, La Jolla, CA).

3. Results

3.1 General description of study animals

All animals were clinically healthy throughout both studies. There was no difference in final weights between the anesthesia, off-point, and acupuncture groups ($P = 0.2$). Mean body weight was 1040.2 g (95% CI 985.59 – 1094.7 g) in the anesthesia group, 1082.6 g (95% CI 978.25 – 1187.0 g) in the off-point group, and 1001.0 g (95% CI 959.72 – 1042.3 g) in the acupuncture group. Femur lengths of the anesthesia (mean 42.24 mm, 95% CI 41.61 – 42.88 mm), off-point (mean 41.63 mm, 40.53 – 42.73 mm), and acupuncture (mean 42.34 mm, 95% CI 41.30 – 43.37 mm) groups were similar ($P = 0.5$), indicating there were no changes in skeletal growth between animals.

3.2 Treadmill-based gait analyses

Movement-related joint nociception was evaluated at a constant speed using treadmill-based gait analysis. Stride length, defined as the distance between successive placement of the same paw, was selected as the main parameter of interest. There were no significant intra- or inter-group differences in stride length after each weekly treatment session (Figure 4.1a). Additionally, there were no significant differences between groups in the change in stride length over time (Figure 4.2b).

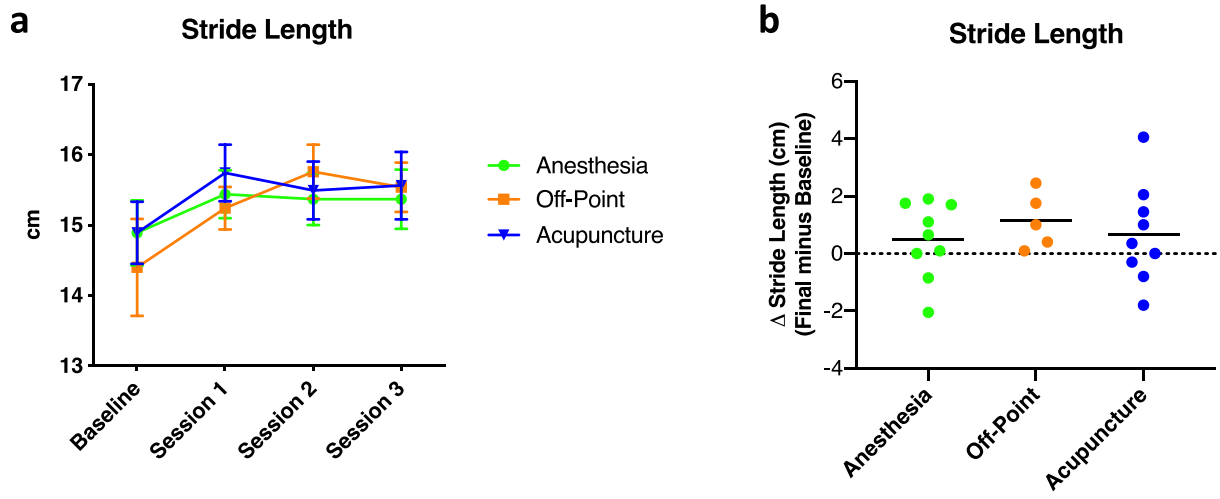

Figure 4.1. Gait analysis results. Average ± SEM stride length at baseline and after each treatment session in anesthesia, off-point, and acupuncture groups (a). Ordinary two-way ANOVA with Tukey's multiple comparisons showed no differences in stride length between time points or treatment groups. Final minus baseline measurements of stride length in anesthesia, off-point, and acupuncture groups (b). Black lines represent mean values. Ordinary one-way ANOVA determined no significant difference in the change in stride length between groups.

3.3 Overhead enclosure monitoring

Overhead enclosure monitoring was used to assess changes in voluntary mobility and behavior parameters between acupuncture-treated and control groups. There were no significant differences in parameters over time or between groups (Figure 4.2). Additionally, there was no significant difference between groups in the final minus baseline measurements of any parameter.

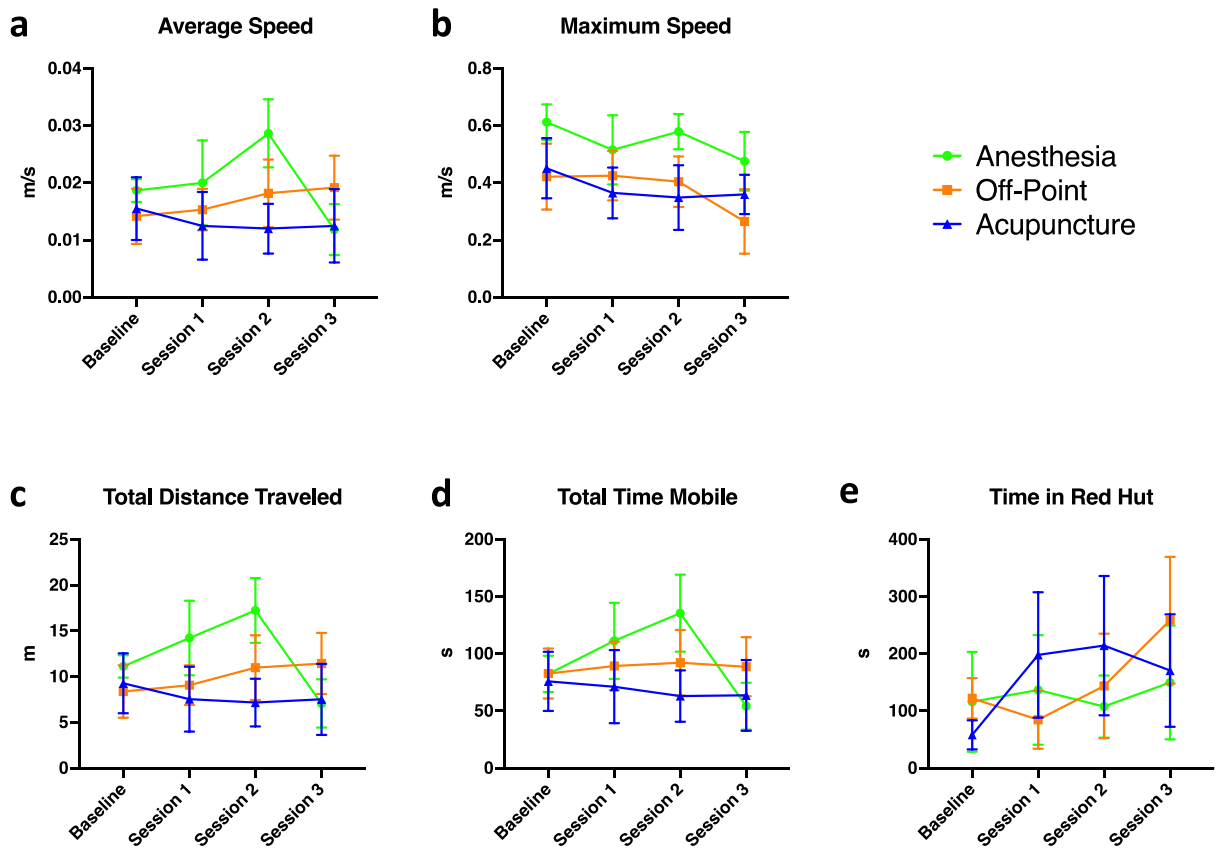

Figure 4.2. Longitudinal enclosure monitoring results. Average ± SEM average speed (a), maximum speed (b), total distance traveled (c), total time mobile (d), and time in red hut (e) in anesthesia, off-point, and acupuncture groups over time. Ordinary two-way ANOVA with Tukey's multiple comparisons tests determined no statistical differences (Figure 4.3).

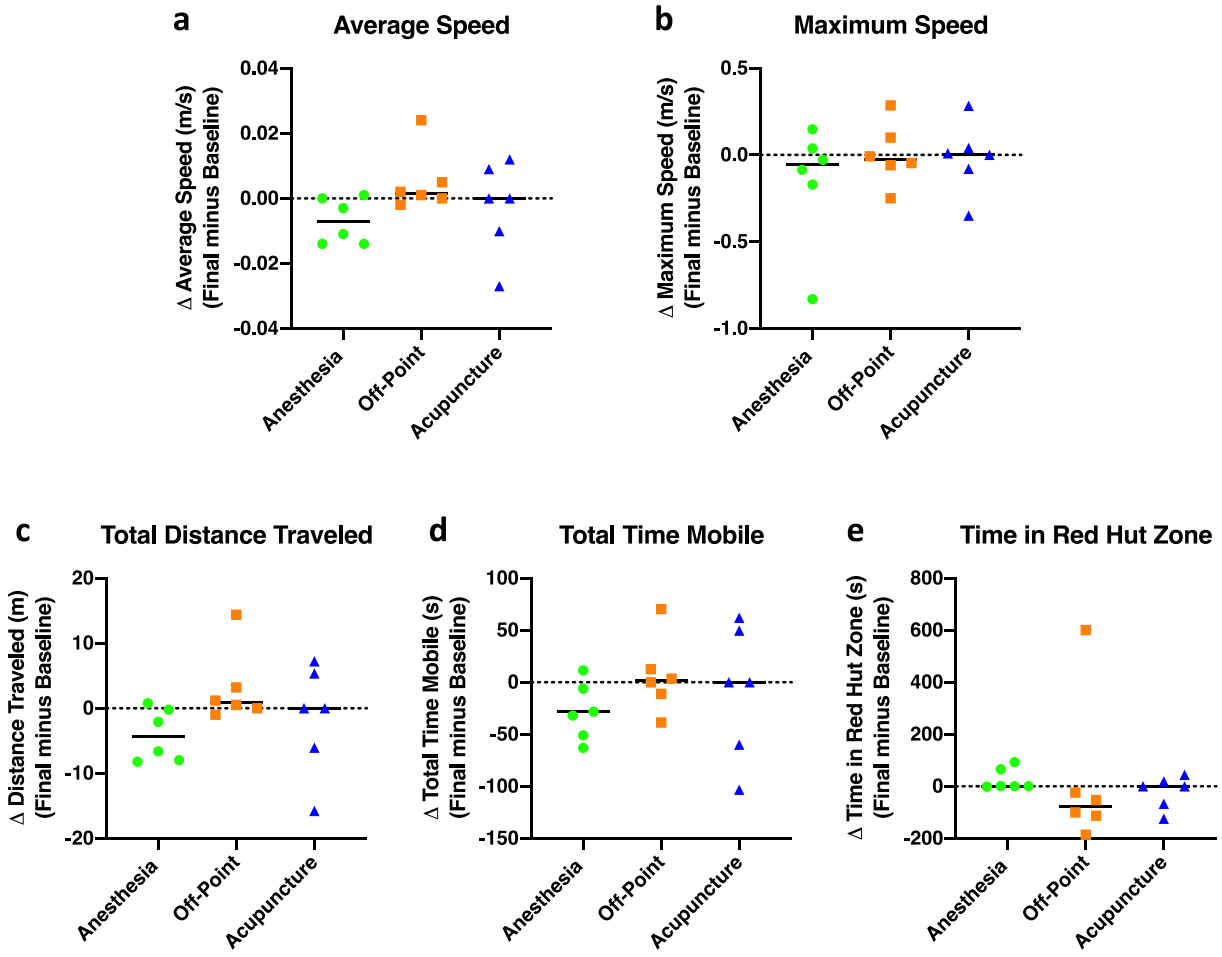

Figure 4.3. Change in enclosure monitoring parameters. Final minus baseline measurements of total distance traveled (a), average speed (b), maximum speed (c), total time mobile (d), and time in red hut (e) in anesthesia, off-point, and acupuncture groups. Black lines represent mean values for normally distributed data (time in red hut) and median values for non-normally distributed data (total distance traveled, average speed, maximum speed, total time mobile).

3.4 Complete blood count, serum biochemistry, and serum protein measurement

CBC, serum biochemistry, and serum protein concentrations of C3 and PGE2 were assessed to evaluate systemic inflammation. There were no significant differences in hematologic or biochemical parameters between groups (data not shown). Concentrations of C3 were significantly decreased in the acupuncture group compared to the anesthesia control group

(Figure 4.4a). PGE2 concentrations were significantly decreased in the acupuncture group compared to both anesthesia and off-point control groups (Figure 4.4b).

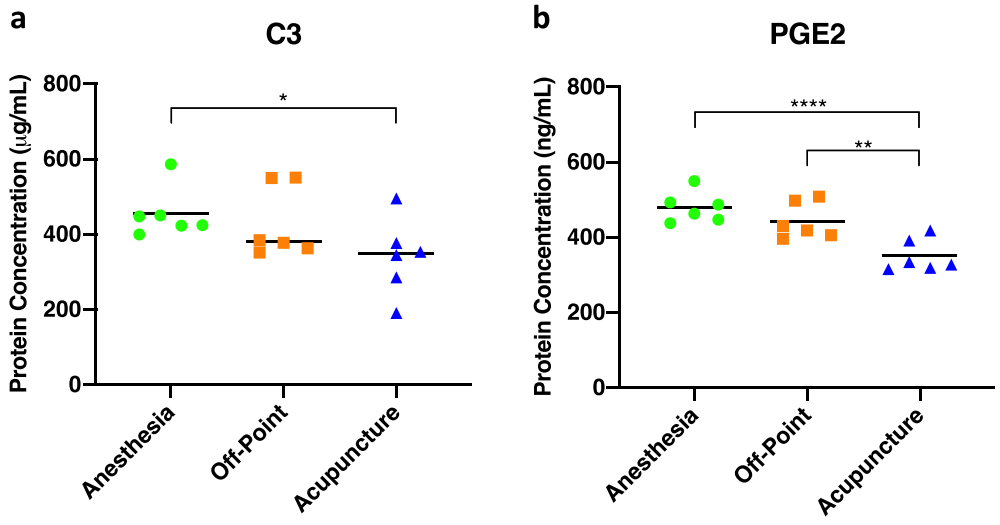

Figure 4.4. Serum protein concentrations of C3 (a) and PGE2 (b) in anesthesia, off-point, and acupuncture groups. Black lines represent mean values. *$P < 0.05$, **$P < 0.01$, ****$P < 0.001$

3.5 Histologic assessment of OA

Histologic assessment of the knee joint structure was performed according to the OARSI grading scheme. Groups from both studies were combined to evaluate differences in joint scores. Total joint scores, as well as scores separated by the medial and lateral joint compartments, are shown in Figure 4.5. While there were no significant differences between groups, there was a trend toward a decrease in OA scores of the acupuncture group compared to the control groups. Additionally, cartilage lesions appeared to differ in severity between groups, as shown in Figure 4.6. In the joints of the anesthesia and off-point groups, there are large areas of cartilage and proteoglycan loss, as well as regions of hypocellularity. In the acupuncture-treated joint, the articular cartilage surface is still intact with minimal proteoglycan loss and changes in cellularity.

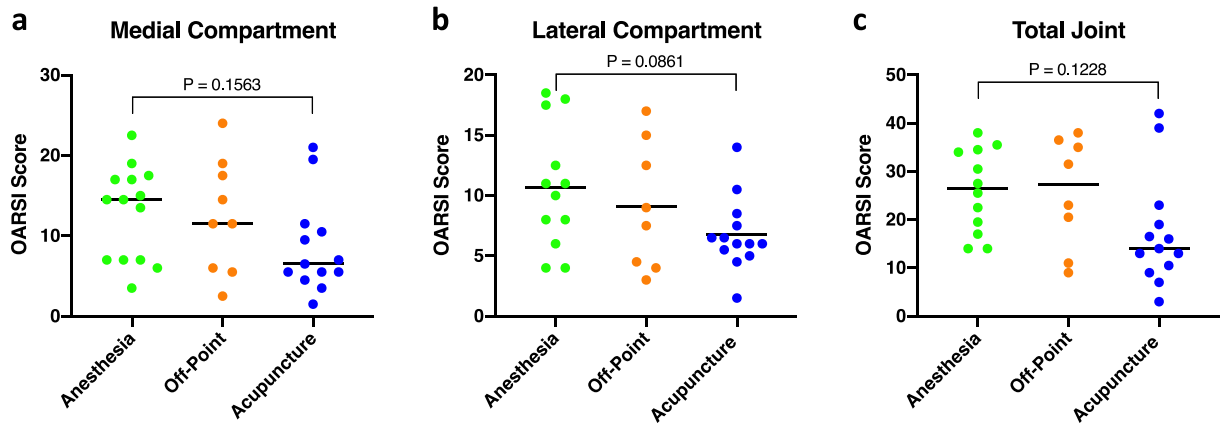

Figure 4.5. Histological scoring. OARSI histology scores of the medial compartment (a), lateral compartment (b), and total knee joint (c) from anesthesia, off-point, and acupuncture groups. Black lines represent mean values for normally distributed data (lateral compartment) and median values for non-normally distributed data (medial compartment, total joint). P-values were determined by an ordinary one-way ANOVA with Tukey's multiple comparisons for normally distributed data and nonparametric Kruskal Wallis test with Dunn's multiple comparisons for non-normally distributed data.

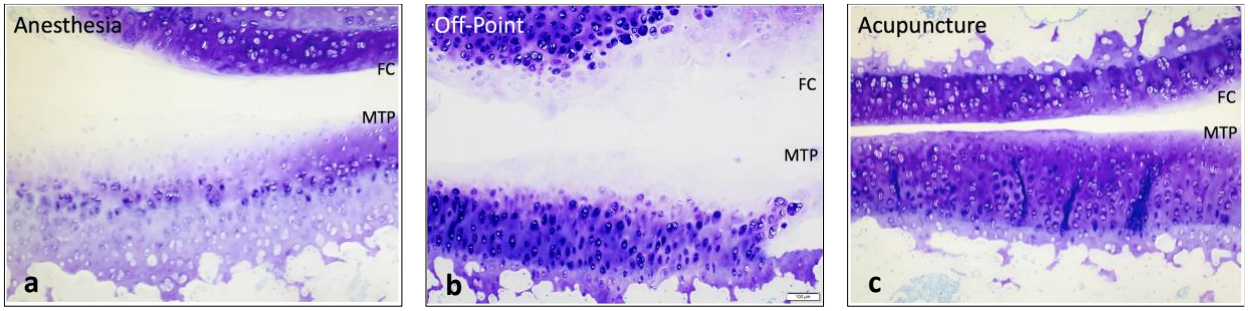

Figure 4.6. Histology images. Representative photomicrographs of toluidine blue-stained medial compartments of knee joints from guinea pigs in anesthesia (a), off-point (b), and manual acupuncture (c) groups. In knee joints of the anesthesia and off-point groups, there is cartilage loss extending into the middle zone, regional to diffuse proteoglycan loss, and regions of hypocellularity. In the acupuncture-treated joint, the articular cartilage surface is mildly undulated with minimal loss of proteoglycan in the superficial zone.

4. Discussion

As the major symptoms of OA are decreased and painful mobility, our primary objective was to determine the efficacy of manual acupuncture for symptom modification in the Dunkin Hartley guinea pig model of primary OA. In this study, acupuncture-treated guinea pigs showed no significant improvement in stride length over time compared to control groups. Further, in open-field assessments, acupuncture-treated guinea pigs did not have significant changes in activity-based parameters. Despite no improvements in symptoms, knee joint OA scores trended toward a decrease in the acupuncture-treated guinea pigs. In contrast, previous studies have demonstrated acupuncture to significantly alleviate pain and inflammation, as well as improve joint structures, in various rodent models of OA. For example, electroacupuncture decreased hyperalgesia, paw edema, and inflammatory histologic scores in rodent models of adjuvant-induced arthritis.[153,154,156,185,186] Electroacupuncture also decreased arthritis scores in rat models of adjuvant-induced arthritis,[154,156,186] as well as surgically-induced arthritis.[93,157]

However, many important differences should be noted between these studies and the current study. Prior studies have used induced models of arthritis, via chemicals or surgery, whereas our study used a natural model of OA to most accurately represent the pathology of humans. Additionally, acupuncture was performed at least 3 days a week for as long as 12 weeks in these studies, compared to only once weekly for 3 weeks in the current study. Another important difference is that guinea pigs received treatment at approximately 1 year of age, by the time moderate OA has already developed in this model. Other rodent studies began acupuncture treatment at the onset of OA. In rats intra-articularly administered monosodium iodoacetate, early administration of electroacupuncture alleviated pain and preserved the joint structure, whereas delayed acupuncture treatment had no beneficial effects. If manual acupuncture was

initiated earlier in the disease stage of the guinea pig model, perhaps it would have been more effective in reducing pain.

It should also be noted that prior studies evaluated electroacupuncture, rather than manual acupuncture. Electroacupuncture is typically favored in basic research as it is able to provide a reproducible stimulus.[184] We previously demonstrated electroacupuncture to improve symptoms in the guinea pig model of OA, providing evidence that electroacupuncture may be more effective than manual acupuncture in alleviating pain. Similarly, many studies comparing the two modalities have shown the analgesic effect of electroacupuncture to be superior to that of manual acupuncture. For example, electroacupuncture, but not manual acupuncture, was shown to effectively reduce capsaicin-induced paw edema in rats.[187] Using pressure algometry, electroacupuncture was determined to induce a significantly greater analgesic effect than manual acupuncture during brief needle application in humans.[188] Further, a meta-analysis of randomized controlled trials determined electroacupuncture to be more effective than pharmacological treatment and manual acupuncture in reducing pain and improving physical function in patients with knee OA.[98] However, a randomized controlled trial found no difference between immediate effects of a single session of manual acupuncture and electroacupuncture on pain, mobility, and muscle strength in humans with OA.[189] Despite conflicting evidence, there may be differences in mechanisms of manual acupuncture and electroacupuncture due to electroacupuncture's higher stimulation intensity. Still, the potential differences between the two methods are poorly understood and frequently unaddressed.[184]

In the current study, we also assessed systemic inflammation using CBCs and serum biochemistries. Mice have shown a decrease in the inflammatory response after manual acupuncture, evidenced by a reduction in neutrophils and eosinophils.[190] However, there were no

differences in CBC or serum biochemistry parameters between groups in the current study. A lack of differences in white blood cells may be due to a relatively short-term effect of manual acupuncture, as it was only performed once weekly for 3 weeks in the current study. Thus, more specific biomarkers of systemic inflammation, C3 and PGE2, were also evaluated. C3 is an acute phase protein in guinea pigs.[158] Levels of acute phase proteins, such as C reactive protein, have been shown to be increased in humans with OA.[159,191] C3 was shown to be significantly decreased in the acupuncture group compared to the anesthesia group, but only trended toward a decrease in the off-point group. As the off-point group had needle stimulation, albeit at non-designated acupuncture points, this may have still caused unknown systemic effects. PGE2 is considered to be the major contributor to inflammatory pain in OA.[192] Carrageenan-induced PGE2 inflammation was previously shown to be decreased by electroacupuncture in rat paws.[185] In the current study, PGE2 was significantly decreased in the acupuncture group compared to both control groups, but this did not appear to significantly alleviate pain.

In general, there are many potential variables in acupuncture research, including the duration and frequency of treatments, type of needle stimulation, and acupuncture point selection. Manual acupuncture treatments may need to be performed more frequently and for a longer duration in order to see improvement in symptoms and pathology. Another potential limitation of the current study was the use of anesthesia. In order to reduce stress and ensure accessibility of acupuncture points, all guinea pigs were anesthetized with isoflurane. However, it is still unknown if anesthesia has an effect on acupuncture mechanisms.

In summary, 3 weekly sessions of manual acupuncture did not appear to improve symptoms of OA in this model. However, manual acupuncture decreased markers of systemic inflammation and trended toward an improvement in the articular cartilage structure. Future

studies should investigate mechanisms of long-term structural modification in this model of primary OA.

CHAPTER IV.

CONCLUSIONS

The primary goal of this project was to determine the efficacy of acupuncture for knee osteoarthritis (OA) using the Dunkin Hartley guinea pig model of primary OA. First, a study was completed to determine hematology and serum biochemistry reference intervals in this guinea pig strain. Although the Dunkin Hartley guinea pig is routinely used for studies of infectious disease and inflammatory disorders, such as OA, comprehensive reference intervals have not yet been established for this strain. Sex-specific reference intervals were created for complete blood counts (CBCs) and serum biochemistry tests, and numerous age- and sex-related differences were identified. Therefore, these factors must be considered when evaluating CBCs and serum biochemistries for laboratory or clinical data.

Further, two studies were performed in Dunkin Hartley guinea pigs to evaluate the efficacy of electroacupuncture and manual acupuncture for knee OA. Guinea pigs that received electroacupuncture had significant improvements in stride length as determined by gait analysis, as well as improvements in mobility parameters evaluated in open-field enclosure monitoring. However, there was no significant difference in knee joint histology scores between electroacupuncture and control groups. Serum inflammatory mediators trended toward a decrease in the electroacupuncture group. Additionally, gene expression revealed several upregulated structural and antioxidant genes in the cartilage of guinea pigs treated with electroacupuncture. However, different results were seen in guinea pigs administered manual acupuncture. There were no significant improvements in gait or mobility-based parameters in acupuncture-treated guinea pigs. However, there was a trend toward a decrease in their total joint

histology scores compared to control guinea pigs. Manual acupuncture did not have an effect on hematologic or biochemical parameters, but significantly decreased serum inflammatory proteins. Gene expression for this project will be completed at a later date.

These results provide evidence that short-term treatment with electroacupuncture is capable of modifying symptoms, but not the joint structure, of guinea pigs with primary OA. In contrast, while short-term treatment with manual acupuncture did not improve symptoms of OA, there was a potential effect on improving the joint structure. Continued work is still needed to demonstrate possible mechanisms capable of explaining the efficacy of these modalities for symptom and disease modification. Additionally, these studies were only performed for 3 weeks and were carried out after significant OA pathology had already been developed in this model. Future studies should investigate efficacy of long-term treatment initiated prior to the development of disease. Lastly, as humans do not typically need anesthesia for acupuncture, further studies should evaluate if guinea pigs will tolerate acupuncture in the absence of sedation or anesthesia.

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